University of Nevada, Reno

Intimations on the Pathophysiology of Human Preterm Labor: The Unique Actions of Nitric Oxide in the Myometrium and the Consequences of its Dysregulation

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in
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by

Scott D. Barnett

Dr. Iain L.O. Buxton/Dissertation Advisor

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SCOTT D. BARNETT

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Iain L.O. Buxton, Pharm.D., Advisor

Cherie Singer, Ph.D., Committee Member

Normand Leblanc, Ph.D., Committee Member

Josh Baker, Ph.D., Committee Member

Patricia Berninsone, Ph.D., Graduate School Representative

David W. Zeh, Ph. D., Dean, Graduate School

December, 2017

Abstract

Approximately 12% of all infants are born prematurely in the United States, costing in excess of 26 billion dollars annually. About half of those preterm births are the result of spontaneous preterm labor (sPTL), which is idiopathic in nature. One of the reasons so many cases of sPTL result in preterm birth is because tocolytics, which are drugs that prevent or halt labor, are only effective at delaying birth by 48-hours. This failure of tocolytics is due in part to the unique nature of uterine smooth muscle. Specifically, we have found that global cGMP accumulation, or depletion, has little effect on nitric oxide-mediated myometrial relaxation. This observation has generally been overlooked during tocolytic development in favor of pursuing therapeutics that modulate canonical pathways; however, this peculiarity of the myometrium may reveal the importance of the direct action of nitric oxide to modify proteins via S-nitrosation, a labile posttranslational modification whose dysregulation is associated with many diseases. Unlike term human myometrium, nitric oxide's effects are not only blunted in sPTL myometrium, but global protein S-nitrosations are also diminished, suggesting a dysfunctional response to nitric oxide-mediated protein S-nitrosation. Our study of S-nitrosoglutathione reductase (GSNOR), an enzyme that degrades the common endogenous form of nitric oxide, S-nitrosoglutathione (GSNO), reveals increased expression of the enzyme in sPTL myometrium, associated with decreased total protein S-nitrosation. Inhibition of GSNOR by N6022 relaxes myometrial tissue,

indicating the importance of nitric oxide donors and protein S-nitrosation in myometrial quiescence. GSNO, which can trans-S-nitrosate proteins, also alters acto-myosin ATP-ase activity, increases TREK-1 outwardly rectifying potassium currents, and increases myosin light chain kinase activity. Taken together, these findings offer novel explanations for nitric oxide-mediated relaxation in myometrium, and provide evidence for the effectiveness of a new class of tocolytics.

Dedication:

To Dharma, for everything that matters

To Ma & Pa Barnett, for endless encouragement and boundless love

To Iain, for patience

and...

To Monica, for friendship

Acknowledgements:

Simply put, a Ph.D. would not be possible without the help of a great many people. As they say, it takes a village, and I would like to acknowledge those who kept my pointed into the wind along the way.

For many people, myself included, the pursuit of a Ph.D., has, at times, felt like a terminal exercise in masochism; an academic run on sentence, if you will. Failed experiments are often bookended by rejected manuscripts and unscored grants, enough to make any person question his or her choices. That's not to say that there were not moments of elation and cautious optimism along the way, because there were...many of them! Passing my "qualifier," receiving notification that my 1st first-author manuscript had been accepted, and countess other small victories, have undoubtedly made this trek worth it. To abuse an over-used analogy, earning a doctorate is often compared to running a marathon. A marathon would be much harder, or impossible, without a team to provide support along the way. I would like to acknowledge those who were most integral to providing this emotional and academic sustenance.

It should come as no surprise that my wife, Dharma, would top this list. Throughout this process, she has tolerated the late nights, long weeks, and maybe even a brief melt down or two, with nary a complaint. She fundamentally recognized that this was my path, and her support never wavered. At the end of the day, she was always there to lift my spirits when I was down, or share in the

happiness of my victories. I could not have asked for a better partner in life. All this from a girl that I met online, imagine that!

I would also like to recognize my mom and dad. Interestingly, they hold the Guinness World Record for being the world's greatest parents. No hyperbole, they're in there, you should look it up. They are my biggest fans and they've always supported my every interest. From a young age they instilled in me such an (over)abundance of confidence, and I have never once thought that there was a goal beyond my grasp.

I would like to recognize my doctoral advisor, Dr. Iain L.O. Buxton. I came to his lab as an undergraduate looking to bolster my resume for medical school. He took me under his wing, helped me to understand that medical school was for people who couldn't hack it in graduate school (kidding, he didn't say that), and he has spent the last eight years teaching me how to become a scientist (if you know me you'll appreciate this is no small task). I truly could not have asked for a better mentor and teacher. Docendo discimus. Thank you!

I would also like to thank the Pharmacology department and its staff. In particular, Anette Christiansen, who is one of the unsung heroes of the department. She has a vast administrative expertise and has always been there to help. Also, thank you to all of the other members of our lab, to include Sarah Thompson, Veronica Arinze, and Connie Spelius for collecting myometrial samples critical to my research. I can't forget Dr. Heather Burkin, for her boundless energy and for

never hesitating to go out of her way to teach, write a LOR, or read and provide feedback on a manuscript. Thank you all!

My penultimate acknowledgement is to my committee members. Academic research is challenging on so many levels. There's the need to secure grants, the need to teach, the need to mentor undergraduates and grad students, and of course, the need to research and publish papers. It is a hard job on the easiest of days. So, it comes as no surprise that I am grateful that these five people, who, without hesitation, offered to serve on my committee and provide guidance over the years. It was an additional responsibly that none of them were required to do, and that is not lost on me.

Lastly, I would like to acknowledge Monica Rice. Monica was a good friend in the CMPP program and was killed by a drunk driver in early 2017, only a few months before she was to defend her own dissertation. She was one of the nicest people you'd ever hope to meet, and your day was guaranteed to be a little brighter in your paths crossed. It is unacceptable that her life was taken away so callously. I am so sorry you are gone Dr. Rice.

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Chapter 1

Introduction

Preterm Labor: *The failure of tocolytics and the search for a new path forward.*

Introduction:

Managing spontaneous preterm labor (sPTL) should be a simple problem. It is however, a problem with no tenable solution despite decades of intensive research (Berkman et al., 2003; Elvira et al., 2014; Giles and Bisits, 2007; Gyetvai et al., 1999). A cursory investigation into the conundrum that is sPTL quickly drives one towards a common-sense solution — since contraction of the uterus is the proximate cause of preterm birth, simply stop the uterus from contracting. There exists a host of therapeutic interventions that can inhibit smooth muscle contraction, so why are there no drugs to prevent the early delivery of a child? The answer to this question is not entirely known, but progress has been made. Here we investigate the idiosyncrasies of the myometrium and propose a novel approach to treat sPTL that leverages the unique relationship between nitric oxide (·NO) and myometrium.

History of Preterm Labor:

Long before modern pharmacological methods were available to treat preterm labor, its consequences were well known. Depictions of pregnancy and birth can be found in ancient Egyptian hieroglyphics (Figure 1), and in 400 B.C.E., Hippocrates described several maladies associated with pregnancy and its complications in his work *Aphorisms* (Hippocrates and Chadwick, 1950). In the

early 16th century, Leonardo da Vinci undertook intense physiological studies to better understand pregnancy and parturition (Gilson, 2008). One of the earliest written descriptions of preterm birth (PTB) was of Sir Isaac Newton, born in 1642. He was portrayed as being born so premature that “he could have been put into a quart mug” (Simpson, 1907). Unfortunately, in the intervening centuries following Sir Isaac Newton’s premature birth, our ability to treat preterm labor (PTL) and PTB remains limited.

PTL, and by extension PTB, impact millions of lives each year. PTB places a massive financial burden on society estimated at $26.2 billion annually in the United States alone (Behrman and Butler, 2007). Adjusted for inflation, this figure has risen to 30 billion. Estimates of the cost of PTB cannot accurately account for many of the costs of ongoing medical care for premature infants with unwanted outcomes such as chronic lung disease, cardiovascular disease or cerebral palsy, or track these costs to adulthood. PTB remains the primary cause of neonatal morbidity and hospitalization during pregnancy (D’Onofrio et al., 2013; Miniño et al., 2006; Rundell and Panchal, 2017) and accounts for a majority of pediatric care worldwide (Howson et al., 2013). In the United States alone, greater than 12% of infants are born prematurely, resulting in 20,000 deaths annually (Martin et al., 2011). About half of those premature births are attributed to sPTL (Goldenberg et al., 2008). Worldwide, the statistics are even more disconcerting. Approximately thirteen million infants across the globe are affected by premature birth each year (Behrman and Butler, 2007). Sub-Saharan Africa is of particular concern, where

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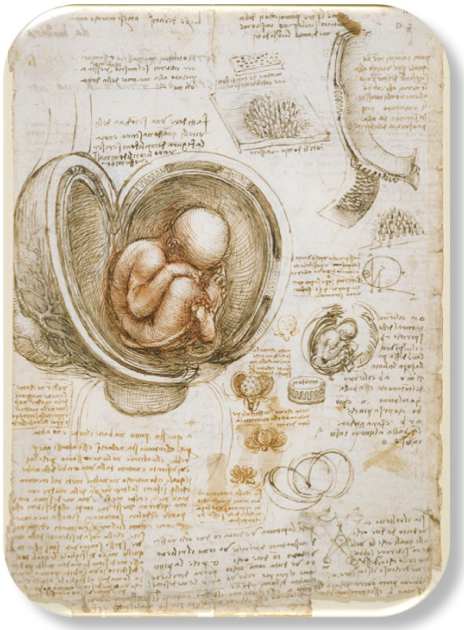

Figure 1: *Depictions of pregnancy and birth in antiquities:* (a) Hieroglyphic depictions of a woman giving birth from The Temple of Kom Ombo in Aswan Governorate, Egypt, which was constructed during the Ptolemaic dynasty, 180–47 BC. Used with permission (CC). (b) Leonardo da Vinci – 'Studies of the foetus in the womb', 1510-1512. Black chalk, sanguine, pen, ink wash on paper. Public domain image ⊘. Written examples of the perinatal disorders have been recorded as far back as Hippocrates in 400 B.C.E., in his book, Aphorisms. 2400 years later and we still have exceedingly few tools to prevent the early delivery of a child, despite decades of intense evidence-based research.

as many as 336,000 of the 1.2 million (28%) births each year result in newborn death (Kinney et al., 2010). In fact, women of African descent are 50% more likely to deliver preterm than women of European descent (CDC, 2015). While some cohorts are disproportionately affected by sPTL and PTL, the emotional and physical consequences of early labor and delivery span all races, nationalities, ages and socioeconomic groups. It is because of this that solving this complex problem is of paramount importance.

PTL is defined as labor between 20 and 37 weeks of gestation, with extreme PTB between 22-28 weeks ("Preterm labor," 1995). sPTL differs from PTL in that it is idiopathic in nature, meaning no root cause for the labor has been established. PTB can adversely affect fetal development; in particular, the heart, lungs, and brain. (Saigal and Doyle, 2008). Approximately 75% of neonatal mortality and 50% of long-term neurologic impairment are the result of PTB (McCormick, 1985). Amillia Taylor, born in 2006 at 21 weeks and 6 days, is the earliest known surviving preterm infant. This is an extraordinary success of modern medicine considering a full-term pregnancy in humans is 40 weeks. While the medical community continues to enhance its ability to decrease morbidity and mortality in extreme preterm infants (H.C. et al., 2015), 60% of all neonatal deaths still occur in infants born prior to 34 weeks of gestation (H.C. et al., 2015). Identifying effective methods to prevent PTB altogether would better serve the infant and our global community at large.

It wasn't until half way through the 20th century that PTB was systematically defined. In 1950, the World Health Organization (WHO) formally described PTB as any infant weighing less than 2,500 grams upon delivery (Camacho, L., Cross M., Lelong M., Levine S., Magnussen E., 1950). For comparison, Amillia Taylor weighed less than ten ounces (283 grams) when born. Amillia's survival is all the more astonishing when we consider that Extremely Low Birth Weight (ELBW) infants, weighing less than 1000 grams at the time of delivery, experience mortality levels of 30–50%, and morbidity rates of 20–50% (Figure 2) as compared to their full term counterparts (H.C. et al., 2015).

In 1961 the WHO refined the definition of PTB to its current definition of birth prior to 37 weeks, and instead used 2,500 grams to define 'low birth weight' (WHO, 1961). This change was made to better offset the observation that infant weight can vary drastically, even in full-term deliveries, but still recognizes that low birth weight can adversely affect postnatal outcome independent of the developmental issues associated with PTB (Ghosh and Daga, 1967).

In the subsequent decades, our ability to care for extreme preterm infants has increased markedly. Morbidity and mortality rates continue to decline (Cooke, 2006), yet approximately 12% of all pregnant women will still experience PTL (Martin et al., 2011), meaning that millions of infants are born each year with no tenable solution to prevent their early delivery. Our inability to keep the fetus *in utero* until term stems from an incomplete understanding of how the body's many

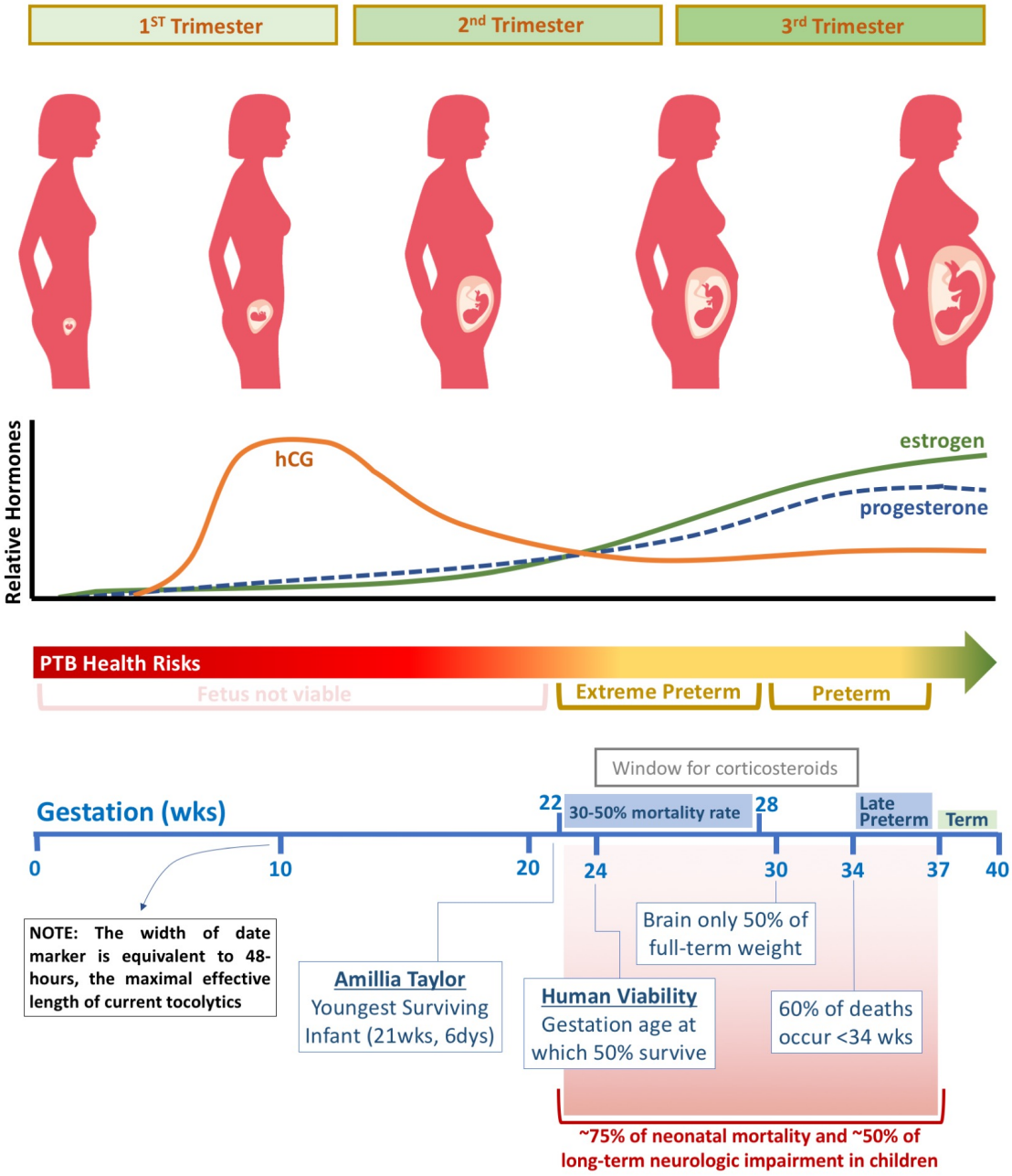
1ST Trimester
2nd Trimester
3rd Trimester
Relative Hormones
hCG
estrogen
progesterone
PTB Health Risks
Fetus not viable
Extreme Preterm
Preterm
Window for corticosteroids
Gestation (wks)
22
30-50% mortality rate
28
Late Preterm
Term
0
10
20
24
30
34
37
40
NOTE: The width of date marker is equivalent to 48-hours, the maximal effective length of current tocolytics
Amillia Taylor
Youngest Surviving Infant (21wks, 6dys)
Human Viability
Gestation age at which 50% survive
Brain only 50% of full-term weight
60% of deaths occur <34 wks
~75% of neonatal mortality and ~50% of long-term neurologic impairment in children

Figure 2: *Preterm birth risks: Morbidity and mortality*: The progression of pregnancy and the associated health risks associated with preterm birth. Tocolytics are ineffective at delaying preterm birth beyond 48-hours after the onset of preterm labor. Mortality and morbidity decrease precipitously as the pregnancy progresses from weeks 22-34, therefore, it is imperative that we identify novel approaches to delay preterm birth beyond current capabilities.

disparate systems converge to initiate and maintain labor. At the core of this process, regardless of which upstream factors trigger the labor, is the myometrium.

The Myometrium and Pregnancy:

The myometrium, a smooth muscle that encapsulates and protects the developing fetus, and provides the force needed to separate infant from mother during birth. Smooth muscles are a specialized class of myocytes found throughout the body. While they serve many roles, they most commonly aid in the function of hollow structures and organs such as the bladder, airway, blood vessels, intestines, and the uterus (Jonathan D. Kibble and Colby R. Halsey, 2015). Most smooth muscles communicate to adjacent cells through gap junctions. These important channels propagate contractile signals between myocytes (Laird, 2006). This efficient cell-to-cell communication allows for an amplified response to modest stimuli. An atypical exception to this paradigm are multiunit smooth muscles, such as those that control pupil diameter and require individual autonomic nerve stimulation to each muscle cell (Bose and Bose, 1977), but here we will focus our discussion on the function on the myometrium. Like skeletal and cardiac myocytes, smooth muscle requires a depolarization, followed by an influx of extracellular Ca^{2+}, to stimulate muscle contraction. To better understand the unique nature of smooth muscle, a brief overview of generalized muscle contraction is warranted.

Generalized Smooth Muscle Contraction:

Like skeletal and cardiac muscle, Ca^{2+} initiates contraction of smooth muscle, albeit through a vastly different mechanism. The stimulation of a smooth muscle contraction varies depending on the type of the muscle group. For example, enteric neurons drive smooth muscle contraction in the digestive tract (Kunze and Furness, 1999). Vascular and airway smooth muscle responds strongly to adrenergic stimulation (Barnes, 1995), and the myometrium relies heavily on hormones and prostaglandins (Gimpl and Fahrenholz, 2001). While there is some overlap in the mechanisms that drive contraction in these different smooth muscle types, such as with common GPCR signaling pathways and depolarization-driven Ca^{2+} entry, minor phenotypic variations dictate unique responses to stimuli and drugs. This also means therapeutics intended for one type of smooth muscle may not be effective in other types; an observation that has become exceedingly clear when examining the failure of tocolytics, drugs used to halt or prevent labor (Giles and Bisits, 2007). This concept holds true for the myometrium and has proven problematic in the treatment of sPTL, as there are significant differences in myometrial physiology when compared to other types of smooth muscle.

The Myometrium:

The myometrium is a powerful smooth muscle that acts as the principle expulsive driving force during labor. It is phasic in nature, as opposed to tonic, meaning that contractions are balanced by relaxation a short time after stimulation (Szal et al., 1994). An endogenous myometrial stimulator is oxytocin (OT), a peptide hormone primarily generated in the hypothalamus and secreted into the bloodstream via the posterior pituitary gland (Kimura et al., 1992). OT binds to oxytocin receptors (OXTR) in the myometrium (Gimpl and Fahrenholz, 2001). When stimulated, these GPCRs ($G\alpha_{q/11}$) activate phospholipase C, in turn generating inositol triphosphate (IP_3) and diacylglycerol (DAG) from membrane stores of phosphatidylinositol 4,5-bisphosphate (PIP_2). This activates protein kinase C (PKC), as well as other downstream effectors of smooth muscle contraction (Figure 3a) (Wray, 1993).

As with skeletal and cardiac myocytes, Ca^{2+} is every bit as critical to contraction in the myometrium, but for very different reasons. GPCR stimulation activates voltage-gated Ca^{2+} channels, which in turn triggers calcium-induced calcium release (CICR) from the sarcoplasmic reticulum, further depolarizing the membrane. Unlike in skeletal muscle, Ca^{2+} binds to calmodulin (CaM), which activates myosin light chain kinase (MLCK) (Bursztyn et al., 2007). MLCK phosphorylates S18/S19 of the regulatory light chain (MYL9) of smooth muscle myosin (SMM) (Frearson et al., 1976; Hong et al., 2011), sometimes called RLC20,

referring to its ~20kDa molecular weight. Of note, the currently accepted nomenclature for RLC20 is MYL9, and will hence force be referred to as such. It has been hypothesized that MLCK isoforms are differentially expressed in non-pregnant, term, and preterm myometrium (Moore and Bernal, 2001), which may affect the catalytic efficiency of this enzyme.

It is widely accepted that the phosphorylation of MYL9 is the 'master switch' that drives smooth muscle contraction, and that the kinase activity of MLCK is balanced by the dephosphorylation of MYL9 by myosin light chain phosphatase (MLCP) (Figure 3b). Class II SMM contains two interacting heavy chains, each with their own actin-binding head domain along with an essential light chain and a regulatory light chain (MYL9). In its unphosphorylated state, one head of the SMM complex binds to the actin filament, while the complementary actin-binding site on the second head is "blocked" due to interactions with the first head (Baumann et al., 2012). As a result, cross-bridge cycling is prevented, and a contraction cannot occur. Phosphorylation of MYL9 causes a conformation change to the myosin head formerly bound to its "sister" head, which in turn permits actin binding, and cross-bridge cycling can occur. Many drugs seek to prevent the influx of Ca^{2+} into the cell, thereby preventing MLCK activation. Our research proposes an alternative mechanism through protein S-nitrosation, that may compliment this well-established pathway by altering the structure and function of these and other proteins associated with the acto-myosin complex

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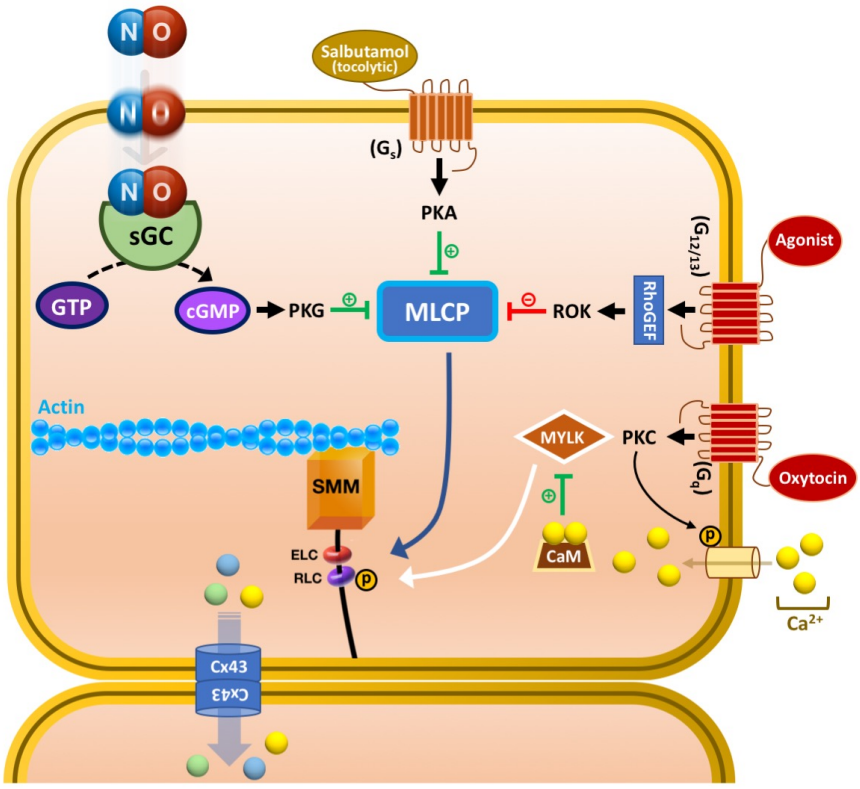

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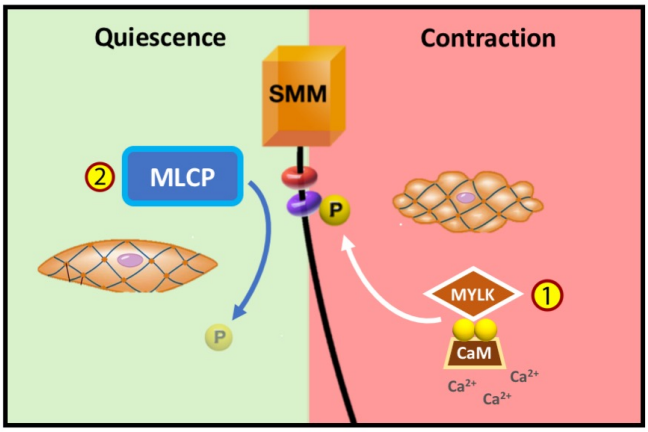


$$\frac{1}{2}\quad \frac{\text{MLCK activity}}{\text{MLCP activity}} \approx [RLC_{20}(P)]$$

Figure 3: *Canonical contraction and relaxation pathways in smooth muscle*: (a) The canonical contraction and relaxation of smooth muscle is mediated by transmembrane receptors, ion channels, small molecules, hormones, and thousands of other molecules. (b) Despite the complex intertwining of pathways that control smooth muscle contraction and relaxation, it is the phosphorylation of MYL9, the 20kD regulatory light chain of smooth muscle myosin that acts as the 'master switch' to contraction, and it is the ratio of kinase (MYLK) to phosphatase (MLCP) that dictates contraction in the cell.

Canonical Smooth Muscle Relaxation:

Unlike skeletal muscle, in which high levels of cytosolic calcium are required to maintain contraction, smooth muscles maintain contractile force until MYL9 is dephosphorylated (Bursztyn et al., 2007). Dephosphorylation of MYL9 is primarily driven by the myosin phosphatase targeting subunit, MYPT1, of MLCP, the predominant phosphatase in smooth muscle. MLCP contains a PP1cδ catalytic subunit, a MYPT1 targeting subunit, as well as an M20 subunit with unknown function (Hudson et al., 2012). MYPT1 itself is phosphorylated at S695, T696, S852 and T853, and is constitutively active when not phosphorylated, albeit with low activity, so some contractile stimulation is needed to maintain contraction (Ito et al., 2004). This modulation of MLCP activity is a major component of Ca^{2+} sensitization/desensitization (Somlyo and Somlyo, 2003), and it allows for complex regulation of smooth muscle contraction and relaxation.

MYPT1 is phosphorylated by cAMP dependent PKA and cGMP dependent PKG, which activate the enzyme, as well as Rho-Kinase (ROCK or ROK), which inhibits MLCP (Puetz et al., 2009). The relationship among these kinases and their activation pathways is complex. In smooth muscle, one common route to PKA activation is through β2 adrenergic (Gs) driven cAMP production. PKG is also activated through cyclic nucleotide production, in this case cGMP, which is created as the result of ·NO-stimulated soluble guanylyl cyclase (sGC). ROK, an inhibitor

of MLCP, is activated by the RhoGEF complex, as a result $GPCR_{12/13}$ stimulation (Figure 3a). While all three of these kinases act upon MYPT1, it should be noted that each of them have a number of phosphorylation targets that either enhance or inhibit contraction (Puetz et al., 2009). PKG action halts when either of its upstream activating factors, ·NO or cGMP, are exhausted. ·NO itself is generally depleted by either endogenous metabolizers of ·NO, such as S-nitrosoglutathione reductase (GSNOR), or the thioredoxin system, while cGMP is metabolized by phosphodiesterases (PDEs). As such, tocolytics that target these systems have been used with varying claims of success, and will be discussed further. Confounding MLCP function in smooth muscle is the finding that disparate functional isoforms of MLCP exist; specifically, it has been discovered that a leucine-zipper variant of MLCP affects PKG activity and cGMP-mediated relaxation in the cell (Dou et al., 2010; Yuen et al., 2011). Thus, neither the mechanism whereby PKG activates MLCP, nor the nature of the interaction between these two proteins are completely understood.

Another important mediator of pMYL9 is Telokin, the 17kD terminal fragment of MLCK that binds to SMM. Telokin is not only a functional domain of MLCK, but also serves as an autonomous, independently-translated, protein that is transcribed by a separate promoter from MLCK (Smith et al., 1998). Telokin serves two known functions. For one, it has an inhibitory effect on MYL9 phosphorylation via competitive binding with MLCK to SMM (Khromov et al., 2006). Second, it aids in MYPT1 activation (Komatsu et al., 2002). As will be discussed

further, Telokin is known to be differentially S-nitrosated depending of the state of pregnancy, which may affect its function (Figure 7b).

Nitric Oxide (·NO): One of the most important mediators of smooth muscle relaxation is •NO, a small molecule produced by the family of nitric oxide synthases (NOS) through the conversion of L-arginine to L-citrulline (Figure 4) (Wink and Mitchell, 1998). ·The discovery of ·NO as endothelium-dependent relaxing factor (EDRF) by Robert Furchgott, its chemical identification as ·NO by Louis Ignaro, and its signaling through cGMP, was the subject of the 1998 Nobel Prize in Physiology or Medicine (Furchgott et al., 1998). ·NO is an uncharged, yet highly reactive molecule, due to an unpaired electron (Valko et al., 2007). These characteristics were thought to allow it to readily cross the membrane to interact with intracellular proteins. This assumption notwithstanding, there is evidence that ·NO is transported from sites of production to sites of action as S-nitrosoglutathione (GSNO) (K A Broniowska et al., 2013; Gaston et al., 1993).

Canonically, ·NO stimulates sGC in smooth muscle, catalyzing the formation cGMP, a second messenger much like cAMP. cGMP activates PKG, which phosphorylates S695 on the catalytic subunit of MLCP, MYPT1 (Puetz et al., 2009). As has been mentioned, phosphorylation does not appear to be an obligate requirement for MLCP activation, and thus some unknown action may serve to activate MLCP, orchestrated by PKG activation. Interestingly, and as will expanded upon in the coming pages, ·NO also activates the inducible form of cyclooxygenase-2 (COX2), increasing the synthesis of the prostaglandin PGE_2

(Kim, 2011; Salvemini et al., 1993) which promotes quiescence through its GPCR (G_s) stimulation. PGE_2 is also an important mediator of cervical ripening (Keelan et al., 2003), indicating that ·NO's role in myometrial pregnancy and parturition is multilayered and nuanced. Another important distinction of uterine smooth muscle is that cGMP action can be compartmentalized (Iain L O Buxton et al., 2010a), and relaxation of the tissue in response to ·NO is independent of global cGMP accumulation (I. L. Buxton, 2004a). The reason behind this phenomenon has not been fully elucidated, but our lab has made substantial progress on this front. This signaling exception may have far reaching consequences, and affect how we approach the treatment of sPTL in the future, a topic that will be covered at length in chapter 2.

Within the family of smooth muscles, the myometrium has a particularly complex and varied response to stimuli that drive contraction and relaxation. It is not surprising that therapies used to treat sPTL are ineffective, as there are multiple compensatory and complimentary pathways which work independently to initiate contractions and mediate quiescence.

What Causes Spontaneous Preterm Labor?

Nitric Oxide and S-nitrosation

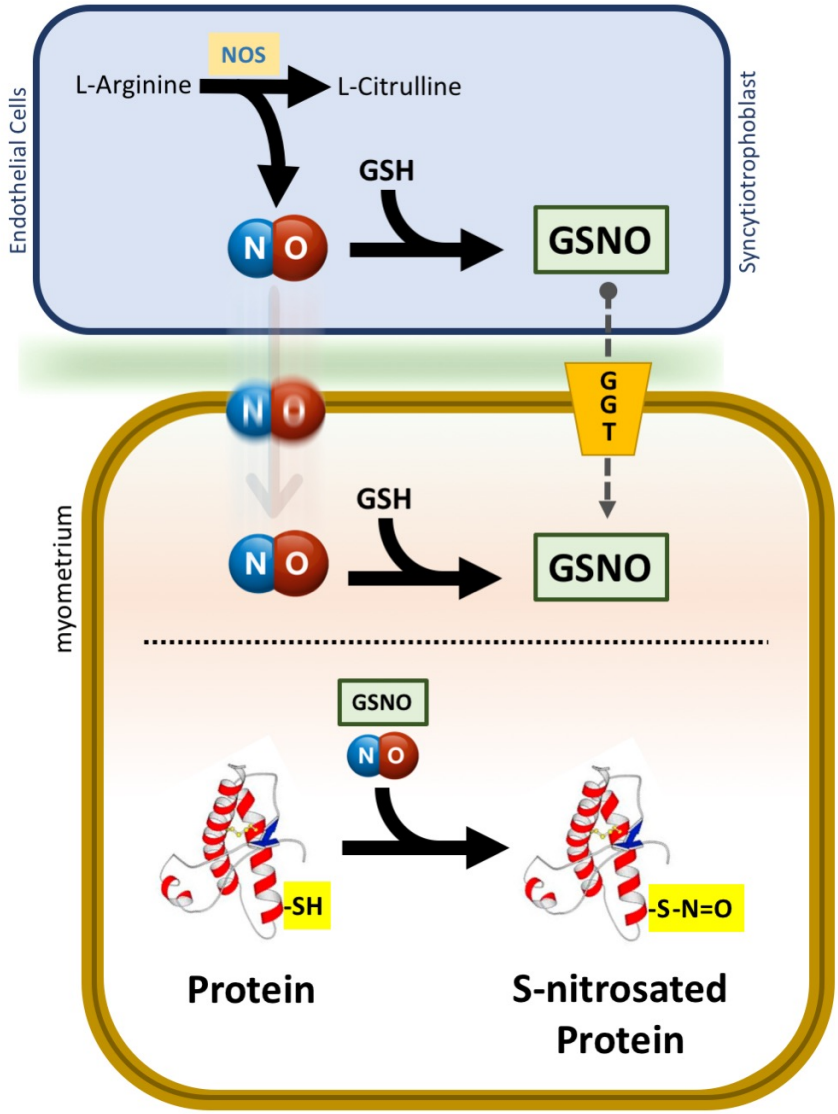


Figure 4: *Nitric Oxide and protein S-nitrosation*: Nitric oxide, an important mediator of smooth muscle relaxation, is created by the NOS family of enzymes. Nitric oxide that is relevant to the myometrium during pregnancy and parturition is believed to originate in either endothelial cells or from the syncytiotrophoblast. NO moves free through the membrane or may be transported by proteins such as γ-glutamyltransferase (GGT). NO is often stored in the cell as stable GSNO. Free NO or GSNO then react with thiol group of cysteines. This PTM called S-nitrosation

Despite a wealth of knowledge concerning this process, the short answer is that we do not know what causes the myometrium to contract spontaneously at times preterm. From an obstetrical point of view, the natural birth of a child requires two important physiological changes to occur; (1) remodeling of the cervix (effacement and dilation), and (2) myometrial contractions. In a normal full-term birth, biochemical factors originating from the fetus (Chard et al., 1971; Gao et al., 2015) and mother (Tribe, 2001), coupled with stretch-induced gene regulation in the myometrium (Buxton et al., 2011a; C. L. Cowles et al., 2015; Hua et al., 2013), work in concert to initiate labor. In the case of generalized PTL, several well-known maladies and dysfunctions, such as preterm premature rupture of the membrane (PPROM), microbial invasion of the amniotic cavity (MIAC), bacterial colonization and/or inflammation of the choriodecidua, among others, can result in PTB through cytokine/prostaglandin cascades that initiate contractions (Agrawala and Hirscha, 2012; Park et al., 2005). sPTL, on the other hand, is idiopathic in nature, and we currently lack the mechanistic insight to determine the root cause(s) of this disorder.

Before we can answer the question of what causes the sPTL, we must first determine whether there is a difference between sPTL and ordinary term labor (TL). An important initial question is whether or not spontaneous labor is simply early onset labor? This distinction is important if we are to identify therapies that target sPTL. It turns out that sPTL is different from TL, although the distinctions

are nuanced. One important difference between sPTL and TL is the concentrations of the prostaglandin precursor, PGHS-2, as well as cytokine IL-8 levels in the lower segment of the myometrium (Tattersall et al., 2008). Other important distinctions, such as protein S-nitrosation, and the expression of contractile proteins, will discussed in further detail.

Biomarkers for PTL and PTB:

A lingering question regarding PTL is whether or not tocolytics are ineffective at preventing PTB beyond 48-hours simply because they are administered too late? That is to say, once the mechanistic underpinnings that drive labor have reached a tipping point, it may not be possible to put the proverbial genie back in the bottle. Due to the inherent risks to the fetus associated with tocolytics, combined with the fact that ~90% of women do not experience preterm labor, it would be irresponsible and cost-prohibitive to administer prophylactic tocolytics (maintenance tocolytics) to all pregnant women. The logical alternative to this approach would be to identify either correlative or causal factors (i.e., biomarkers) that can be used to distinguish women at high risk for preterm birth from those at low risk.

Fortunately, there are many well-known positive predictors of PTL and PTB (Table 1) (Sayres, 2010; Weismiller, 2000). Several common contributing factors to PTL and PTB, such as illicit drug use, alcohol abuse, and smoking, are easy to mitigate, at least conceptually, although their cessation is more difficult in practice

Table 1: Common Risks Factors for Preterm Birth

- Ethnicity
- Maternal Age (<18 or >35)
- Stress
- Uterine Physiology
- Drugs/alcohol/smoking/pollutants
- High/Low BMI
- Infection
- Hypo/hypertension
- Multiple Pregnancy
- Geographic Location
- PPROM
- Access to Healthcare
- Low Socioeconomics
- Cervix (short/insufficient)

Table 1: *Common risk factors for preterm birth*: There are dozens of known risk factors that correlate to preterm labor (PTL) and preterm birth (PTB). While useful for indicating those at higher risk for PTL and PTB, and in some cases, provide a route to decrease the overall risk of PTL and PTB, these factors generally do not provide the mechanistic underpinnings of spontaneous PTL.

(Donatelle et al., 2004; Kaltenbach et al., 1998). Others, such as race (Culhane and Goldenberg, 2011), and low socioeconomic class (Whitehead, 2012), are much more perplexing; however, in both cases access to healthcare, at least in the United States, is more limited than with other cohorts (Olah et al., 2013), indicating a potential contributing factor. High levels of stress also contribute to PTL, which is well-known to increase proinflammatory cytokines (Field, 2017; Karlsson et al., 2017). That being said, these positive predictors of PTL are correlative, and are not in themselves true biomarkers.

If a biomarker for sPTL is to be useful in a clinical sense, it must be consistent and unambiguous. Fetal fibronectin is a protein produced by the fetus and found at the interface of the chorion and the decidua (Cunningham et al., 2014). Fibronectin acts as a cervical adhesive and can be found in higher levels in the vagina as the pregnancy nears term (Crane et al., 1999). Using the presence of fetal fibronectin in the vagina as a positive predictor of PTL has been contentious (Esplin et al., 2017; Roman et al., 2005; Stafford et al., 2008), but the evidence does not currently support its use as a biomarker to predict labor (Jwala et al., 2016). More recently, it has been proposed that the levels of cell-free fetal DNA (cffDNA) found in maternal blood plasma may be used as a marker for PTL (Phillippe, 2014; Romero et al., 2014), as cffDNA can activate TLR-9 and induce an inflammatory response (Scharfe-Nugent et al., 2012). Also, miRNA has been investigated as a biomarker for PTL. Interestingly, miR-143 and miR-145 see a 12.3 and 11.5-fold change, respectively, in cervical cells of women who

experienced PTB (Elovitz et al., 2014). These miRNAs target several hundred genes, to include TLR-2 and fibronectin production, as well as the translation of proteins associated with phenotypic switch in smooth muscle and cytoskeleton dynamics. MicroRNAs may ultimately prove to be useful as PTL/PTB biomarkers, but more research is needed.

Single Nucleotide Polymorphisms (SNPs) and Genetics: SNPs, while not biomarkers in the classical sense, do provide a minimally-invasive means to identify modifications to genes that may contribute to early onset labor. SNPs may ultimately prove to affect proteins and pathways that alter the body's response to pregnancy. Over the past several decades, the importance of SNPs in human health and disease has become evident (Frazer et al., 2009). Due to SNPs tendency to alter the structure, function and/or expression of proteins, it is only logical that they be investigated in relation to PTL and PTB.

Several SNPs in genes important to pregnancy maintenance and labor have been identified over time. For instance, SNPs that alter expression of metalloproteinase (MMP1/MMP9), β_2 adrenergic receptors (β_2ARs), TNF-alpha and IL-1β were all positively correlated to PTB (Crider et al., 2005). An investigation of SNPs in a Latin American cohort of women found SNPs in KCNN3, the gene encoding for Small Conductance Calcium-Activated Potassium Channel 3 (SK3), Corticotropin Releasing Hormone Receptor 1 (CRHR1), and F3, a Coagulation Factor associated with PPROM driven PTB (Gimenez et al., 2017). A

2017 GWAS study of more than 43,000 women found six genes strongly associated with PTB, including the gene for adenylyl cyclase, WNT4, as well as other genes with SNPs relevant to labor (Zhang et al., 2017). Of interest, SNPs in *ADH5*, the gene that encodes GSNOR, an enzyme that modulates ·NO availability in the myometrium, has been found in some groups who experience asthma (Wu et al., 2007). As ·NO is important to both airway smooth muscle and uterine smooth muscle relaxation, it would be of interest to determine if these SNPs are present in women who experience PTL and PTB. While SNPs most likely play an important role in pregnancy and parturition, much more work is needed to determine how they specifically effect this complex process.

Like many complex disorders, there may not be a “magic bullet” to identify women at risk for, or who are experiencing, preterm labor. SNPs and other genetic anomalies may serve as important contributing factors, that when combined with others, “kick-start” the process of sPTL. To this point, women with a history of spontaneous preterm delivery are 1.5 to 2x more likely to have a subsequent preterm delivery (Rundell and Panchal, 2017). While in some cases this may be the result of lifestyle or other contributing factors, it is not unreasonable to hypothesize that a genomic influence may also contribute. Researchers have begun to identify methodologies to systematically categorize how genetics drive the phenotypic endpoint that is PTL (Manuck et al., 2015; Villar et al., 2012), and progress has been made on identifying specific maternal and fetal genes that may be in play (Allen and Founds, 2013). To this point, our laboratory has found that

splice variants of the outward-rectifying K^+ channel, TREK-1, affect trafficking of the protein to the membrane, and diminish the ability of the cell to maintain the negative membrane potential needed for myometrial quiescence (Chapter 2) (C. L. Cowles et al., 2015).

While the list of biomarkers considered in this review is by no means exhaustive, the unfortunate fact of the matter is that biomarkers which reliably identify risk of PTL or PTB do not exist. An extensive 2011 review of biomarkers associated with PTB over 4 decades (Menon et al., 2011), as well as a more recent 2014 review of the data (Kacerovsky et al., 2014), found no reliable trend between biomarkers and PTB. That being said, our ability to use the power of genomic analysis to identify those at risk for PTL is still in its fledgling stage. As whole genome sequencing becomes more cost effective (Herper, 2017), and as 'big data' analysis becomes more sophisticated (Bellazzi, 2014; Raghupathi and Raghupathi, 2014), we may see significant advances in identifying genes and other biomarkers important to PTL and PTB. Due to the potential benefits of recognizing a true biomarker of sPTL, researchers and clinicians are highly incentivized to continue their searches. The identification of biomarkers that recognize women who are at high risk for PTL and PTB would represent a major leap forward in obstetrics. It would allow us to monitor appropriate individuals more closely, and might open the door to create patient specific tocolytics to be used as prophylactic agents.

Tocolytics

Drugs that suppress or terminate uterine contractions in an effort to treat premature labor are called tocolytics. The word tocolytic is derived from the Greek *tokos* = contraction, and *lysis* = to untie or dissolve (Keirse, 2003; Lewis, 1983). The term tocolytic did not emerge until the mid-1960s when there was a dramatic increase in research to treat premature labor (Mosler, 1966). The first modern compound used specifically as a tocolytic was relaxin in 1955 (Abramson and Reid, 1955). Relaxin is a peptide hormone which is generated by the corpus luteum and activates GPCRs in the myometrium. Despite its encouraging namesake, relaxin was quickly determined to be ineffective at mitigating contractions during labor (Babcock and Peterson, 1959). In the intervening years dozens of drugs that target a multitude of contractile pathways have been introduced into the market (Keirse, 2003). One of the reasons the myometrium is such an attractive therapeutic target is because labor can be initiated by many factors, such as: hormones, prostaglandins, cytokines and other immunological factors, stress, infection, among others. As such, it is no surprise that a wide-range of drugs have been tested for therapeutic potential as tocolytic agents.

The crux of sPTL, and the reason why the search for new drugs continues, is that currently available tocolytics are not effective at delaying birth beyond 48-hours (Weismiller, 2000). A systematic review of tocolytic research from 1966-1998 revealed a simple startling observation, “Although tocolytics prolong

pregnancy, they have not been shown to improve perinatal or neonatal outcomes and have adverse effects on women in preterm labor" (Gyetvai et al., 1999). The only known exception to this paradigm is in women with a short cervix or a history of PTB, where progesterone can delay birth longer than 48-hours (Navathe and Berghella, 2016); however, this treatment regime does not hold for sPTL. Despite these findings, tocolytics are routinely used in an attempt to maintain uterine quiescence in high-risk pregnancies, as well as for acute treatment of labor, for the simple reason that more effective therapies do not exist. Because of their limited efficacy, tocolytics are primarily used to provide a window for which to administer antepartum corticosteroids, between 24 and 34 weeks of gestation (Borders and Gyamfi-bannerman, 2017), which serve to accelerate fetal lung development (Roberts et al., 2017), rather than to halt labor altogether.

This observation is not surprising when we consider that tocolytics, in general, are borrowed pharmacology. That is to say, nearly every drug used to treat PTL was designed to treat maladies in other types of smooth muscle, such as vascular, colonic and airway. Even atosiban, a selective oxytocin–vasopressin receptor antagonist designed specifically to mitigate uterine contractions (Åkerlund et al., 1985), is not approved for use in the United States, despite demonstrating initial positive results (Coomarasamy et al., 2002; Romero et al., 2000). Importantly, atosiban does not reduce the risk of preterm birth beyond 48-hours or improve neonatal outcome (Papatsonis D, Flenady V, Cole S, 2005).

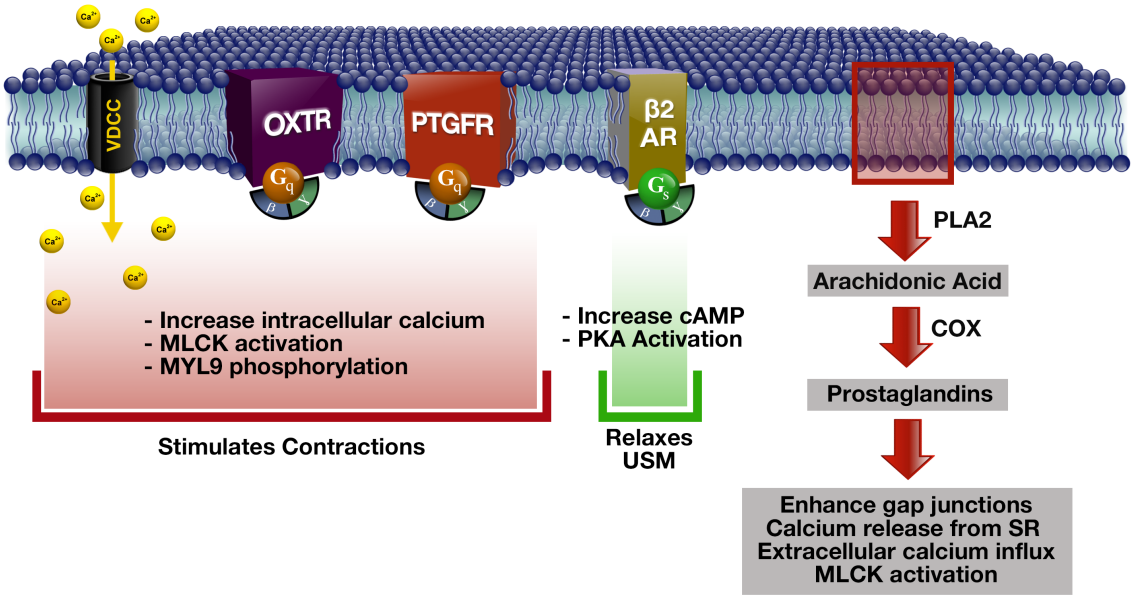
Generally speaking, modern tocolytics fall into four major classes: Calcium channel blockers, hormone modulators, prostaglandin pathway antagonists, and beta-2 adrenergic receptor (B_2AR) agonists (Figure 5), although dozens of tocolytics exist. With the exception of B_2AR agonists, most therapies seek to prevent contractions by inhibiting the "front-end" of the contractile pathway. In other words, they attempt to mitigate the onset of contractions, which begins with the influx in cations into the cell. This approach is logical, though not entirely effective, as we will see. To better understand why the current line of tocolytics perform so anemically, let us first look closer at some of the most common drugs used as tocolytic agents.

Magnesium Sulfate: $MgSO_4$ was first used clinically as a tocolytic in 1966, and is still used off-label by some physicians today. $MgSO_4$ limits calcium influx into the cell (Altura et al., 1987) through the action of the Mg^{2+} ion, a group IIA divalent cation similar to Ca^{2+}, albeit with a smaller diameter. Mg^{2+} decreases the tone and frequency of smooth muscle contraction, in part by competing with Ca^{2+} for entry into the cell (Altura and Altura, 1974; Karaki, 1989). Mg^{2+} is readily absorbed by the fetus that can lead to hypermagnesemia, depression of the central nervous system, heart rate variability (Ramsey P., 1983), and brain lesions (Mittendorf et al., 2006). $MgSO_4$ administered antenatally can decrease the incidence of moderate-to-severe cerebral palsy (Rouse et al., 2008), in particular in preterm

infants (Rundell and Panchal, 2017), but it does not delay birth (Crowther et al., 2014).

β_2 Agonist and Betamimetics: β_2 adrenergic receptor agonists, as well as betamimetics - named for their ability to 'mimic' endogenous β_2 ligands - have proven popular as tocolytics. Second generation β_2 agonist such as ritodrine, fenoterol, salbutamol, terbutaline, buphenine, and hexaoprenaline are poor tocolytic agents due to their limited specificity (i.e. β_1 activation), rapid metabolism or renal clearance, as well as unacceptable side-effects that include: fetal tachycardia, chest pain, headache, difficulty breathing, hypokalemia, nausea and/or vomiting (Lewis, 1983). In 2014, a full review of 12 betamimetics trials between 1966 and 2010, involving 1367 women, found that while this class of drugs could delay birth by up to 48-hours, they do not reduce the incidence of preterm birth, nor is there improvement to neonatal outcome or perinatal death (Neilson et al., 2014a). Furthermore, in 2011, after a 14-year review, the FDA issued a safety announcement warning against the use of terbutaline for the prevention or prolonged treatment of preterm labor due to a lack of efficacy and the potential risks to the mother and child (FDA, 2011). More recent studies have also found that PKA activation is only a partial contributing factor to cAMP-mediated myometrial relaxation, and that prolong exposure to cAMP agonist does not maintain relaxation (Lai et al., 2016), questioning the importance of cAMP (and cGMP, see chapter 2), in myometrial quiescence.

Common Therapeutic Targets for PTL



Tocolytics

Azapeptide: Prostaglandin Receptor Antagonist

$MgSO_4$: Competes with Calcium for Ion Channel

Terbutaline: Beta2 Adrenergic Receptor Agonist

Nifidepine: VDCC Antagonist

Atosiban: Oxytocin Receptor Antagonist

Indomethacin: COX1/COX2 Inhibitor

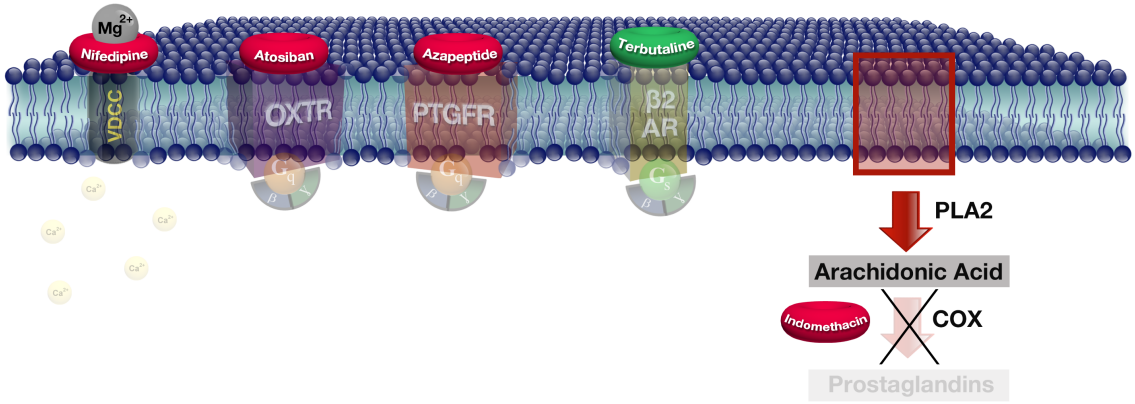


Figure 5: *Common tocolytics used to treat preterm labor*: Tocolytics are drugs used to prevent or to halt labor. Tocolytics generally target essential pathways to contraction, such as the oxytocin receptor (OXTR), prostaglandin receptors (PTGFR) and prostaglandin synthesis (COX), adrenergic receptors (B2AR) and voltage-dependent calcium channels (VDCC). Currently available tocolytics are not able to delay preterm birth beyond 48-hours.

Calcium Channel Blockers: Calcium channel blockers (CCBs) are commonly used in adults to treat hypertension. As the name implies, CCBs function by preventing the influx of calcium into the cell. Nifedipine and $MgSO_4$ are commonly prescribed tocolytics in this class. Nifedipine, a voltage-gated calcium channel antagonist, is used to treat high blood pressure by acting upon vascular smooth muscle (Olivari et al., 1979), which is a tonic, and not phasic, smooth muscle. The most significant issue with using CCBs as tocolytic agents is not that they are ineffective at reducing contractions, rather it is that these drugs pose life-threatening consequences to the fetus. As with most tocolytics, nifedipine only provides a modest reduction in the birth rates within 48-hours, and does not improve neonatal mortality or respiratory distress rates (Gyetvai et al., 1999). Furthermore, severe hypotension and an increased fetal mortality has been reported with nifedipine use (Veen A.J., Pelinck M.G., 2005). Nifedipine significantly decreases maternal systolic and diastolic blood pressure, which is to be expected, but it also decreases the uterine artery pulsatility index, and important indicator of fetal circulation (Guclu et al., 2006).

Antibiotics/Anti-inflammatories: Pregnancy is a unique immunological state in which the mother's immune system must balance the need to protect itself from infection, yet must also avoid rejecting the fetus (Racicot et al., 2014). A woman's

immune system naturally fluctuates as the pregnancy progresses (Gillespie, Porter, 2016), but in certain cases immunological factors, like cytokines, leukotrienes, and prostaglandins can increase the risk of PTL and PTB (Goldenberg et al., 2008; Mor and Cardenas, 2010; Romero et al., 2006). In the first century A.D., the Roman scientist Celsus documented the four cardinal signs of inflammation: rubor (redness) tumor (swelling) calore (heat) dolore (pain) (e Silva, 1978). We now know that inflammation is the inventible consequence of either infection or other exogenous/endogenous proinflammatory stimuli. Given the consequences of the inflammatory response during pregnancy (Belt et al., 1999; Racicot et al., 2014; Romero et al., 2014; Scharfe-Nugent et al., 2012), much research has been conducted to mitigate infection and proinflammatory immune modulators.

Intrauterine infection is a well-known cause of PTL (Agrawala and Hirscha, 2012), but sub-clinical, or “occult” infections stemming from floral dysregulation in the uterus and vagina have long been thought to act as contributing factors to sPTL (Gibbs et al., 1992; Potkul et al., 1985). Unfortunately, prophylactic antibiotics do not alter the incidence of chorioamnionitis, postpartum endometritis, or placental infection (Matsuda et al., 1993). Most importantly, the treatment of vaginosis with antibiotics does not decrease the rate of PTB, and prophylactic antibiotics do not decrease the rates of PTL or PTB (King et al., 2002; McDonald et al., 2007). The one exception to this paradigm are cases in which preterm premature rupture of the membranes (PPROM) has occurred (Hubinont and Debieve, 2011). This is not

to say that infections do not cause PTL and PTB; however, the evidence is clear that prophylactic administration of antibiotics does not prevent the early delivery of the fetus. The American College of Obstetricians and Gynecologists goes so far as to state, “antibiotics should not be used to prolong gestation or improve neonatal outcomes in women with pre-term labor and intact membranes” (ACOG, 2016).

Prostaglandins: Prostaglandins are not only deeply entwined with the inflammatory response, but they are also integral to pregnancy and parturition. In fact, the term ‘prostaglandin’ was first used almost a century ago after it was determined that seminal fluid could initiate uterine contractions (Kurzrok and Lieb, 1930). The two primary prostaglandins associated with pregnancy and labor are PGE_2 and PGF2α. These prostaglandins drive cervical ripening and bolster the contractile response in the myometrium during labor (Figure 6) (Bakker et al., 2017). PGE_2 is a ligand for receptor EP4, a GPCR (Gs) that activates cAMP through adenylyl cyclase, promoting quiescence, but also contributes to cervical ripening during labor. These functions may appear to oppose each other, but β_2AR expression decreases by half in the myometrium during labor, helping to minimize the quiescence effect of PGE_2 (Chanrachakul et al., 2003). PGF2α, on the other hand, is a ligand for FPa/b, a G_q, which increases phospholipase C activity (Katzung et al., 2004). Azapeptide and compound PDC113.824, both PGF2α receptor antagonists, both mitigate contraction, but they have not been thoroughly tested for safety and efficacy in a clinical setting (Bourguet et al., 2011; Goupil et al., 2010).

Cytokine Inhibition: Proinflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNFα), are not only generated in response to injury or infection, but they also function to promote cervical ripening and help to initiate labor (Nadeau-Vallée et al., 2016; Rhee et al., 2015; Tattersall et al., 2008; Unal et al., 2011). IL-1β induces COX2 expression through NF-κB and PKC activity in the myometrium (Belt et al., 1999), which then leads to the synthesis of the prostaglandins PGE_2 and PGF2α. Researchers are in the early stages of testing compounds that inhibit IL-1β (compound 101.10) while avoiding deactivation NF-κB (Nadeau-Vallée et al., 2016), as well as drugs that target the PGF2α-mediated Rho-Kinase activation (Goupil et al., 2010), a process that deactivates MYPT1. Investigation into the use of NF-κB inhibitors, like Sulfasalazine, have also proven fruitful in women with infection (Sykes et al., 2015), but it is unknown if NF-κB inhibition would be useful to treat sPTL. While it is too early to determine if these approaches will yield significant dividends in the field of tocolytic development, it does provide interesting insight into novel therapies to treat PTL.

Due to the integral role of cytokines and prostaglandins in parturition, researchers have used nonsteroidal anti-inflammatory drugs (NSAIDs), which are inhibitors of the COX pathway, to mitigate the response of the body to cytokines and prostaglandins. Indomethacin, a nonspecific COX1/COX2 inhibitor, when used alone (Ehsanipoor et al., 2011), or in concert with other tocolytics (Vogel et al., 2014), provides a modest tocolytic effect, but does not extend PTB by more

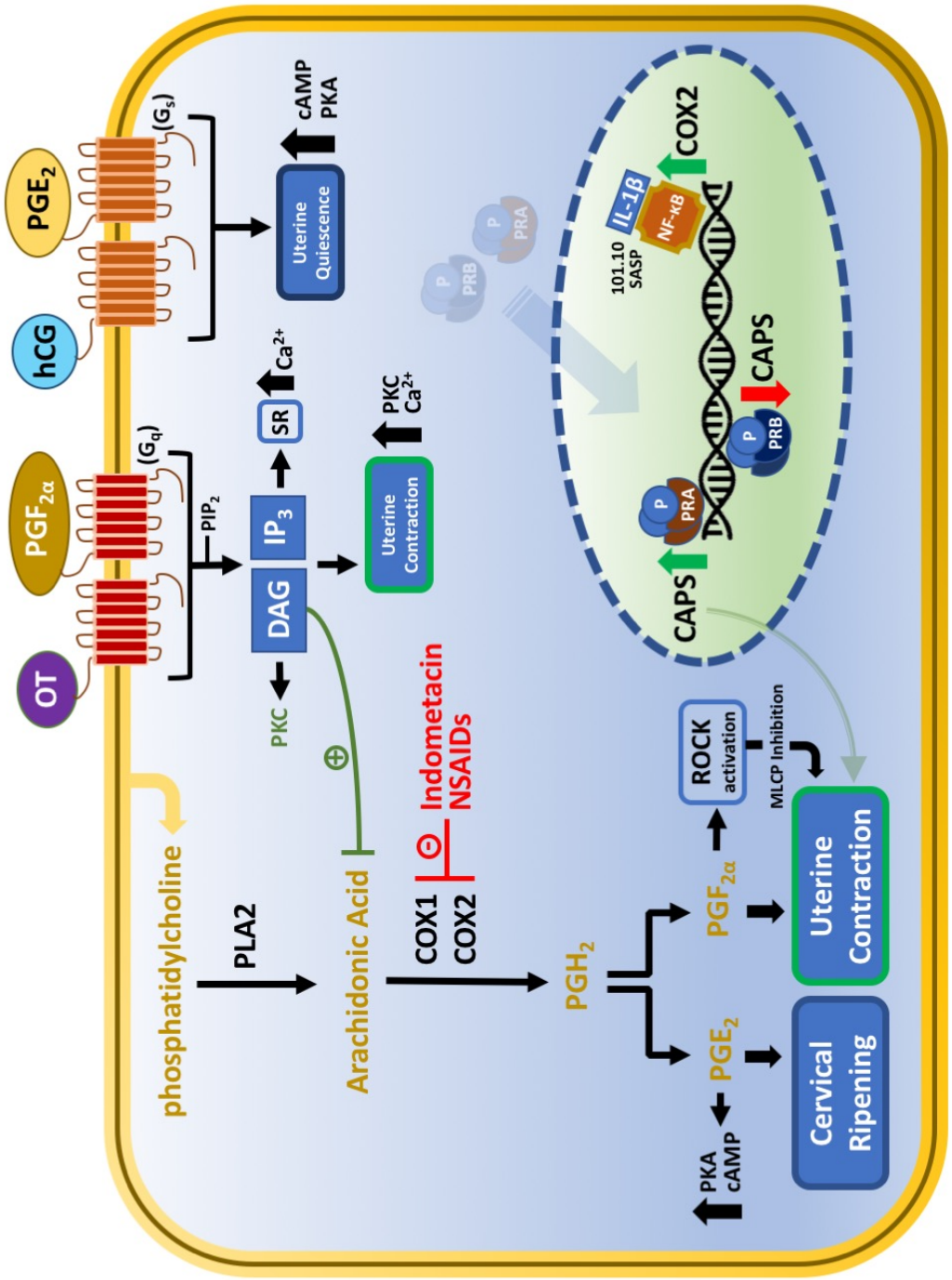

OT
PGF2α
hCG
PGE2
(Gq)
(Gs)
PIP2
DAG
IP3
SR
Ca2+
PKC
PKC
Ca2+
cAMP
PKA
Uterine Quiescence
Uterine Contraction
phosphatidylcholine
PLA2
Arachidonic Acid
COX1
COX2
Indometacin
NSAIDs
PGH2
PGF2α
PGE2
ROCK activation
MLCP Inhibition
Uterine Contraction
Cervical Ripening
PKA
cAMP
P
PRA
P
PRB
101.10
SASP
IL-1β
NF-κB
COX2
CAPS
CAPS
P
PRB
P
PRA

Figure 6: *Prostaglandin and hormone pathways during pregnancy:* Hormones and prostaglandins play an integral role in pregnancy and parturition. Oxytocin (OT) and prostaglandin F2-alpha (PGF2a) stimulate uterine contraction through GPCR (Gq) activation. Human chorionic gonadotropin (hCG), and the prostaglandin PGE2, conversely, quiesce the cell through GPCR (Gs) activation. Prostaglandin synthesis is drive by cyclooxygenase activity (COX), which converts arachidonic acid to prostaglandins. Prostaglandin synthesis is inhibited by NSAIDS. Progesterone is an important pregnancy maintenance hormone. PR-A and PR-B differentially regulate the expression of contractile-associated proteins (CAPS).

than 48-hours. More significantly, there are potentially serious side effects to the fetus when using Indomethacin. Premature closure of the patent ductus arteriosus, as well as renal complications, are known outcomes of Indomethacin administration (Moise, 1993), limiting its use. To avoid the adverse effects associated with COX1 inhibition (Reinebrant et al., 2015), researchers have also targeted the inducible isoform of cylcooxygenase, COX2, which is upregulated during labor (Loudon et al., 2003). Unfortunately, COX2 inhibitors have undesirable side effects as well, and a comment regarding their usefulness was aptly put forth by Vermillion and Landen in 2001 when they stated, “They [COX-2 Inhibitors] may join the long list of medications that inhibit uterine activity but are not clinically useful” (S.T. and C.N., 2001).

Oxytocin Receptor (OXTR) Antagonist: To date, the only drug designed specifically as a tocolytic is Atosiban, an oxytocin receptor (OXTR) antagonist. Atosiban, trade name Tractocile® (Ferring Pharmaceuticals), is a derivative of the peptide hormone, oxytocin (OT) (Williams and Pettibone, 1996). Early studies hinted at the potential of Atosiban as a tocolytic (Åkerlund et al., 1985; Coomarasamy et al., 2002); however, a systematic review of Atosiban in 2005 involving two trials with 651 women showed that Atosiban neither reduced the risk of preterm birth, nor improved neonatal outcomes (Papatsonis D, Flenady V, Cole S, 2005). Atosiban was withdrawn from a phase III clinical trial in 2015 (ClinicalTrials.gov Identifier: NCT01796522), but its use is still approved for use in

the European Union, Australia and New Zealand. It may not be entirely surprising that Atosiban is not highly effective as a tocolytic when we consider that OT null mice (-/-) have no deficits in fertility, reproduction, gestation or parturition (Nishimori et al., 1996). Moreover, blockade of the actions of OT do not completely relax the term laboring myometrium (I. L. Buxton et al., 2001). While there is no doubt that OT can initiate strong and regular contractions to induce labor (Erickson et al., 2017) and increase prostaglandin synthesis (Blanks and Thornton, 2003), it is possible that OT's function role in parturition is complimentary, not obligatory.

hCG and Progesterone: The hormones human chorionic gonadotropin (hCG) and progesterone are critical to implantation and pregnancy maintenance. Unlike luteinizing hormone (LH), which is a close analog to hCG (J G Pierce and T F Parsons, 1981) produced in the anterior pituitary of the mother, hCG is generated by the syncytiotrophoblast, and later the placenta, and stimulates progesterone production by the corpus luteum for the first 5-6 weeks of gestation (Norman and Litwack, 1997). hCG levels drop considerably after the placenta begins producing appreciable quantities of its own progesterone (Figure 2). Despite the diminished presence of hCG in the second and third trimester, it has been proposed that hCG may still be useful as a maintenance tocolytic. hCG receptors, which are GPCRs (G_s) and stimulate cAMP production and PKA activity, have been identified on the membrane of uterine smooth muscle cells (Kurtzman et al., 2001). In practice, while hCG administration is effective at reducing the peak force of contractions,

hCG paradoxically increases the frequency of contractions (Eta et al., 1994), and is therefore not a practical tocolytic.

Progesterone, on the other hand, due to its critical role in the maintenance of pregnancy, would appear to have obvious potential as a tocolytic. Progesterone is an important transcriptional modulator that inhibits the expression of many genes encoding contraction-associated proteins (CAP) during pregnancy (Figure 6). Because of this, progesterone serves as an obligate hormone for pregnancy maintenance and it increases in concentration continually throughout the pregnancy (Pepe and Albrecht, 2008). In the 3rd trimester placental production of progesterone peaks at 210 mg/day, or ~600 nM (Lin et al., 1972), a notably high concentration for any steroid hormone. Circulating progesterone below 10 ng/ml will result in abortion in 80% of women (Nygren et al., 1973). To this point, the drug mifepristone (RU486), a progesterone receptor antagonist, is used clinically to terminate pregnancies by inducing premature labor (Avrech et al., 1991).

The most obvious problem with using progesterone as a tocolytic is that humans, along with a handful of other mammals such as non-human primates and guinea pigs, do not experience an appreciable decrease in circulating progesterone until after the delivery of the placenta (Tulchinsky et al., 1972). The initiation of labor in many mammals, including mice and rats, is triggered by a fall in progesterone. This seemingly minor deviation from other mammals, called progesterone withdrawal, is an important differentiator that is often minimized. Therefore, methodology of studies touting progesterone as a maintenance

tocolytic must be analyzed closely to ensure their findings align with human studies.

Those who favor progesterone's use as tocolytic often point to the actions of progesterone on secondary pathways. For instance, it has been proposed that progesterone metabolites may act as a OXTR antagonist (S Thornton, V Terzidou, A Clark, 1999), which would enhance its role as a tocolytic, but other groups have refuted this finding (Astle et al., 2003). Also of consideration, is the fact that the two primary receptors of progesterone, PR-A and PR-B, serve contradictory roles during pregnancy. PR-A promotes labor by inhibiting the anti-inflammatory actions of PR-B, and the ratio of these two receptors can vary drastically in the myometrium during pregnancy (Tan et al., 2012). This may explain the numerous contradicting reports of progesterone's efficacy in treating preterm labor.

In general, studies using progesterone as a maintenance tocolytic show promise, but lack broad applicability. Notably, In 2011, the FDA approved Makena®, an injectable progestin "for use in women with a singleton pregnancy who have a history of singleton spontaneous preterm birth and a short cervix" (*NDA 21-945 Makena PI 3Feb2011Clean - FDA*, 2011). It should be noted that Makena's® approval was granted using the less stringent guidelines outlined in the Orphan Drug Act (Norwitz and Greenberg, 2011). Makena® holds the distinction as being the only FDA-approved tocolytic, although with the aforementioned stipulations.

Progesterone serves many important roles during pregnancy and it may contribute in unknown pathways to promote quiescence. Due to the breadth of potential targets of progesterone, it is difficult to predict all of the interactions that progesterone may regulate in the realm of uterine quiescence; therefore, more studies are needed to fully elucidate progesterone's potential as a tocolytic.

Nitric Oxide: It is well known that ·NO is the predominate canonical mediator of smooth muscle relaxation in the body. Because of this, its use to halt contractions is both logical and obvious. Therapeutically, ·NO is most generally administered using transdermal glyceryl trinitrate patches, as intravenous nitroglycerin, or an inhaled gas to treat bronchopulmonary dysplasia in preterm infants, although its efficacy in this sense unclear (Hasan et al., 2017). While some have reported success using nitroglycerin as a tocolytic (Shaikh et al., 2012), a comprehensive meta-review of twelve trials, including a total of 1227 women, concluded that ·NO does not prevent PTB (Duckitt et al., 2014). One of the problems with the systemic administration of ·NO donors is that the ·NO readily acts on all the smooth muscles of the body. More specifically, when used as a tocolytic, off-target effects are felt by both mother and child, and include: hypotension, increased heart rate, and intense headaches (Nankali et al., 2014). Furthermore, ·NO donors can amplify cervical ripening (Ghosh et al., 2016), a result that is contradictory to the desired outcome of delaying birth.

Taking the aforementioned information into consideration, it becomes clear that a systemically delivered therapeutic dose of ·NO exerts unacceptable side effects, and it is not an appropriate tocolytic. Additionally, and as will be later explained in detail, the sPTL myometrium exhibits a blunted response to ·NO, further limiting its effectiveness as a tocolytic. Of note, therapies that promote a global increase in plasma ·NO should not be confused with amplifying the cell's endogenous capability to modulate ·NO, as with what occurs with GSNOR inhibition or eNOS activation. ·NO is an important contributor to smooth muscle relaxation through both canonical cGMP-mediated pathways, and as proposed in this dissertation, through protein S-nitrosation.

Other Tocolytics: Tocolytics comprise a wide range of drugs that, regardless of their mechanisms of action, seek to delay PTB by halting or minimizing uterine contractions. To this point, there are many pathways, proteins, and enzymes that contribute to uterine contractions and quiescence outside of the common pathways described thus far.

One of the first drugs used as a tocolytic was ethanol (Haas et al., 2015), although it is unclear whether its popularity was due to its proposed efficacy, or because of its well-known calming and analgesic effects. There were several studies in the 1960s and 1970s that recommended ethanol's continued use as a tocolytic, including the discovery that ethanol negatively regulates OT release (Fuchs and Wagner, 1963; Gibbens and Chard, 1976); although more recent

studies have called into question the importance of OT in labor (Nishimori et al., 1996). It was also discovered that ethanol mutes the contractile response in isolated myometrial strips (Wilson et al., 1969), and that it slows the phasic wave-like propagation of depolarizations in smooth muscle (Subramanya et al., 2015). However, as with so many other tocolytics, a multitude of carefully controlled studies over the past 30 years leaves little doubt to ethanol's inadequacy as a tocolytic (Abel, 1981; Haas et al., 2015).

In recent years, a more thorough biochemical understanding of smooth muscle structure and function has led researchers to investigate phosphodiesterases (PDEs) as tocolytics. PDEs are important enzymes in smooth muscle that aid in the degradation of cyclic nucleotides, such as cAMP and cGMP. PDE inhibitors are an interesting target for the treatment of PTL as they can increase PKG and MLCP activity, and it has been proposed that PDEs may serve as effective tocolytic agents (Montorsi et al., 2004). The combination of nifedipine and sildenafil citrate, a PDE5 inhibitor commonly known as Viagra®, is in phase I clinical trials for the treatment of acute preterm labor (NCT02337881, 2016) in Saudi Arabia. However, it should be considered that of the many isozymes of PDEs, it is PDE4 rather than PDE5, that is the predominant form of the PDE in the myometrium near term (Méhats et al., 2007). Furthermore, and as will be expanded upon in the coming pages, our data indicates that this treatment will be ineffective due to myometrium's muted response to cGMP accumulation.

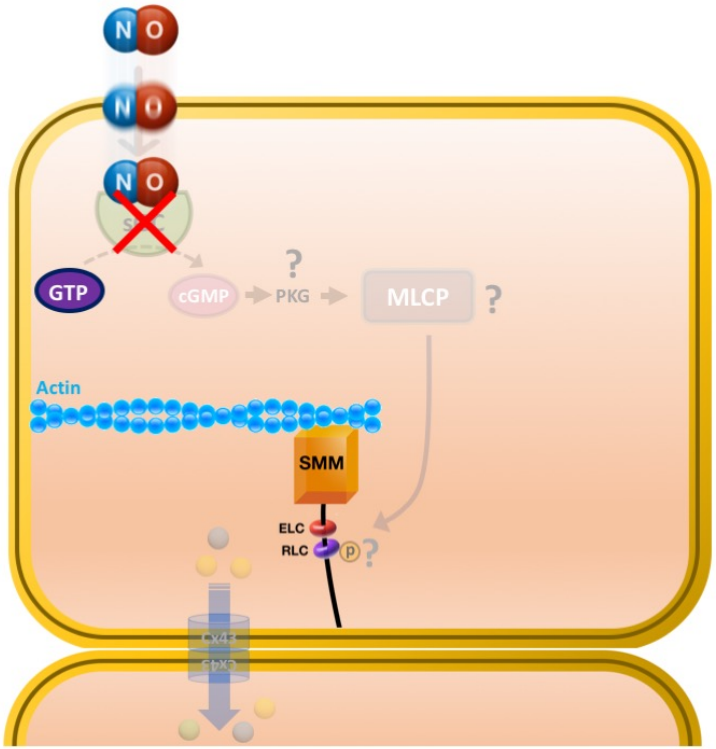
Diazepam, or Valium®, is a potent benzodiazepine known for its anxiolytic properties. It principally acts upon GABA receptors in the brain (Griffin et al., 2013); however, research has shown this drug has an affinity for many other targets. In smooth muscle diazepam relaxes the tissue through inhibitory effects on voltage-dependent Ca2+ channels (Kim et al., 2000; Yamakage et al., 1999). In rat uterus, diazepam has been shown to inhibit α1-adrenergic receptors, promoting relaxation (Zupkó et al., 2003). In chapters 2 and 5, the importance of K^+ in uterine quiescence, through TREK-1 activity, will be addressed.

Lastly, we consider the inhibition of the Rho-associated kinases, ROK-1 and ROK-2. Rho-kinases, or ROKS, are important mediators of MLCP function (Figure 3a) (Mitchell et al., 2013). ROKs phosphorylate MYPT1 (T696 & T853), inhibiting MYPT1's primarily catalytic function of dephosphorylating MYL9 (Puetz et al., 2009). This facilitates the smooth muscle's ability to maintain contraction in the absence of continued Ca^{2+} influx (calcium sensitization). Early research has indicated that inhibiting ROK with either AS1892802, or fasudil, a drug used in the treatment of cerebral vasospasm, decreases contractile force in the myometrium (Ergul et al., 2016). While there is no clinical data available to evaluate the efficacy of ROK inhibition as a tocolytic, this approach may be of importance in the future.

Interestingly, there is a dearth of clinically available pharmacological inhibitors that target two critical proteins critical to smooth muscle contraction, CaM and MLCK. If either these proteins were prevented from acting upon their targets, MYL9 phosphorylation would cease, and the myometrium would relax. While

·NO Still Relaxes Uterine Smooth Muscle In the Absence of cGMP Accumulation

a)



Protein S-nitrosation Provides an Alternative Mechanism for Relaxation

b)

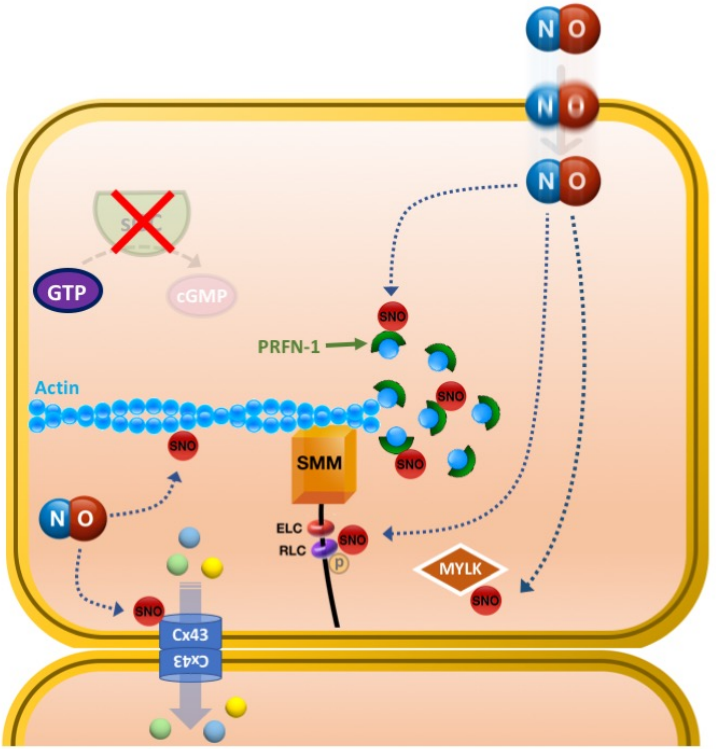


Figure 7: *S-nitrosation as an alternative pathway to myometrial quiescence*: (a) Nitric oxide relaxes myometrial tissue independently of cGMP accumulation. (b) We hypothesize the S-nitrosation of important Contractile-Associated Proteins (CAPS) by nitric oxide may contribute to the cGMP-independent relaxation of the tissue.

inhibitors of these proteins exist (Eikemo et al., 2016; Sakurada et al., 2003), it is possible that a major hurdle to their use as tocolytics is the ability to localize their administration to the myometrium, as systemic administration of these inhibitors would most likely have catastrophic effects on other classes of smooth muscle. However, new methods that target drugs to specific tissue types, such as with oxytocin receptor-targeted liposomes which deliver drugs largely to the myometrium (Paul et al., 2017), may provide new opportunities to test these classes of drugs.

Clearly, over the past century there has been an enormous effort to identify, develop, and test a multitude of tocolytics. While it is unfortunate that currently available therapeutics are unable able to delay PTB by more than 48-hours, this shortcoming does shed light on the reality that there is something fundamental to the process of pregnancy and parturition that remains obfuscated. We believe that our research may shed light on this enigma. As will be discussed in detail, our findings reveal that the myometrium has a unique relationship with cGMP and ·NO when compared to other types of smooth muscle (Figure 7). We seek to better understand this relationship and to identify tocolytics that leverage this important distinction.

A New Path Forward:

Three key pieces of information regarding the myometrium and sPTL set the stage for the research described in this dissertation: (1) We do not know what causes idiopathic early onset labor (sPTL); (2) tocolytics are ineffective at delaying PTB beyond 48-hours; and (3) the myometrium responds uniquely to ·NO. It is here that we attempt to lift the veil on these unanswered questions.

It has been well documented that ·NO is not suitable as a tocolytic. While it does relax the term myometrium, systemically administered ·NO produces unacceptable side effects. That does not mean that ·NO is not critically important to natural relaxation of the myometrium. The metabolism of ·NO is regulated by several enzymes in the cell, such as the thioredoxin system (Sengupta and Holmgren, 2012a, 2012b), and carbonyl reductase (Bateman et al., 2008), but the predominant mediator of ·NO availability in the cell is the enzyme S-nitrosoglutathione reductase, or GSNOR (Hou et al., 2011).

GSNOR is a potent negative regulator of S-nitrosoglutathione (GSNO) (Figure 8), a common endogenous form of ·NO in smooth muscle (Que et al., 2009). The aberrant expression of *ADH5*, the gene that encodes GSNOR, is associated with many diseases (S D Barnett and Buxton, 2017; Jelski et al., 2009; Jelski and Szmitkowski, 2008; Laniewska-Dunaj et al., 2013). In fact, deletion of the *ADH5* gene increases both the levels of GSNO and total protein S-nitrosation in vivo (Liu et al., 2001a). GSNOR, like other cysteine containing proteins, can be S-nitrosated by GSNO, which creates a feedback loop that affects GSNOR expression (Guerra et al., 2016) and activity (Brown-Steinke et al., 2010) .

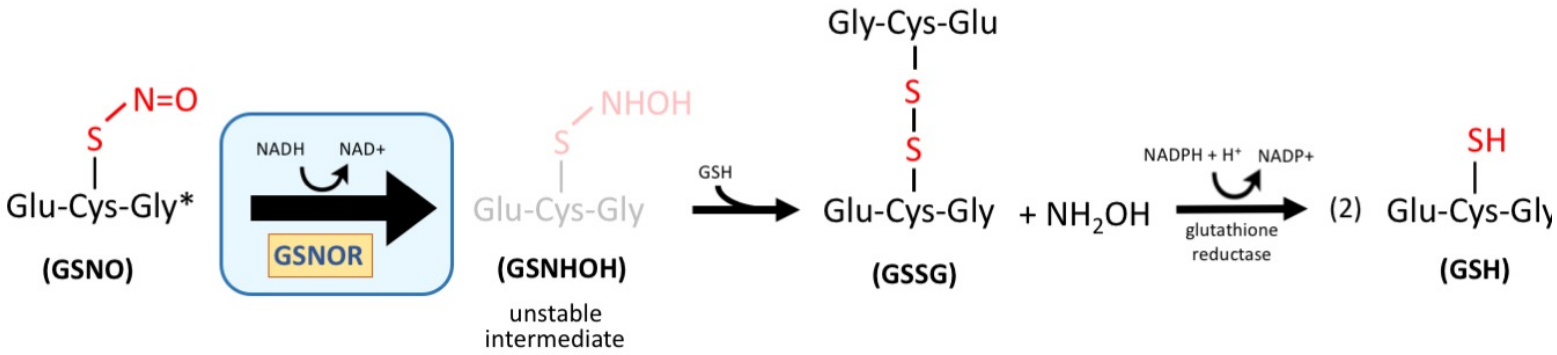

N=O
S
Glu-Cys-Gly*
(GSNO)
NADH
NAD+
GSNOR
NHOH
S
Glu-Cys-Gly
(GSNHOH)
unstable intermediate
GSH
Gly-Cys-Glu
S
S
Glu-Cys-Gly
(GSSG)
+ NH2OH
NADPH + H+
NADP+
glutathione reductase
(2)
SH
Glu-Cys-Gly
(GSH)

Figure 8: *Metabolism of S-nitrosoglutathione by S-nitrosoglutathione reductase*: GSNOR metabolizes multiple substrates. S-nitrosoglutathione (GSNO), one of the primary substrates for GSNOR, is first enzymatically degraded to an unstable intermediate, N-hydroxysulfinamide (GSNHOH). In the presence of additional glutathione (GSH) GSNHOH will be converted to glutathione disulfide (GSSG), then back to GSH (Scott D. Barnett and Buxton, 2017b).

As will be discussed in much greater detail, we hypothesize that the dysregulation of GSNOR in sPTL tissue precipitates contractions through the excess metabolism of endogenous ·NO.

There are very few tocolytics that target relaxation specific pathways in smooth muscle. The inhibition of GSNOR functionally increases the global amount of available ·NO in the cell, making it an attractive therapeutic target. GSNOR inhibitors are currently being tested to treat asthma, cystic fibrosis and interstitial lung disease, and we are testing GSNOR inhibition as tocolytic.

S-nitrosation:

At the core of this research is an attempt to better understand how the myometrium differs from other smooth muscles. Tocolytics are ineffective at extending birth beyond 48-hours; therefore, it is important that we better comprehend the mechanistic underpinnings of myometrial physiology so that we can develop better therapies to treat PTL.

As previously mentioned, ·NO is highly reactive. As such, it often forms a stable intermediate with glutathione (GSH), a modified tripeptide of Glu-Cys-Gly, called S-nitrosoglutathione (GSNO) (Williams, 1999). Glutathione is one of the

most highly expressed peptides in the cell, often in the high mM range (Lushchak, 2012), and GSH is critically important to the redox system.

In the same manner as GSNO formation from GSH and ·NO, the addition of the •NO moiety to a cysteine thiol of a protein is called S-nitrosation (Figure 4). The effects of this posttranslational modification (PTM) on cell signaling and its role in disease have reverberated throughout the medical community for over 25 years (Broniowska and Hogg, 2012a; Foster et al., 2009a; Stamler et al., 1992a). A unique characteristic of protein S-nitrosation is the lability of this PTM, partially due to the fact that S-nitrosation and de-S-nitrosation of proteins is not enzymatically driven. In fact, the ·NO moiety can easily be transferred to a cysteine on another protein, as with GSNO mediated S-nitrosation, through a process called trans-S-nitrosation (Broillet, 1999). It is intracellular availability of nitric oxide and its functional derivatives, like GSNO, that enable protein S-nitrosation (Broniowska and Hogg, 2012a; Hess et al., 2005a; Thomas and Jourd'heuil, 2012a). To this point, there is a direct stoichiometric relationship between intracellular ·NO concentrations and total protein S-nitrosation (Katarzyna A Broniowska et al., 2013; Broniowska and Hogg, 2012b; Hess et al., 2005b; Thomas and Jourd'heuil, 2012b). Protein S-nitrosation is of intense interest to researchers and clinicians as the hypo/hyper-S-nitrosation of a diverse set of proteins, spanning nearly every tissue type, can have drastic effects on disease states (Foster et al., 2009a)(Barnett, et. al., 2017). Some of these include: Type 2 diabetes (Akt et al., 2005), sickle cell anemia (Bonaventura et al., 2002, 1999), ventricular arrhythmia

in individuals with Duchenne muscular dystrophy (Fauconnier et al., 2010), cell death and survival pathways (Anand Krishnan V. Iyera, Yon Rojansakulb, 2011), post-infarct cardio-protection (Methner et al., 2014), and preterm labor (Ulrich et al., 2013b), among others.

Protein S-nitrosation is highly regulated during pregnancy and parturition. Our laboratory has discovered that proteins are differentially S-nitrosated based on the state of labor in women (Ulrich et al., 2013b). When GSNO is applied to the lysates of PTL and TL tissue, many proteins important to contraction, such as MYL9, telokin, actin, and profilin-1, are either up-S-nitrosated or down-S-nitrosated (Figure 7b). This is an unexpected observation that suggests something endogenous to the cell is mediating the differential S-nitrosation of proteins between the two labor states. Whether these changes are due to conformational changes to the protein, or perhaps some other mitigating factor, is yet to be determined.

One of the principal inquiries addressed in this dissertation is whether or not protein S-nitrosation alters protein function. It is one thing to know that proteins are differentially S-nitrosated based on the state of labor (Ulrich et al., 2013c), but it is entirely another to determine if those S-nitrosations are functionally relevant. We know that the myometrium relaxes independently of cGMP accumulation (Figure 7a) (Bradley et al., 1998a; I. L. O. Buxton, 2004; Tichenor et al., 2003). The question remains as to what other roles, complementary or otherwise, ·NO plays during pregnancy and parturition? We propose that protein S-nitrosation imparts

a functional effect on important CAP proteins, and provide evidence to support this hypothesis.

Conclusion

There is little doubt that sPTL is a complex multifactorial disorder. The cause(s) of sPTL most likely include genetic, environmental, and social factors. When developing therapies to treat sPTL we must not forget that the broader goal of tocolytics are not only to prevent preterm labor, but more importantly, to delay or prevent preterm birth. Infant mortality and morbidity decreases precipitously as the third trimester progresses, (H.C. et al., 2015); however, an extensive analysis of extreme preterm births (22-28 wks) by the National Institute of Child Health and Human Development found that while 92% of infants survive if delivered by week 28, 93% of them had serious and life-threatening health complications (Stoll et al., 2010). These data amplify the need for a new class of tocolytics that not only mitigate contractions, but extend the time to birth beyond the current limit of 48-hours.

Over the past century researchers and physicians have labored extensively to identity tocolytics that target the many pathways that drive labor, yet there are still unexplored avenues to investigate. Excluding beta-agonists, there are an exceedingly small number of therapeutics that bolster the cell's active "relaxation" response, rather than just prevent contraction. This is not in a pedantic distinction.

Inhibiting smooth muscle contraction (i.e. VDCC, $G\alpha_{q/11}$, etc.) is quite different functionally from promoting relaxation (i.e. MLCP, CPI-17, telokin, etc.). As evidenced in the previous pages, currently available tocolytics that inhibit contractions leave much to be desired, therefore new approaches should be investigated.

Our research seeks to leverage the myometrium's unique response to ·NO to identify novel tocolytics. This, coupled with an ever-increasing understanding of how our collective and individual genomes affect pregnancy and parturition, will increase the likelihood of developing new therapeutics to delay PTB. The need for more capable data analysis is further highlighted by the fact that the 'pregnancy system' includes two or more sets of genomes that interact and influence both mother and child. Fortunately, new tools are coming online. IBM's Watson®, which interprets complex health issues by analyzing the treatments and outcomes of millions of patients, is identifying new ways to diagnose and treat diseases in ways previously beyond our capabilities (Brief, 2015; Chen et al., 2016). This, coupled with the use of artificial intelligence (Dilsizian and Siegel, 2014) and better analysis of 'big data' aggregated from massive data sets (i.e. GWAS, RNA-seq, eipigenomics) (Taglang and Jackson, 2016), has huge potential to change the way we identify and treat women at risk for sPTL.

Preterm labor, and by extension preterm birth, are the byproducts of an exceedingly intricate, and poorly understood system of converging pathways that stem from both mother and child. The fruit of 100 years of evidence-based

research has only allowed us to delay the birth of an infant by 48-hours in women who enter labor early. Few would question the need to develop a better understanding of the basic mechanisms of pregnancy and parturition in order to develop new, more effective, therapies to treat this disorder. The research contained in this dissertation seeks to extend our knowledge of preterm labor, however incrementally, by investigating the myometrium's unique response to nitric oxide to identify novel tocolytics to treat preterm labor.

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Chapter 2

The Distinctly Different Myometrium

Abstract:

Spontaneous preterm labor is a pandemic issue with no obvious solution. In order to develop novel tocolytics to stop labor, and prevent preterm birth, we must better understand the mechanisms that drive uterine quiescence. This research characterizes the relevance cGMP signaling in the myometrium through the actions of soluble guanylyl cyclase (sGC) and particulate guanylyl cyclase (pGC-C), and the electrophysiological characteristics of the outward rectifying potassium channel, TREK-1, with and without co-expression of its five splice variants. We have determined that pGC-C, which is activated by uroguanylin, and not nitric oxide, relaxes pregnant myometrium, but not non-pregnant, presumably through compartmentalized cGMP action. Conversely, sGC activation by BAY58-2667, or the addition of 8-bromo-cGMP, does not relax myometrial tissue. The expression of TREK-1 splice variants found in preterm myometrium decreases TREK-1 trafficking to the membrane, indicating dysregulation of this system in preterm tissue. Taken together, these data provide further insight into the underpinning of myometrial function, and open the door to the identification of therapeutics that are specifically targeted to the myometrium.

Introduction:

The uterus is responsible for maintaining an environment nurturing to fetal development and survival over the 40 weeks of gestation. The perpetuation of our species would not be possible, in part, had we not developed mechanisms that were largely affective at maintaining uterine quiescence prior to term. As such, the myometrium has evolved into phenotypically unique tissue (Buxton et al., 2011b; I. L. O. Buxton, 2004; Chad L Cowles et al., 2015). An obvious, and relevant, example of this concept can be seen by examining the myometrial response to oxytocin, which binds to oxytocin receptors to drive contraction (Blanks and Thornton, 2003). Outside the reproductive complex, oxytocin plays a limited role in muscle contraction (Gimpl and Fahrenholz, 2001). Beyond oxytocin-mediated contractions, there exist an extraordinary number of factors that dictate whether or not the myometrium will contract or remain quiescent. Prostaglandins, cytokines, hormones, protein expression and associated posttranslational modifications, kinases and phosphatases, membrane polarization, and more (Challis et al., 2009; Chwalisz and Garfield, 1997; Elvira et al., 2014), each contribute with differing degrees of influence to unbalance the teeter-totter of contraction and relaxation. Here we discuss our findings surrounding two distinct systems in the myometrium that are important to uterine quiescence: The compartmentalization of the cyclic nucleotide cGMP, and the outward rectifying potassium channel, TREK-1 (**T**WIK-**Re**lated **K**+ Channel 1).

cGMP in the Myometrium: Like cAMP, cGMP is an important second messanger responsible for a variety of actions across most cell types (Lucas et al., 2000). cGMP wasn't formally discovered until 1963 (Ashman et al., 1963), but its effects, like other cyclic nucleotides (Blumenthal, 2012), have long been observed. cGMP is generated by the guanylyl cyclase (GC) family of isozymes, and they are degraded by phosphodiesterases (Francis et al., 2010). In smooth muscle, cGMP activates PKG, a kinase with many targets, that include MYPT1, the catalytic subunit of myosin light chain phosphatase (Schmidt et al., 1993). There are two primarily expressed isoforms of GC in the myometrium; soluble (sGC), and particulate type C (pGC-C) (Telfer et al., 2001). As its name implies, sGC exists as a free enzyme in the cytosol. Alternatively, pGC-C is a membrane bound enzyme (Hardman and Sutherland, 1969). Both sGC and pGC-C catalyze the formation of cGMP from GTP, but the ligands that stimulate each enzyme are very different. sGC is principally activated by ·NO, while pGC binds uroguanylin (uGN) (Lucas et al., 2000). ·NO is created by the family of nitric oxide synthases (NOSs), and ·NO is known to activate sGC in smooth muscle (Kuhn, 2016). uGN, on the other hand, is a 16-amino acid peptide excreted by many cell types, binds to an extracellular domain on pGC-C, and is upregulated in rat myometrium (Girotti and Zingg, 2003). pGC-C has long been known to play a crucial role in intestinal and kidney function (Kuhn, 2016), and we propose that it may be important to uterine physiology as well.

A logical question is whether or not these disparately dispersed enzymes, both of which generate the same product, exert a differential influence in the myometrium (Figure 1)? sGC activation increases global intracellular concentrations of cGMP and is known to activate PKG type I (Salvador Moncada, 1994), a kinase that is downregulated during pregnancy (Word and Cornwell, 1998). The canonical relaxation of smooth muscle by ·NO is traditionally thought to be driven by sGC activation, yet we have previously shown that inhibition of sGC by LY-83583 (Bradley et al., 1998b) or 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), does not prevent NO-mediated relaxation in the myometrium (I. L. O. Buxton et al., 2001). Conversely, by artificially increasing global cGMP levels in the myometrial tissue, without sGC activation, through the addition of the cell permeable cGMP analog, 8-bromo-cGMP, contractile dynamics are not altered (Bradley et al., 1998b). This finding highlights an important peculiarity of the myometrium and questions the role of sGC and cGMP in myometrial relaxation. One of the unanswered questions we further investigate here is whether or not sGC activation, rather than its inhibition, plays any role in myometrial relaxation.

Apart from sGC, the other major generator of cGMP in the myometrium is the aforementioned pGC-C, the membrane-bound isoform of GC. In 2004, Dr. Iain Buxton proposed the idea that cGMP signaling in the myometrium may be compartmented (I. L. O. Buxton, 2004). The importance of cAMP and cGMP compartmentalization has been well documented (Houslay, 2010; Maurice et al., 2014). Both PKG type II (Schlossmann et al., 2005) and pGC-C are membrane-

Effects of global vs. local cGMP levels in the myometrium

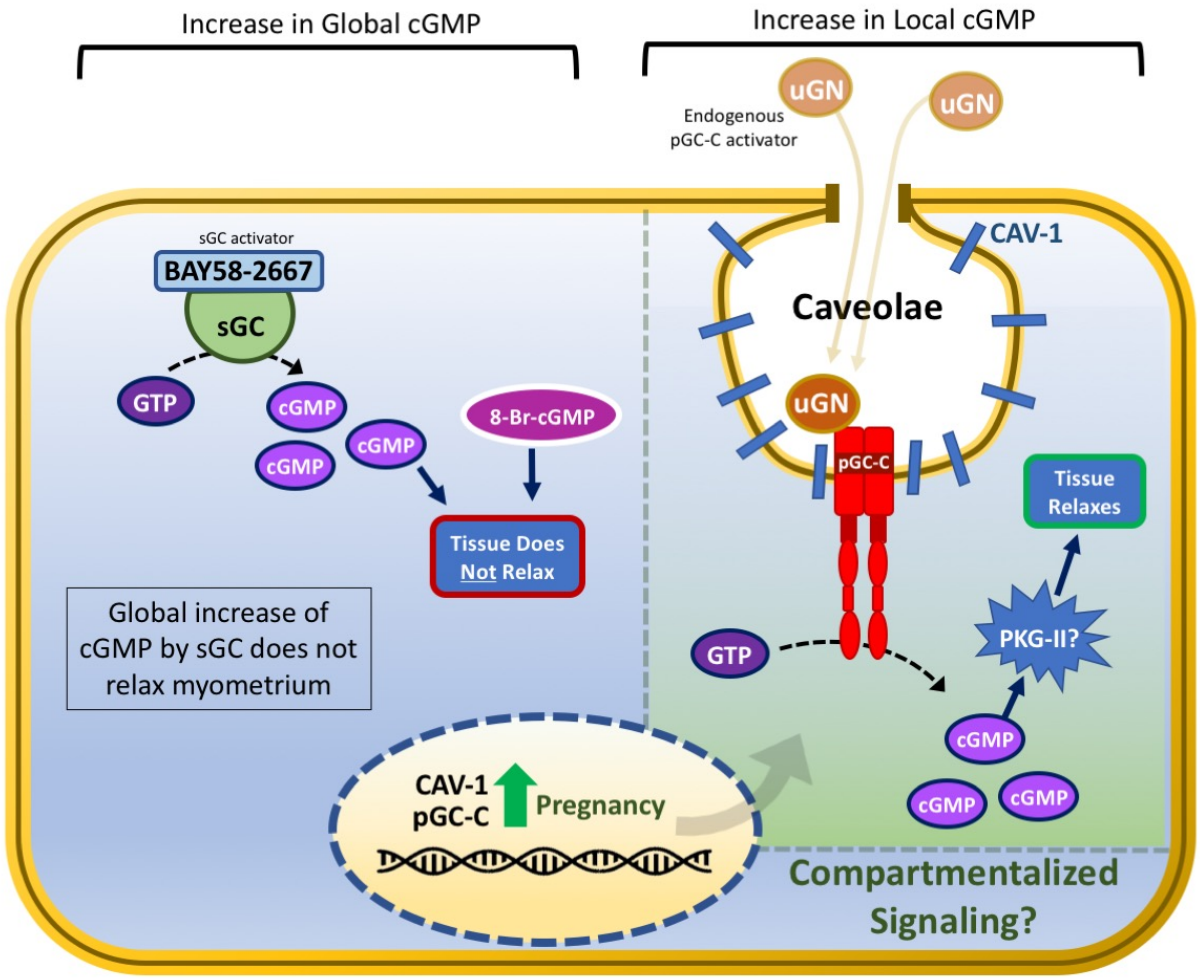


Figure 1 *Global cGMP elevation does not relax myometrium where localized elevation does*: Compartmentalized actions of cGMP in the Myometrium. In the myometrium, the cyclic nucleotide cGMP can be generated by either soluble guanylyl cyclase (sGC), or by particulate guanylyl cyclase type C (pGC-C). A global increase in cGMP by sGC stimulation with BAY58-2667 does not relax the tissue. On the other hand, pGC-C activated by uroguanylin (uGN) does relax pregnant myometrium. pGC-C is known to form complexes in cholesterol-rich caveolae. Both pGC-c and CAV-1, and important structural component of caveolae, are upregulated during pregnancy. It may be this localization with other mediators of relaxation, such as PKG-II that promotes relaxation of the tissue.

bound (Iain L O Buxton et al., 2010a; Schlossmann et al., 2005), and in epithelial cells PKG-II and pGC-C work in concert to increase ion flow across the membrane (Forte et al., 2000; Vaandrager, 2002). A reasonable question is to ask whether or not the actions of pGC-C are relevant to myometrial quiescence? Based on previous findings that the signaling domain of pGC-C relies on cholesterol (Buxton and Vittori, 2005; Zhang et al., 2007), we propose that pGC-C signaling may be heightened when in proximity to caveolae lipid rafts. We have shown that both CAV-1, an important structural protein of caveolae, and pGC-C, are upregulated during pregnancy (Iain L O Buxton et al., 2010b), further bolstering this hypothesis. Here we investigate whether cGMP generated by pGC-C relaxes myometrial tissue where sGC generated cGMP cannot.

TREK-1 and Myometrial Function: Another critical mediator of contractile dynamics in the uterus is the myometrial membrane potential. Like all muscles, a negative membrane potential is required to maintain uterine quiescence. The depolarization of the membrane is the result of an influx of positively charged ions, including Ca^{2+}, that drives contraction. Generally speaking, the resting potential of the myometrium in a non-pregnant woman is between -40 mV and -50 mV (Aguilar and Mitchell, 2010). By mid-pregnancy this drops to as low as -60 mV, before returning to approximately -45 mV near term (Parkington and Coleman, 1990; Patel and Honore, 1998). Clearly there are mechanisms at play that ensure a robust negative membrane potential during the critical phases of fetal development

prior to the onset of parturition. One such mediator of myometrial negative membrane potential is TREK-1.

TREK-1 is a unique 4TMS/2P member of the KCNK family of potassium permeable channels that regulate the myometrial cell membrane potential by shuttling K^+ ions to the extracellular environment (Lotshaw, 2007). Each K2P channel subunit is made up of four transmembrane segments and two pore-forming domains, which are arranged in tandem, and function as either homo or heterodimeric channels (A Dedman et al., 2009). KCNK channels were not discovered until the mid-1990s (Fink et al., 1996), so in a modern biochemical sense, these channels are quite new, and there is no doubt more to be learned about their function. TREK-1 exist alongside the inward rectifiers and the voltage and/or calcium-dependent K^+ channels. Potassium channels, in general, play an important role in smooth muscle contraction and quiescence (Nelson and Quayle, 1995). An important distinguisher of TREK-1 when compared to other potassium channels, such as TASK-1 and TASK-3, which are constitutively active “leak” channels (Goldstein et al., 2001), is that TREK-1 requires stimulation before permitting ion flow (A Dedman et al., 2009). Both the C-terminus and N-terminus of TREK-1 are located intracellularly and TREK-1 can be activated by many stimuli, to include: phosphorylation of S333 on the C-terminal domain by PKA, S351 phosphorylation by PKG, unsaturated fatty acids such as arachidonic acid, stretch (important as the uterus expands during pregnancy), intracellular acidification at

E306 (Patel and Honore, 1998), and potentially PTMs such as glutathionylation and S-nitrosation (see chapter 5) (Buxton et al., 2011a).

As one might expect, there are many ion channels important to pregnancy in the myometrium (Sanborn, 1995). TREK-1 has long been known to be important as a modulator of vascular tone (Mongahan et al., 2011) and neuronal activity (Alexandra Dedman et al., 2009), and we have shown that TREK-1 expression increases during gestation in the myometrium, then trends towards baseline as the pregnancy nears term (I L O Buxton et al., 2010c). Even more compelling is that TREK-1 is downregulated in in preterm laboring myometrium as compared to term laboring and term non-laboring tissue, making it an important ion channel to investigate (I L O Buxton et al., 2010c).

The significance of TREK-1 in the myometrium during pregnancy is further amplified when we consider the possibility that the presence of TREK-1 splice variants (SVs) may affect trafficking and function of the channel. SVs are formed in eukaryotic organisms by the combination of multiple alternatively spliced exons, allowing for a much greater diversity of expressed proteins in the cell (Modrek and Lee, 2002; Pan et al., 2008). Previously, our laboratory identified the transcripts of five unique SVs of TREK-1 in myometrial samples (Wu et al., 2012). Each variant either lacks either pore domains, transmembrane domains, or both, and these variants are known to co-exist with wild-type TREK-1 (wtTREK-1), potentially altering trafficking and cellular distribution.

The recent identification of TREK-1 SVs, coupled with TREK-1's relevance to myometrial quiescence during gestation, confers a significant role for TREK-1 as an important mediator of contractile dynamics. Here, for the first time, we investigate the physiological characteristics of TREK-1 in the myometrium, and we determine the impact on channel activity when TREK-1 SVs are co-expressed with wtTREK-1.

Note: A portion of these Methods and Results are from (I L O Buxton et al., 2010a; C. L. Cowles et al., 2015; N S Heyman et al., 2013)*, for which I was a co-author. Permission to use is contained in Appendix C*

Material and Methods:

Primary culture pregnant human uterine smooth muscle cells: With informed consent obtained in writing and approved by the University of Nevada, Reno Biomedical Institutional Review Board, samples of term pregnant (non-laboring) uterine tissue from the superior aspect of the incision were obtained from women undergoing Cesarean section. Women were selected based on the surgery schedule when a clinical decision was made to deliver a normal term pregnancy by Caesarian section. Exclusion criteria were age less than 21 years, multiple pregnancies, known illicit drug use, or HIV, or hepatitis C infection. Within 20 min of their removal, fresh tissue samples were transported to the laboratory in cold

physiological buffer containing (in mM): NaCl (120), KCl (5), KH_2PO_4 (0.587), Na_2HPO_4 (0.589), $MgCl_2$ (2.5), Dextrose (20), $CaCl_2$ (2.5), Tris (25), and $NaHCO_3$ (5), adjusted to pH 7.4. Uterine smooth muscle (myometrium) was dissected under the microscope from tissue samples and prepared as thin strips (2 x 2 x 8 mm) devoid of blood vessels in a modified Krebs'-HEPES buffered solution (in mM: 118 NaCl, 4.75 KCl, 1.2 KH_2PO_4, 0.25 $NaHCO_3$, 1.2 $MgSO_4$, 20 dextrose, 25 Na HEPES, pH 7.4). Minced tissue underwent three 30 min incubation periods in a 50 ml conical tube containing 2 mg/ml collagenase type II and 1 mg/ml trypsin at 37^{o}C. Each successive supernatant was collected in growth media with 10% fetal bovine serum (FBS), antibiotics (penicillin, 60 μ g/ml; streptomycin, 100 μg/ml; fungizone, 5 μ g/ml), pH 7.4, with trypsin inhibitor (1 mg/ml). Cells were centrifuged at 400g, resuspended in Dulbecco's growth media containing 10% steroid free FBS (FBS_{SF}) with estrogen 15 ng/ml - progesterone 200 ng/ml to mimic third trimester human pregnant plasma concentrations and plated on tissue culture dishes. For freshly isolated patch clamp studies, the cells were immediately plated on glass cover slips coated with Type 1 collagen from rat tail (Sigma) and employed in electrophysiological experiments within 8-12 hours. Other cells were grown through passage 5 in primary culture and were combined in equal numbers from three Caucasian donors at term (24-29 yr) and telomerized to establish a pregnant myometrial cell model.

Tissue collection for sGC contractile studies. Human tissue collection and research was approved by the University of Nevada Biomedical Review

Committee for the protection of human subjects. Human uterine myometrial biopsies were obtained with written informed-consent from mothers undergoing Cesarean section without infection or rupture of membranes. Tissues were transported to the laboratory immediately in cold Krebs buffer containing: NaCl (118mM), KCl (4.75mM), $CaCl_2$ (2.5mM), KH_2PO_4 (1.2mM), $NaHCO_3$ (25mM), $MgCl_2$ (1.2mM), dextrose (20mM), and adjusted to pH 7.4. Tissues were microdissected under magnification to isolate smooth muscle, employed in contractile experiments or snap frozen in liquid nitrogen, and stored at -150°C. The average age for patients in the pregnant laboring group was 28.9 ± 5.6 yr and in the preterm laboring group 30.8 ± 10.2 yr. Pregnant laboring patients ranged from 39 to 41 wk gestation, with the mean at 39 wk. Preterm laboring patients without evidence of infection, PROM or preeclampsia ranged from 29.2 to 36 wk of gestation, with the mean being 33.5 wk. Patients represented a range of ethnicities and were 52% Caucasian, 30% Hispanic, 7.4% African American, and 11% other. Animal studies were approved by the University Institutional Animal Care and Use Committee. Dunkin-Hartley Guinea pigs (Elm Hill, Chelmsford, MA) were purchased as either virgin juveniles (300-350g) and bred on site, or as timed-pregnancies (30-35d). Non-pregnant guinea pigs were estrogen primed (3mg/kg β-estradiol) 48-hours prior to tissue collection to ensure alignment of estrous cycles. Virgin female guinea pigs and timed-pregnant animals were sacrificed under isoflurane anesthesia. Uterine tissue was dissected and used immediately as previously described (Iain L O Buxton et al., 2010b).

Contractile studies: Virgin female guinea pigs or timed pregnant animals at different stages of gestation were sacrificed under isofluorane anesthesia according to an Institutional Animal Care and Use Committee-approved protocol. Virgin animals were estrogen-primed with 1 mg/kg of estradiol-17 in corn oil (0.5 ml) injected subcutaneously each day for 2 days, and the animals were sacrificed on day 3. Estrogen treatment has the effect of bringing animals into estrus so that control comparisons are uniform. Uterine horns were removed and placed in HEPES-buffered Krebs' solution without Ca^{2+}, containing 118 mM NaCl, 4.75 mM KCl, 1.2 mM KH_2PO_4 , 0.25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 20 mM dextrose, and 25 mM Na HEPES, pH 7.4. Tissues were opened longitudinally in a dissecting dish. For pregnant samples, fetuses were removed along with their placenta, and regions of uterus between placentas in the upper third of the horn were dissected for contractile experiments. Muscle was dissected away from the decidua (DEC), and the myometrium was cut to 4-mm strips and mounted between a fixed point and a force transducer (Kent Scientific, Torrington, CT) in 5 ml of tissue baths filled with Kreb's buffer (118 mM NaCl, 4.75 mM KCl, 1.8 mM $CaCl_2$, 1.20 mM KH2PO_4, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, and 20 mM glucose, pH 7.4). Transducer voltages were amplified and converted to digital signals by an analog-to-digital board mounted within a computer system running the DaisyLab 10 data acquisition system (I/O Tech, Norton, MA). Strips were maintained at 37°C, aerated with 95% O2/5% CO2, and loaded with initial tensions of 1.2-g force as described previously (Kuenzli et al., 1996). During the course of a 1-h equilibration period, tissues were

routinely challenged first with high potassium (60 mM KCl) and subsequently with 100 nM oxytocin (OT) followed by washout. Tissues were allowed to equilibrate for 1 h before experiments. Uroguanylin (Sigma-Aldrich, St. Louis, MO) was added to organ baths (1:1000) from a concentrated stock solution made daily and protected from light. The effects on both spontaneous and OT-stimulated tissues were studied over 15 min of addition followed by washout and were quantified as area under the force curve for 15 min before treatment and 15 min after treatment. Additions of ascorbate (blank) or peptide were blinded from the experimenter to serve as control. The putative pGC-C inhibitor 2-chloro-ATP (2Cl-ATP) was dissolved in Kreb's buffer and added to the tissue bath from a 1:1000 working stock. 1H-[1,2,4]ox-adiazolo[4,3-a]quinoxalin-1-one (ODQ) was dissolved in dimethyl sulfoxide and diluted 1:10,000 from a working stock. Isatin (1H-indole-2,3-dione; (Frolova V.Kh.; Biyushkin,V.N.; Chumakov,Yu.M.; Belkova,O.N.; Malinovskii,T.I., 1988)) was dissolved in hot ethanol (70°C) and diluted 1:100,000 from a working stock. Dimethyl sulfoxide at a final concentration of 0.01% or ethanol at 0.001% did not have any effect on contraction.

Patch clamp wtTREK-1: Cells were plated on glass coverslips 4-48 hours before experiments, placed in a chamber for recording mounted on top of an inverted microscope and currents typically were recorded in the standard whole-cell variant of the patch clamp technique using pCLAMP software (V9.2; Axon Instruments/Molecular Devices Inc; Sunnyvale, CA). Currents were amplified with an Axopatch200B amplifier (Axon Instruments/Molecular Devices Inc.; Sunnyvale,

CA), digitized using a computer interfaced with a Digidata 1322A acquisition system (Axon Instruments/Molecular Devices Inc.; Sunnyvale, CA), filtered at 1kHz and digitized at 2kHz for whole cell recording and filtered at 1kHz and digitized at 4kHz for inside out patch recordings. The standard external solution contained (in mM): NaCl (140), KCl (5.4), $CaCl_2$ (1.8), HEPES (10), $MgCl_2$ (1), and TEA (2) adjusted to pH 7.4 with NaOH and with osmolarity adjusted to 310 mOsm/L with D-mannitol (measured with Model 3320 Osmometer/ Advanced Instruments; Norwood, MA). The standard pipette solution contained (in mM): KCl (140), K_2ATP (3), NaGTP (0.2), HEPES (5), $MgCl_2$ (1), and BAPTA (10; minimize large-conductance Ca^{+2}-activated K^+ currents), adjusted to pH 7.4 with KOH with osmolarity adjusted to 310 mOsm/L with D-mannitol. Solutions were delivered by gravity through a manifold perfusion system. Pipettes were made of borosilicate glass (Sutter Instrument Co; Novato, CA) pulled on a two-stage vertical puller (pp-83; Narishige International USA, Inc.; East Meadow, NY) and had a resistance of 2-4 mΩ when filled with standard pipette solution. Cell capacitance and series resistance were measured using the membrane test feature of pCLAMP. Series resistance was then compensated ≈70%. Cell capacitance was later used for normalization of whole cell current to capacitance to yield current density (pA/pF) for each cell. Whole cell currents were monitored by running a pulse/ramp protocol every 15 seconds stepping to +80mV for 100 milliseconds, ramping from +80 to -80mV over 1 second and finally stepping to -80mV for 100 milliseconds. For experiments employing AA, cells were held at -80mV between pulse/ramp

protocols (Meadows et al., 2000). For all other whole cell experiments cells were held at 0mV between pulse/ramps protocols. For inside out patch experiments, the pipette was filled with standard (5 mM KCl) bath solution containing 2 mM TEA, 100 μM DIDS, and 100 μM $GdCl_3$ to block voltage gated K^+, Cl^-, and non-selective cation channels respectively. The bath solution for inside out experiments contained (in mM) KCl (140), EGTA (1), HEPES (5), $MgCl_2$ (1), and TEA (2) adjusted to pH 7.4 with KOH and osmolarity to 310 mOsm/L with D-mannitol. Negative pressure was applied via pipette suction. Suction was measured using a pressure transducer calibrated to mm Hg. Currents were digitized at 4kHz and filtered at 1kHz. Channel activity was measured by taking the mean value of current of ≈10 seconds of recording minus the mean value of baseline current (current when no channels were open).

Patch clamping splice variants: HEK293T were transfected using Lipofectamine 2000, 1 μ g HA-TREK-1 plasmid, and 5 μ g HSV plasmid was used for each of the TREK-variants during co-expression experiments; 1 μ g HA- TREK-1 or 5 μ g of HSV plasmid were used for individual expression studies. The ratio of 1:5 was selected based on previous studies of TREK-1 variants that were discovered in other tissues that directly interacted with TREK-1. Successful transfection was verified using co-expression of fluorescent proteins in the plasmid DNA (green fluorescent protein with HA-TREK-1; and red fluorescent protein with each TREK-1 variant). Transfected cells were trypsin digested, plated on glass coverslips, and utilized for patch clamp experiments within 72 hr of transfection.

Transfected cells on glass coverslip shards were placed in a chamber mounted on top of an inverted microscope. The chamber was continuously perfused with a bath (external) solution that contained: 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, 1 mM $MgCl_2$, 2 mM TEACl (to block voltage gated K^+ channels), 0.1 mM 4,4-Diisothiocyanatostilbene-2,2-disulfonic acid (DIDS; chloride channel blocker), and 0.1 mM $GdCl_3$ (non-specific cation current blocker) with the pH adjusted to 7.4 and osmolarity adjusted to 310 mOsm/L with D-mannitol that was delivered by gravity through a manifold. Currents were recorded in the standard whole-cell variant of the patch clamp technique using pCLAMP software (V9.2; Axon Instruments/ Molecular Devices Inc; Sunnyvale, CA). Pipettes were made of borosilicate glass (Sutter Instrument Co; Novato, CA) pulled on a two- stage vertical puller (pp-83 Narishige International US, Inc.; East Meadow, NY) with a resistance of ~6 MΩ when filled with standard pipette solution. The pipette solution contained: 140 mM KCl, 3 mM K_2ATP, 0.2 mM NaGTP, 5 mM HEPES, 1 mM $MgCl_2$, and 10 mM BAPTA (to minimize Ca^{2+} activated K^+ currents) with the pH adjusted to 7.4 and osmolarity adjusted to 310 mOsm/L. Cell capacitance and series resistance were measured using the membrane test feature of pCLAMP and series resistance was compensated. Currents were amplified with an Axopatch200B amplifier (Axon Instruments/Molecular Devices Inc.; Sunnyvale, CA) and digitized using a computer interfaced with a Digidata 1322A system (Axon Instruments/Molecular Devices Inc.; Sunnyvale, CA). Currents were filtered at 1 kHz and digitized at 2 kHz for whole cell recording. Whole cell currents were

normalized using cell capacitance to yield current density (pA/pF) for each cell. Whole cell currents were monitored by running a pulse or ramp protocol every 15-sec stepping to +80 mV for 0.1 sec, ramping from +80 to -80 mV over 1 second, and finally stepping to -80 mV for 0.1 sec. For all other whole cell experiments, cells were held at 0 mV between pulse/ramp protocols. Currents were also observed using a step protocol during which they were held at 0 mV for 0.25 sec, an input potential of -100 mV was applied for 0.5 sec, and the cells were held again for 0.25 sec at 0 mV, the potentials were increased by 20 mV for each recording until the input reached +100 mV. To elicit TREK-1 currents, a bath solution containing 90 mM sodium bicarbonate was perfused into the chamber resulting in intracellular acidosis, a known activator of TREK-1 currents.

Statistics: Data are expressed as mean ± standard error of the mean. Student's *t* tests were used to compare mean values. Paired tests were used when both conditions were measured on the same cell. Unpaired tests were used when conditions were measured on different cells. One-tailed tests were used when the direction of changes were hypothesized. *P* values of less than 0.05 were taken as a statistically significant.

Results:

pGC-C and sGC on myometrial relaxation

Both soluble (sGC) and particulate (pGC-C) guanylyl cyclase are expressed in the myometrium. Canonically, guanylyl cyclases promote relaxation through the generation of the second messenger, cGMP, which activates PKG. Here we investigate the ability of sGC and pGC-C activation to relax myometrium.

Uroguanylin relaxes pregnant guinea pig myometrium: Addition of 100 nM uGN to guinea pig myometrial tissues from estrogen-primed non-pregnant (NP) guinea pigs failed to reduce oxytocin (OT) (100 nM)-induced contractions (Figure 2, B and C). Despite the appearance of a small regularization of the contraction seen (Figure 2B), no significant effect was measurable when tested in duplicate tissue strips (n=6) (Figure 2C) (Buxton et al., 2010). However, when 10 nM uGN was added to myometrial strips from pregnant guinea pigs (34 days), there was a marked reduction in both the frequency of contractions and peak tension (Figure 2A). The effect of uGN quantified as tension over time (area under the curve, 15 min) was dose-dependent with significant inhibition of OT-induced contractions at 3 nM uGN (Figure 2C). Because uGN is known to stimulate pGC-C in intestinal epithelium, we tested the possibility that uGN was acting via an increase in cGMP accumulation in the myometrium. “When the putative pGC-C antagonists 2Cl-ATP or isatin were added to the tissue bath, followed 3 min later by addition of 10 nM uGN, relaxation was prevented (Figure 2C). The effect of uGN to relax the tissue was not caused by stimulation of the soluble guanylyl cyclase, because addition of the sGC inhibitor, ODQ, had no effect” (Buxton et al., 2010).

Activation sGC Fails to Relax Myometrium. In order to examine the role of cGMP in uterine smooth muscle relaxation in the absence of haem-dependent activation of soluble guanylyl cyclase by ·NO, we have assessed the effect of direct stimulation of myometrial tissue by the haem-independent agonist BAY58-2667 (cinaciguat) on uterine relaxation. BAY58-2667 fails to relax human term myometrium despite the use of concentrations from 0.1 to 10μM, administered in an increasing accumulative dose at 10 min intervals after oxytocin stimulation (8nM) (Figure 3A). Treatment of rat aorta with BAY58-2667 undertaken as a comparative control, resulted in immediate relaxation consistent with the known actions of cGMP in vascular smooth muscle after stimulation with 1 μM phenylalanine (Figure 3B). The EC_{50} for BAY58-2667 activation of the sGC is 6.4 nM and its effect to relax vascular smooth muscle is sub-μM (Stasch et al., 2006). Experiments repeated with term myometrium from multiple human (n=4) and guinea pig (n=3) donors (Figure 3C,3D) confirmed the failure of BAY58-2667 to relax the tissue (simple one-way ANOVA: human p=0.29; guinea pig p=0.92). Addition of 300μM GSNO after application of the final BAY58-2667 dose relaxed the tissue (data not shown).

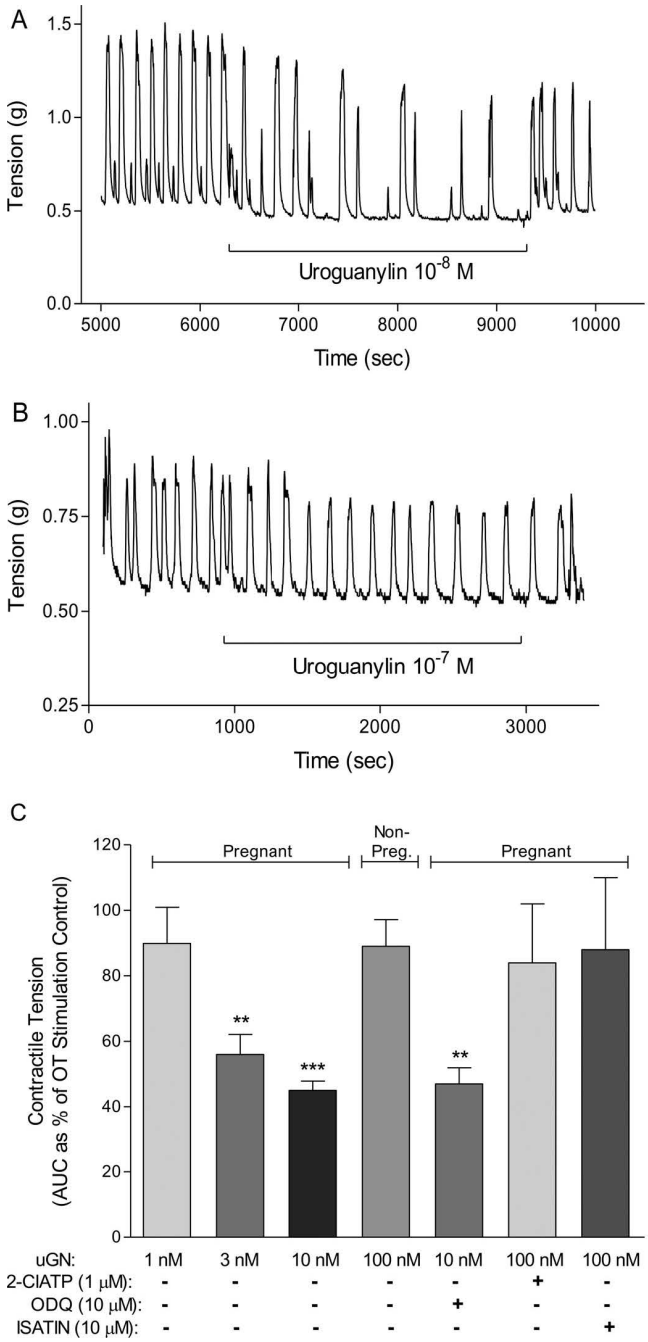

A
Tension (g)
1.5
1.0
0.5
0.0
Uroguanylin 10-8 M
5000
6000
7000
8000
9000
10000
Time (sec)
B
Tension (g)
1.00
0.75
0.50
0.25
Uroguanylin 10-7 M
0
1000
2000
3000
Time (sec)
C
Contractile Tension
(AUC as % of OT Stimulation Control)
Pregnant
Non-
Preg.
Pregnant
120
100
80
60
40
20
0
**
***
**
uGN:
1 nM
3 nM
10 nM
100 nM
10 nM
100 nM
100 nM
2-ClATP (1 μM):
ODQ (10 μM):
ISATIN (10 μM):

Figure 2 *Uroguanylin relaxes myometrium through pGC-C stimulation*: Uroguanylin relaxes oxytocin-stimulated contractions in a dose-dependent, pGC-C-mediated fashion in pregnant guinea pig myometrium. (A) In the pregnant guinea myometrium (50–60 days gestation) 10 nM uGN relaxes the tissue with a reproducible effect on peak height and frequency of contraction. (B), no such effect is seen in tissues from estrogen-primed nonpregnant animals even at 100 nM. Traces are representative examples. Effects were reproducible after washout and were seen both early and late in the recording. (C) contractile tension was measured in grams from area under the curve (AUC) for 15 min of oxytocin- stimulated contractile activity in replicate pregnant guinea pig tissues (n=6) in the presence or absence of 2Cl-ATP, ODQ, or isatin. The uGN relaxation was dose-dependent and significant at 3 nM uGN. Uroguanylin stimulation in the presence of 2Cl-ATP (1μM) or isatin (10μM) prevented the relaxation to 100 nM uGN. ODQ (10μM) had no significant effect on the uGN-mediated relaxation. Data are mean S.E.M. from two replicate tissues from each of six animals (50 – 60 days gestation). , *p* 0.01; , *p* 0.001. (Iain L O Buxton et al., 2010b)

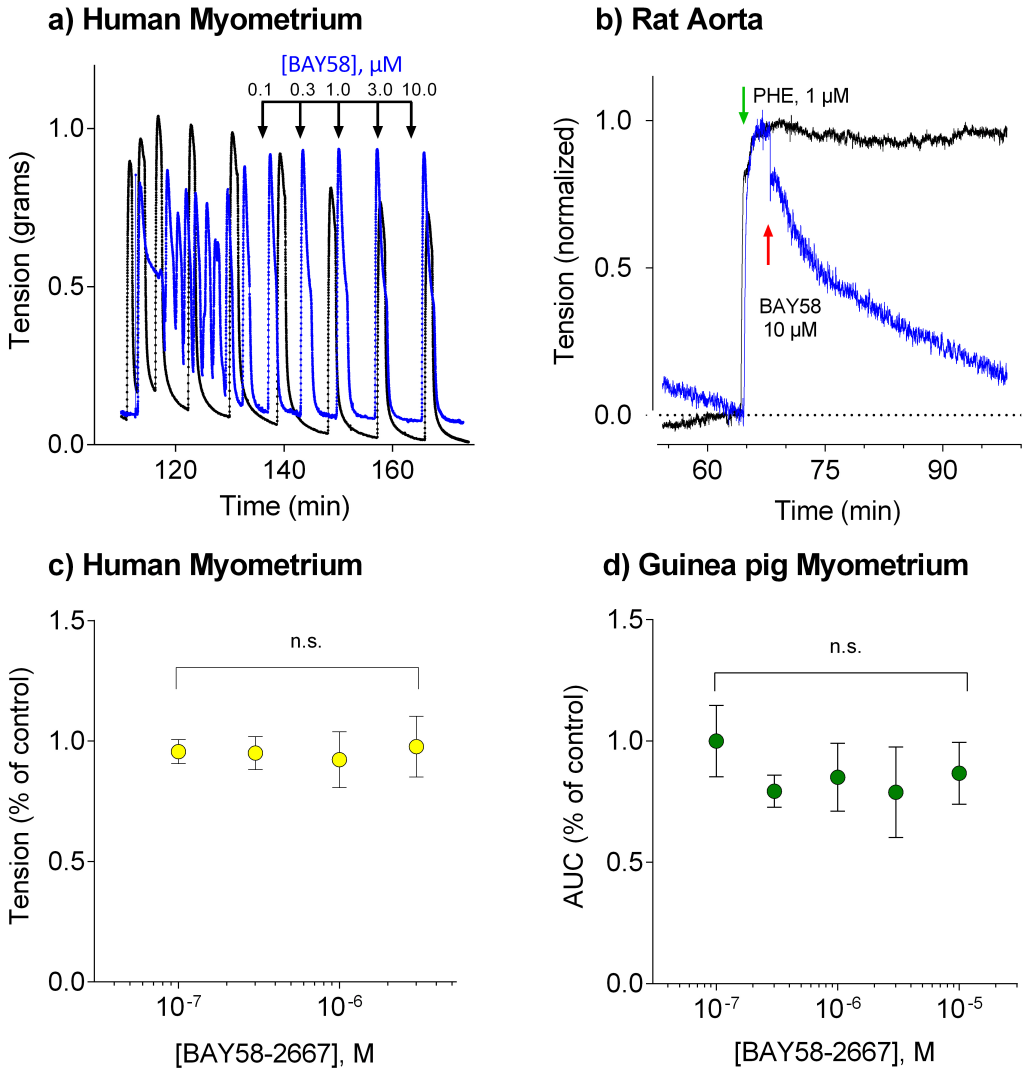

a) Human Myometrium
[BAY58], µM
0.1 0.3 1.0 3.0 10.0
Tension (grams)
1.0
0.5
0.0
120
140
160
Time (min)
b) Rat Aorta
PHE, 1 µM
BAY58
10 µM
Tension (normalized)
1.0
0.5
0.0
60
75
90
Time (min)
c) Human Myometrium
n.s.
Tension (% of control)
1.5
1.0
0.5
0.0
10-7
10-6
[BAY58-2667], M
d) Guinea pig Myometrium
n.s.
AUC (% of control)
1.5
1.0
0.5
0.0
10-7
10-6
10-5
[BAY58-2667], M

Figure 3 *The myometrium does not relax in response to BAY58-2667, an sGC activator:* Smooth muscle response to sGC activation by BAY58-2667. (A) Human pregnant myometrium was hung in tissue baths at 37°C with continuous oxygenation and stimulated to contract in the presence of oxytocin. Following 60 min, tissues were challenged in the presence or absence of BAY58-2667 in a cumulative fashion as indicated in the figure. No response was seen to addition of BAY58-2667. Addition of the NO-donor at the end of the experiment confirmed the ability of the tissue to relax (not shown). (B) Rat aorta was hung in tissue baths with Krebs buffer at 37°C with continuous oxygenation and allowed to equilibrate for 60 min in the absence of drug. Addition of phenylephrine (1 μM) produced immediate contractions that could be relaxed by addition of BAY58-2667 (10 μM). (C) The failure of tissues to relax to BAY58 was repeatable in both human (n=4) and (D) guinea pig (n=3), and quantified as area under the curve over 10 min and demonstrated the absence a dose-dependent effect on tension.

Membrane potential and uterine quiescence

Beyond the role of guanylyl cyclase variants on myometrial relaxation, there are other factors that contribute to contractile/relaxation dynamics in the myometrium. Earlier work by our laboratory has shown that TREK-1, an outward rectifying potassium channel important to membrane polarization, is upregulated during pregnancy, presumably to aid in myometrial quiescence, and is significantly downregulated in preterm tissues (I L O Buxton et al., 2010c). Here, for the first time, we electrophysiologically characterize TREK-1 in uterine SM cells, and we explore effects of TREK-1 splice variants on channel activity (Figure 4).

Intracellular acidification activates TEA-insensitive K^+ current in pHUSMC and HEK293-hTREK-1 cells: The ability of intracellular acidification to activate TEA-insensitive K^+ currents in pregnant human uterine smooth muscle cells (pHUSMC) was tested by measuring whole cell currents in the presence of TEA (2 mM) under two recording conditions resulting in intracellular acidification: *i*) after exchanging 90 mM NaCl for 90 mM $NaHCO_3$ in the bath solution, and *ii*) adjusting the pipette solution from pH 7.4 to pH 6. “Both approaches yielded an increase in outward current in both pHUSMC and HEK293-hTREK-1 cells that resulted in a shift in current reversal potential toward that for K^+. Intracellular acidification by $NaHCO_3$ yielded an increase in outward current at +80 mV from 3.0 ± 0.8 to 11.8

± 2.7 pA/pF (≈4 fold) in pHUSMC (n=4) and from 34.8 ± 8.9 to 218.6 ± 45.0 pA/pF (≈6 fold) in HEK293 hTREK-1 cells (n=8) that was reversible upon returning to standard bath solution" (Figure 5) (Heyman et al., 2013). $NaHCO_3$ bath solutions also shifted the reversal potential toward that predicted for K^+ (≈-85 mV) with a shift from 0.5 ± 6.0 mV to -40.6 ± 3.0 mV (n=4) in standard vs. 90 mM $NaHCO_3$ bath, respectively in pHUSMC; and from -46.1 ± 4.4 mV to -65.1 ± 3.6 mV (n=8) in standard vs. 90 mM $NaHCO_3$ bath, respectively in HEK293 hTREK-1 cells.

"Intracellular acidification using pH 6 pipette solutions resulted in the activation of an outward current that typically took 5-10 min after membrane rupture to reach a maximal steady state level (Figure 6). Acidification by a pH 6 pipette solution resulted in significantly higher outward currents in pHUSMC 10 min after membrane rupture (16.9 ± 3.2 pA/pF, n=5) when compared to pH 7.4 pipette solutions at the same time point (2.5 ± 0.7 pA/pF, n=5; Figure 6). A similar increase was seen in HEK293-hTREK-1 cells 10 min after membrane rupture with pH 6 pipette solution having a mean of 83.2 ± 13.8 pA/pF (n=8) vs. 30.9 ± 20.7 pA/pF (n=4) in pH 7.4 pipette solution. Intracellular acidification by pH 6 pipette solution also resulted in a negative shift in current reversal potential toward that predicted for K^+ (≈-85mV) in pH 7.4 vs. pH 6 pipette solutions from 1.0 ± 5.1 mV to -46.0 ± 6.0 mV (n=5) and from -46.1 ± 4.4 mV (n=4) to -53.7 ± 4.2 mV (n=8) in pHUSMC and HEK293-hTREK-1 cells, respectively" (Heyman et al., 2013). Replacing the standard bath with a high K^+ bath solution after activating current with pH 6 pipette solution

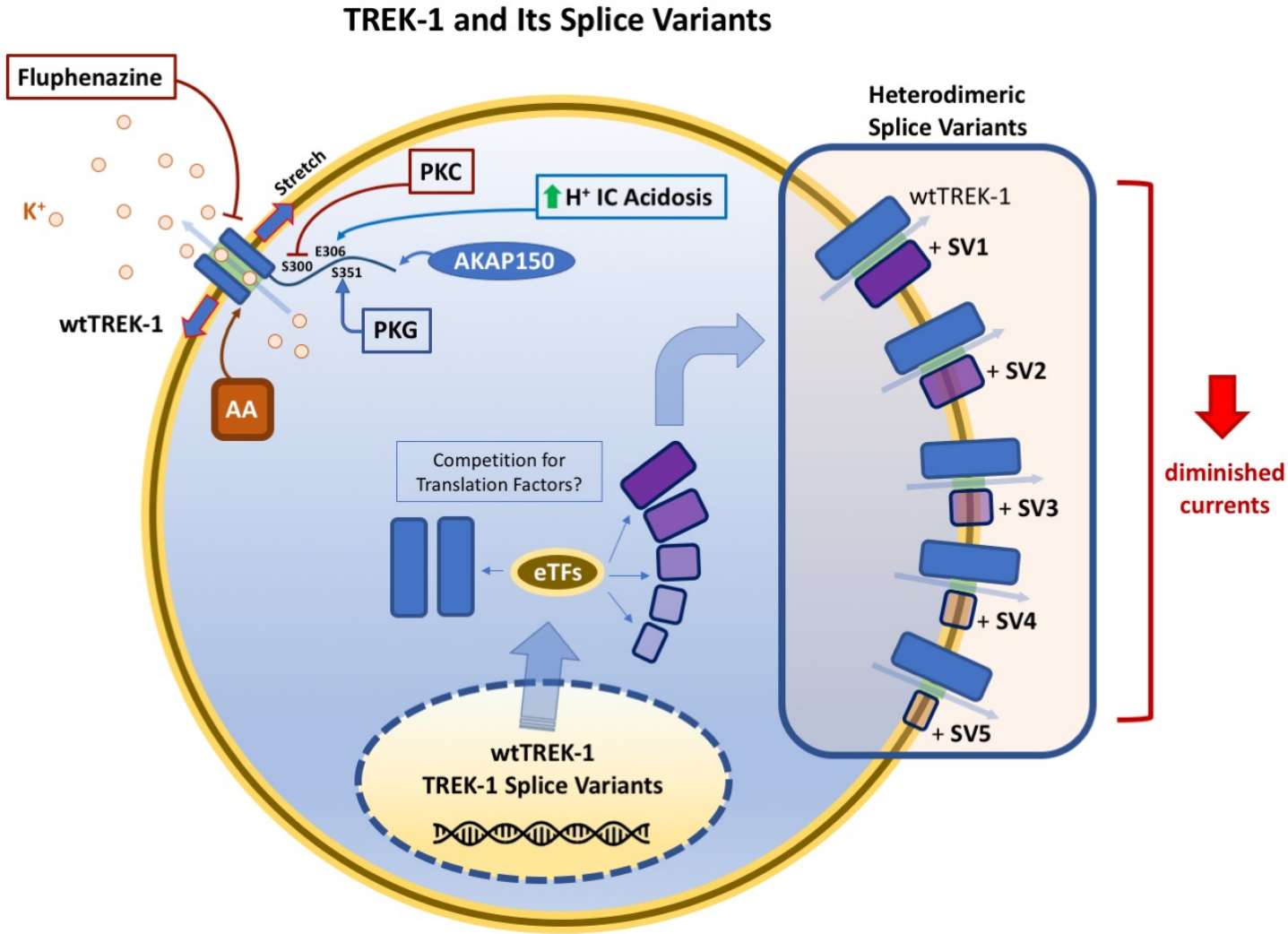

TREK-1 and Its Splice Variants
Fluphenazine
K+
Stretch
PKC
H+ IC Acidosis
E306
S300
S351
AKAP150
PKG
wtTREK-1
AA
Competition for Translation Factors?
eTFs
wtTREK-1
TREK-1 Splice Variants
Heterodimeric Splice Variants
wtTREK-1
+ SV1
+ SV2
+ SV3
+ SV4
+ SV5
diminished currents

Figure 4 *TREK-1 splice variants alter its trafficking and function:* Native TREK-1 assembles as a homodimer and acts as an outward rectifying potassium channel, which in turn hyperpolarizes the membrane, maintaining quiescence during pregnancy. There are five known splice variants of TREK-1 that decrease full-length TREK-1's trafficking to the membrane, resulting in decreased TREK-1 currents.

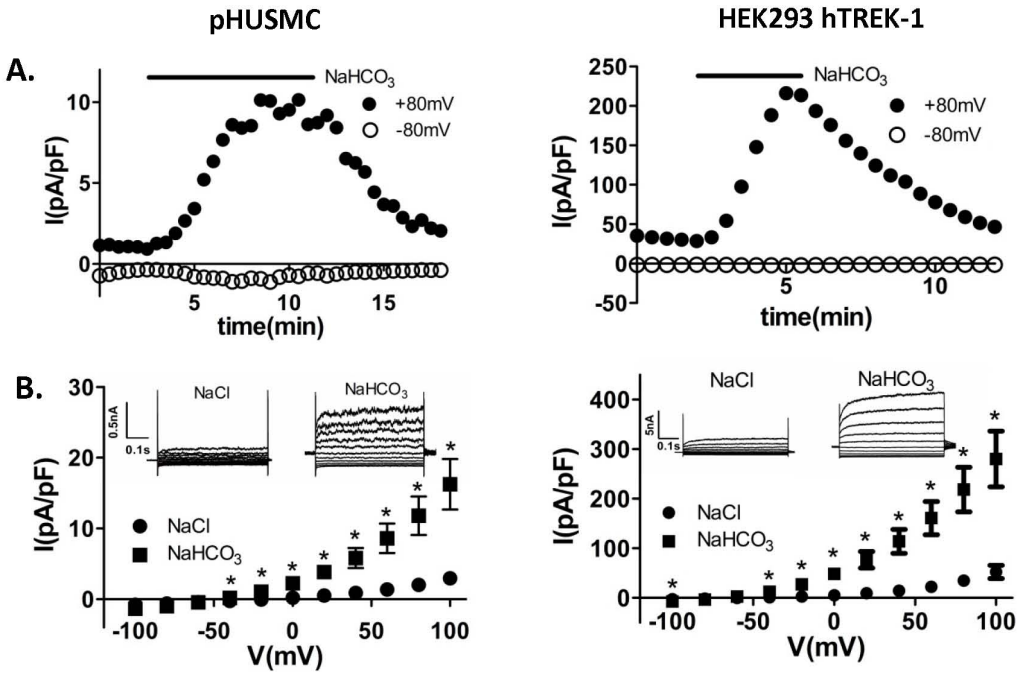

pHUSMC
HEK293 hTREK-1
A.
NaHCO3
+80mV
-80mV
I(pA/pF)
time(min)
B.
NaCl
NaHCO3
0.5nA
0.1s
5nA
V(mV)

Figure 5 *Intracellular activation activates TREK-1:* Intracellular acidification by 90mM NaHCO3 activates a TEA-insensitive K^+ current in pHUSMC and HEK293-hTREK-1 cells. A: time course of whole cell voltage-clamp recording at -80 and +80 mV showing reversible activation of TEA-insensitive outward current after exchange of 90 mM NaCl for 90 mM NaHCO3 in bath solution to cause intracellular acidification in pHUSMC and HEK293- hTREK-1 cells. B: mean current density in response to 20-mV voltage steps from -100 to +100 mV before (NaCl) and after NaHCO3 treatment in pHUSMC and HEK293-hTREK-1 cells. *$P < 0.05$. Insets: representative whole cell current traces before and after NaHCO3 treatment. (Nathanael S Heyman et al., 2013b)

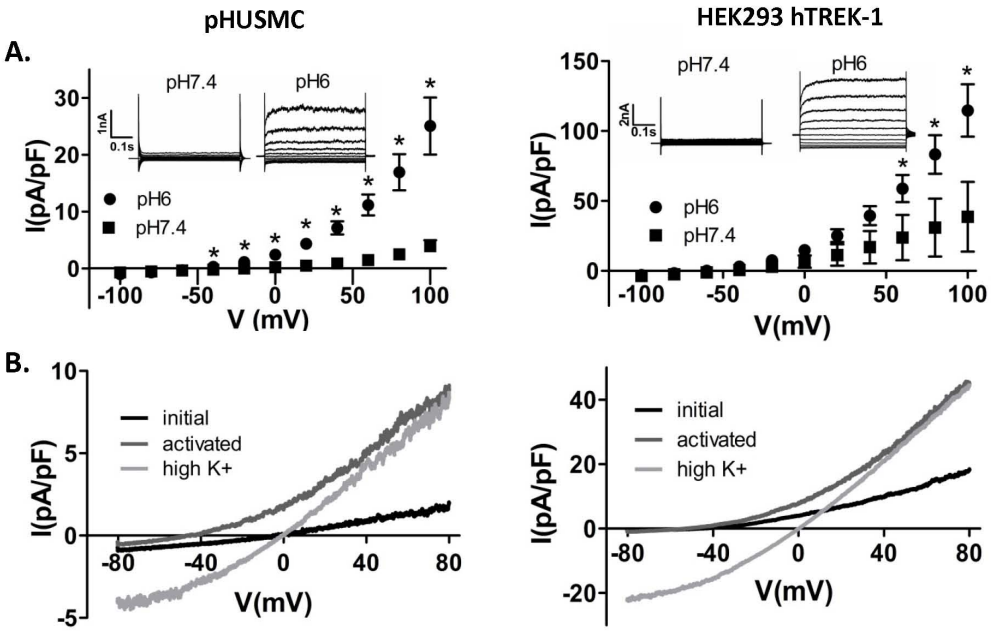

pHUSMC
HEK293 hTREK-1
A.
pH7.4
pH6
1nA
0.1s
2nA
0.1s
I(pA/pF)
V (mV)
V(mV)
pH6
pH7.4
B.
initial
activated
high K+

Figure 6 *Isometric K^+ shift the reversal potential of TREK-1 to 0mV: pH6 pipette solution activates a TEA-insensitive K^+ current:* **A.** Mean current densities measured in response to 20mV voltage steps from -100 to +100mV with pH7.4 (■**)** and pH6 (●) pipette solutions from pHUSMC (left panel) and HEK293 hTREK-1 cells (right panel) (* $p<0.05$). **B.** Representative whole cell current density traces in response to voltage ramps from +80 to -80mV made shortly after gaining whole cell access (initial), after 10 min (activated) and after activation and switching to high K^+ bath solution (high K^+). (Nathanael S Heyman et al., 2013b)

increased inward current and moved the reversal potential near 0 mV (Figure 6B) in both cell types.

Arachidonic acid activates TEA-insensitive K^+ current in pHUSMC and HEK-hTREK-1 cells. "TREK-1 K^+ channels have been shown to be activated by AA (Caley et al., 2005; Meadows et al., 2000; Moha ou Maati et al., 2011) and to be largely insensitive to the classical K^+ channel blocker TEA (53). To test for the presence of an AA-activated and TEA-insensitive K^+ current in pHUSMC, AA (10 μM) was applied during recording of whole cell currents in pHUSMC and HEK293-hTREK-1 cells in the presence of TEA (2 mM). Application of AA resulted in a significant increase in outward current at +80 mV, from 4.8 ± 1.5 to 19.4 ± 7.5 pA/pF (~4 fold) in pHUSMC (n = 7) and from 91.0 ± 23.8 to 247.5 ± 73.3 pA/pF (~3-fold) in HEK293-hTREK-1 cells (n = 6), that was reversible upon washout (Figure 7). In addition, activation of current by AA resulted in a negative shift in reversal potential toward the equilibrium potential for K^+ from 4.3 ± 5.8 to -33.1 ± 2.8 mV (n = 7) in pHUSMC and from -50.3 ± 7.5 to -62.8 ± 4.7 mV (n = 6) in HEK293-hTREK-1 cells. Under the recording conditions used, only K^+ had a Predicted Nernst potential negative to 0 mV. Replacing the standard bath with a high- K^+ bath solution under AA activation increased inward current and moved the reversal potential near 0 mV (Figure 7B) in both cell types. This result indicates that a majority of the current was carried by K^+. The AA-activated current in both cell types showed outward rectification in physiological K^+, slight outward rectification in symmetrical K^+, time-dependent activation at more positive

membrane potentials, lack of inactivation, and a flickering appearance. All these properties have been previously reported for TREK-1. These data demonstrate the presence of a TEA-insensitive K^+ current activated by AA in pHUSMC and HEK293-hTREK-1 cells with properties similar to those previously reported for TREK-1 channels (Caley et al. 2005; Meadows et al. 2000). Importantly, in response to AA, freshly isolated myocytes, pHUSMC, and HEK293-hTREK-1 cells demonstrated similar current activation.

Fluphenazine blocks TEA-insensitive K^+ currents in pHUSMC and HEK293 hTREK-1 cells: The antipsychotic fluphenazine has been shown to block hTREK-1 and hTREK-2 currents but not hTRAAK currents (Patel et al., 1999). In order to test the ability of fluphenazine to block the TEA-insensitive K^+ currents reported here, currents were activated with 10 μM AA, a 90 mM $NaHCO_3$ bath, and pH 6 pipette solution. In each case the current was allowed to fully activate and then 10 μM fluphenazine was applied in the continued presence of each agonist to observe blockade. Fluphenazine significantly blocked current stimulated by all three treatments (Figure 8) in both pHUSMC and TREK-1 overexpressing HEK cells. Currents in pHUSMC at +80 mV were reduced by 51.2 ± 9.8% (n=6), 73.9 ± 4.2% (n=5), 75.6 ± 4.0% (n=6) after activation by 10 μM AA, 90 mM $NaHCO_3$ bath, or pH6 pipette solutions, respectively. Currents in HEK hTREK-1 cells at +80 mV were reduced by 89.5 ± 2.3% (n=3), 91.6 ± 3.4% (n=3), and 89.8 ± 2.2% (n=7) after activation by 10 μM AA, 90 mM $NaHCO_3$ bath, and pH 6 pipette solution respectively. The somewhat smaller block seen in the presence of AA in pHUSMC

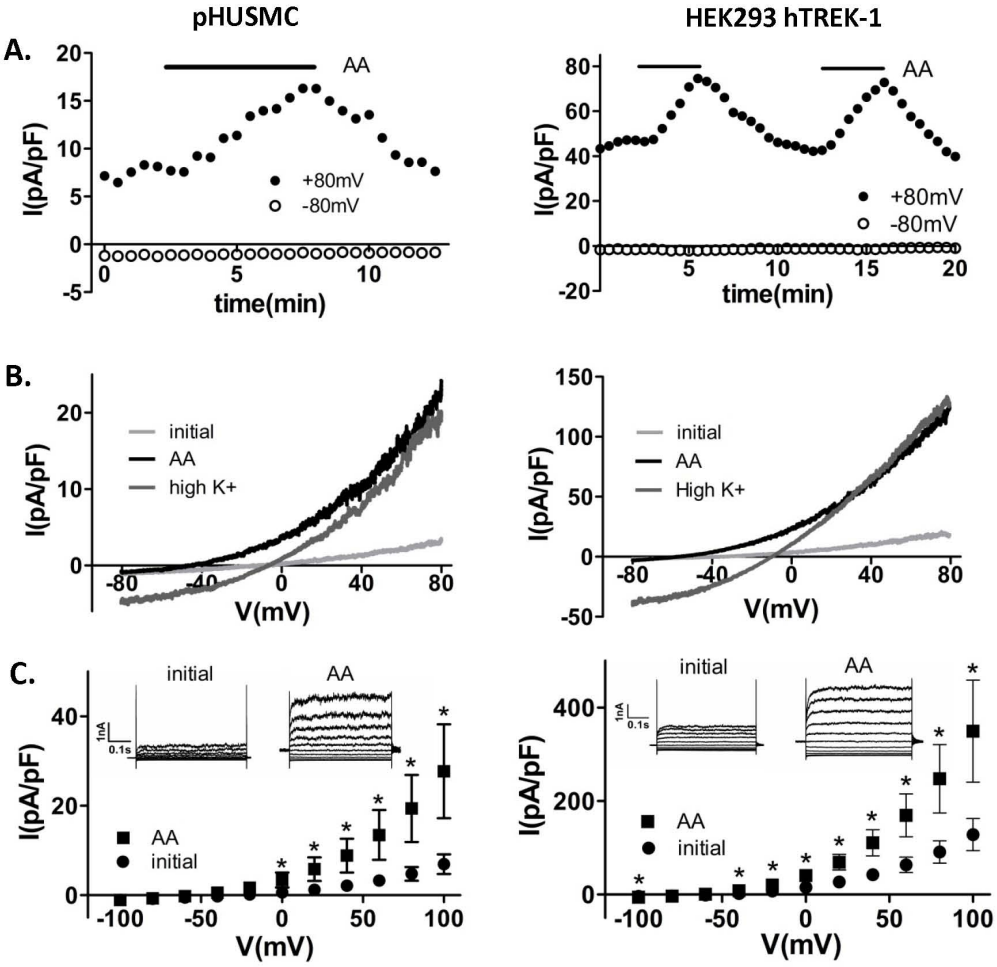

pHUSMC
HEK293 hTREK-1
A.
AA
+80mV
-80mV
I(pA/pF)
time(min)
B.
initial
AA
high K+
High K+
V(mV)
C.
initial
AA
1nA
0.1s
*

Figure 7 *Arachidonic acid activation of TREK-1:* AA activates a TEA-insensitive K current in pHUSMC and HEK293-hTREK-1 cells. *A*: time course of whole cell voltage-clamp recording from pHUSMC and HEK293-hTREK-1 cells at 80 and 80 mV showing reversible activation of outward current by AA (10 M). *B*: representative whole cell current density traces in response to voltage ramps from 80 to 80 mV before treatment (initial) and in the presence of AA in standard and high-K bath solutions in pHUSMC and HEK293-hTREK-1 cells. *C*: mean current density in response to 20-mV volt- age steps from 100 to 100 mV before (initial) and after AA treatment in pHUSMC and HEK293- hTREK-1 cells. **P* 0.05. *Insets*: representative whole cell current traces before and after AA treatment. (Nathanael S Heyman et al., 2013b)

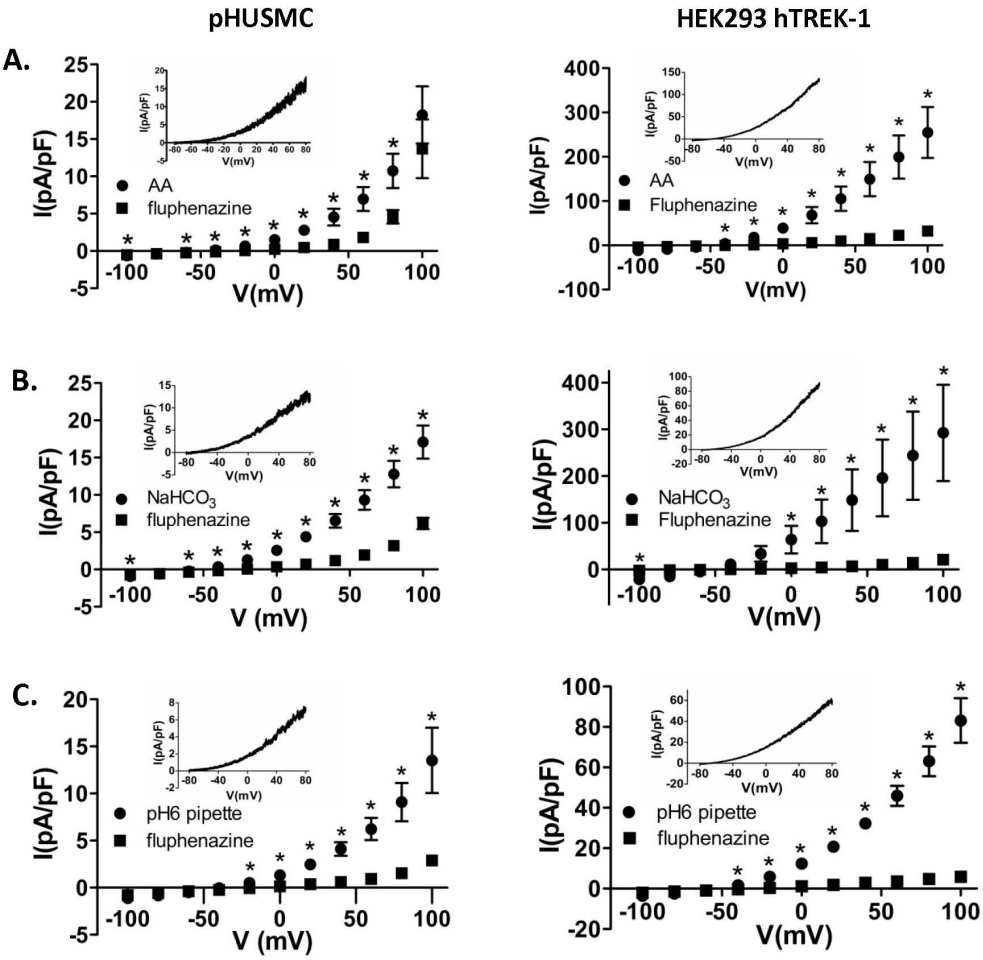

pHUSMC
HEK293 hTREK-1
A.
B.
C.
I(pA/pF)
V(mV)
AA
Fluphenazine
fluphenazine
NaHCO3
pH6 pipette

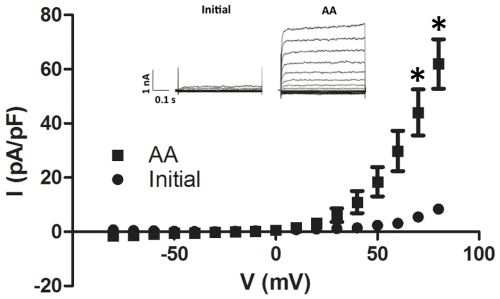
Figure 8 *Fluphenazine inhibits TREK-1 currents:* Fluphenazine block of TEA-insensitive K currents. *A–C*: mean current density in response to 20-mV voltage steps from 100 to 100 mV before and after 10 M fluphenazine in pHUSMC and HEK293- hTREK-1 cells. Currents were first activated by AA (*A*), 90 mM $NaHCO_3$ (*B*), or pH 6 pipette solution (*C*). **P* 0.05. *Insets*: representative whole cell current density traces in response to voltage ramps from 80 to 80 mV of the fluphenazine-sensitive current (fully activated fluphenazine-blocked). (Nathanael S Heyman et al., 2013a)

was likely due to the presence of a contaminating current that reversed at ≈0 mV that sometimes developed in these experiments. Subsequent experiments were carried out in the presence of 100 μM $GdCl_3$ and 100 μM 4,4'diisothio-cyanatostilbene-2,2'-disulfonic acid (DIDS) to reduce non-selective cation and Cl^- currents respectively. The fluphenazine-sensitive currents (*Figure 8 insets*) for all three agonists showed outward rectification in physiological K^+ and reversal potentials (-71.0 ± 3.9 mV (n=6), -77.1 ± 1.3 mV (n=5), and -67 ± 7.6 mV (n=6) for AA, $NaHCO_3$, and pH 6 pipette activated currents respectively in pHUSMC) near that predicted for K^+ (≈-85 mV). HEK293-hTREK-1 cells showed similar fluphenazine-sensitive currents with reversal potentials of -62.4 ± 11.8 mV (n=3), -66.0 ± 5.7 mV (n=6), and -63.2 ± 12.4 mV (n=3), for AA, $NaHCO_3$, and pH 6 pipette activated currents respectively. These data show that the TEA-insensitive K^+ currents activated in pHUSMC and HEK293 hTREK-1 cells by AA and intracellular acidification are significantly blocked by fluphenazine, a known blocker of TREK-1 channels. These findings are consistent with hTREK-1 and not hTRAAK being the primary K^+ current carrier under these conditions.

TEA-insensitive K^+ Current in Freshly Isolated Myocytes: To investigate channel properties under conditions that most closely resemble the physiological environment in which we hypothesize a role for TREK-1, whole cell recordings were performed in myocytes freshly isolated from pregnant and non-pregnant subjects. An outwardly rectifying TEA insensitive current was observed in freshly isolated myocytes (Figure 9) from pregnant subjects that could be activated by AA

(n=4). Moreover, in freshly isolated myocytes from non-pregnant women, AA could not stimulate the same channel activity. Only a small residual outward basal current was detectable in pregnant myocytes in the presence of TEA. The AA-induced current was consistent with the properties of the TEA insensitive K^+ channels that were characterized in both HEK hTREK-1 and pHUSMC cells displaying outwardly rectifying properties, voltage-dependent activation, and lack of inactivation at positive potentials" (Heyman et al., 2013).

TREK-1 Currents are Inhibited in the Presence of Variants: "While TREK-1 splice variants were incapable of generating significant currents, their interaction with TREK-1 and the potential relationship to uterine quiescence was explored using co-transfection. Therefore, HEK293T cells were co-transfected with 1 μg TREK-1 and 5 μ g of each splice variant; TREK-1 was also co-transfected with 5 μg RFP as an expression control. The average current-voltage relationship for basal (NaCl bath) TREK-1 currents in HEK293T cells co-transfected with TREK-1 and each of the variants compared to the basal current of TREK-1 co-transfected with RFP (the positive control) were observed. When TREK-1 was co-transfected with variants, the current was decreased; at 0 mV basal currents were 2.1 ± 0.6, 2.5 ± 0.1, 6.4 ± 0.3, 2.7 ± 0.2, 7.5 ± 2.3 pA/pF for TREK-1 co-transfected with HSV-1 (n=8), HSV-2 (n=10), HSV-3 (n=9), HSV-4 (n=10), and HSV-5 (n=8), respectively, compared to 11.97 ± 1.3 pA/pF for TREK-1 (n=10) co-transfected with RFP (Figure 10). This reduction in basal current was found to be significantly different for HSV-1, HSV-2, HSV-3, and HSV-4 at more positive (> 40 mV) input potentials compared to


Initial
AA
1 nA
0.1 s
80
60
40
20
0
-20
I (pA/pF)
AA
Initial
-50
0
50
100
V (mV)
*
*

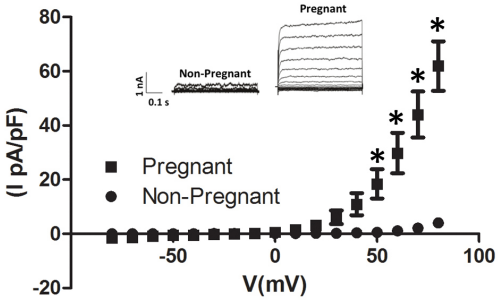

Pregnant
Non-Pregnant
1 nA
0.1 s
80
60
40
20
0
-20
(I pA/pF)
Pregnant
Non-Pregnant
-50
0
50
100
V(mV)
*
*
*

Figure 9 *Arachidonic acid activates TREK-1 in primary pHUSMC cells:* Arachidonic acid (AA) activates tetaethylammonium (TEA)-insensitive current in freshly isolated pregnant uterine myocytes. Whole cell recordings from freshly isolated pregnant myocytes demonstrate a current elicited using AA (*left*). AA activates pregnant, but not non-pregnant, myocytes in freshly isolated cells (*right*). *I*, current; *V*, voltage. **P* 0.05. (Nathanael S Heyman et al., 2013b)

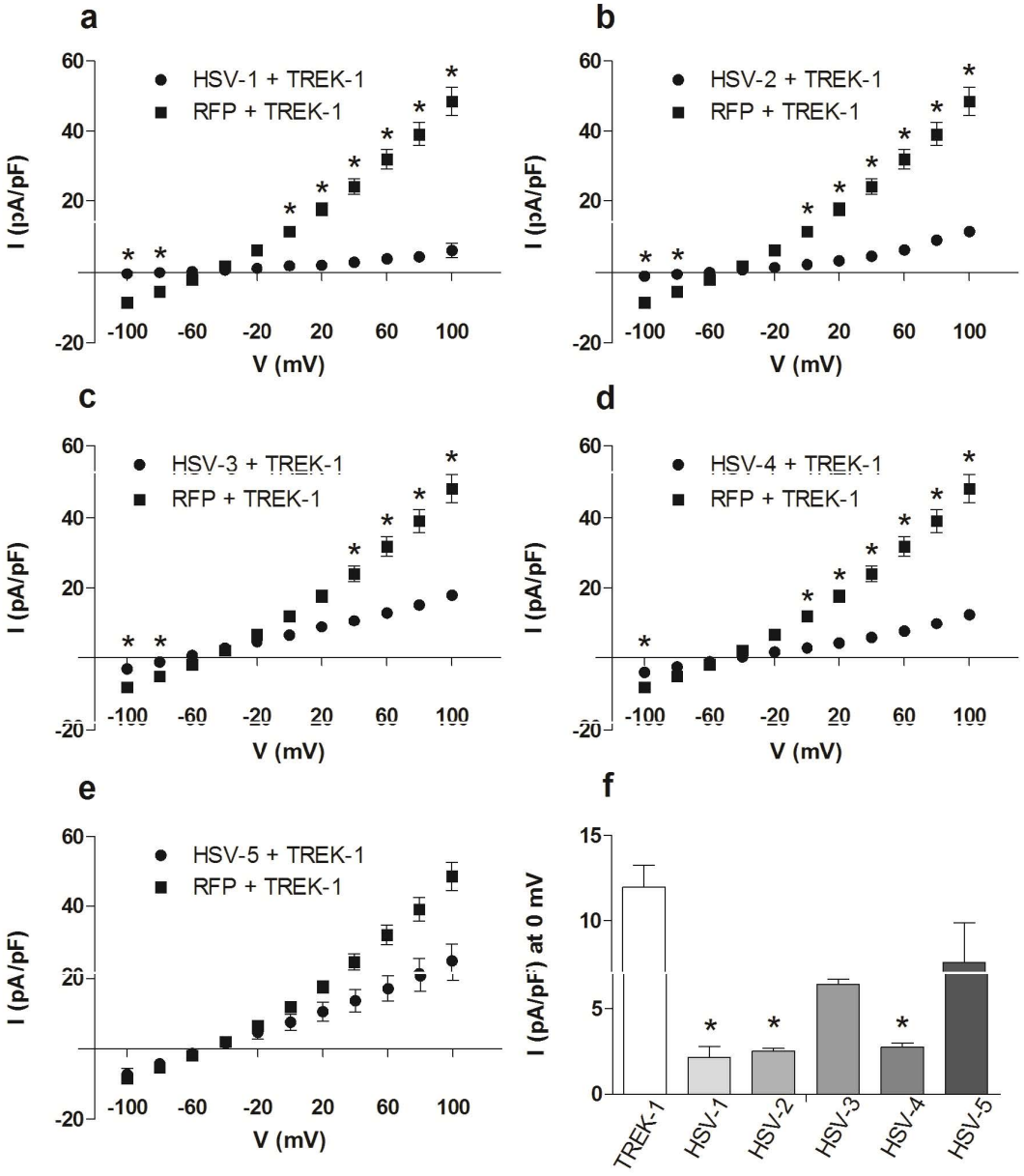

a
HSV-1 + TREK-1
RFP + TREK-1
I (pA/pF)
V (mV)
b
HSV-2 + TREK-1
RFP + TREK-1
I (pA/pF)
V (mV)
c
HSV-3 + TREK-1
RFP + TREK-1
I (pA/pF)
V (mV)
d
HSV-4 + TREK-1
RFP + TREK-1
I (pA/pF)
V (mV)
e
HSV-5 + TREK-1
RFP + TREK-1
I (pA/pF)
V (mV)
f
I (pA/pF) at 0 mV
TREK-1
HSV-1
HSV-2
HSV-3
HSV-4
HSV-5

Figure 10 *Co-expression of TREK-1 splice variants with full-length TREK-1 decreases basal activity:* Co-expression of wt-TREK-1 and TREK-1 splice variants decreases basal currents. HEK293 cells on glass coverslips were transfected (1.0 μg of HA-TREK-1 and 5.0 μg of HSV or RFP) and placed in a recording chamber. Currents were recorded in the whole cell mode of patch clamp. Currents were stabilized in an NaCl bath for 2 min. a-e) Average mean whole cell current densities in response to 20 mV steps from -100 to + 100 mV for TREK-1 co-expressing each individual splice variant compared with TREK-1 co-expressing RFP in standard 90 mM NaCl bath solution (TREK-1+RFP, n=10; TREK-1+HSV-1, n=8; TREK-1+HSV-2, n=10; TREK- 1+HSV-9, n=8; TREK-1+HSV-4, n=10; TREK-1+HSV-5, n=8) (*$p < 0.05$ between average HA-TREK-1+RFP current vs. TREK-1 HSV current). f) Average currents at 0 mV in NaCl bath solution for TREK-1 co-expressing RFP or each individual splice variant *0.05). (Chad L Cowles et al., 2015)

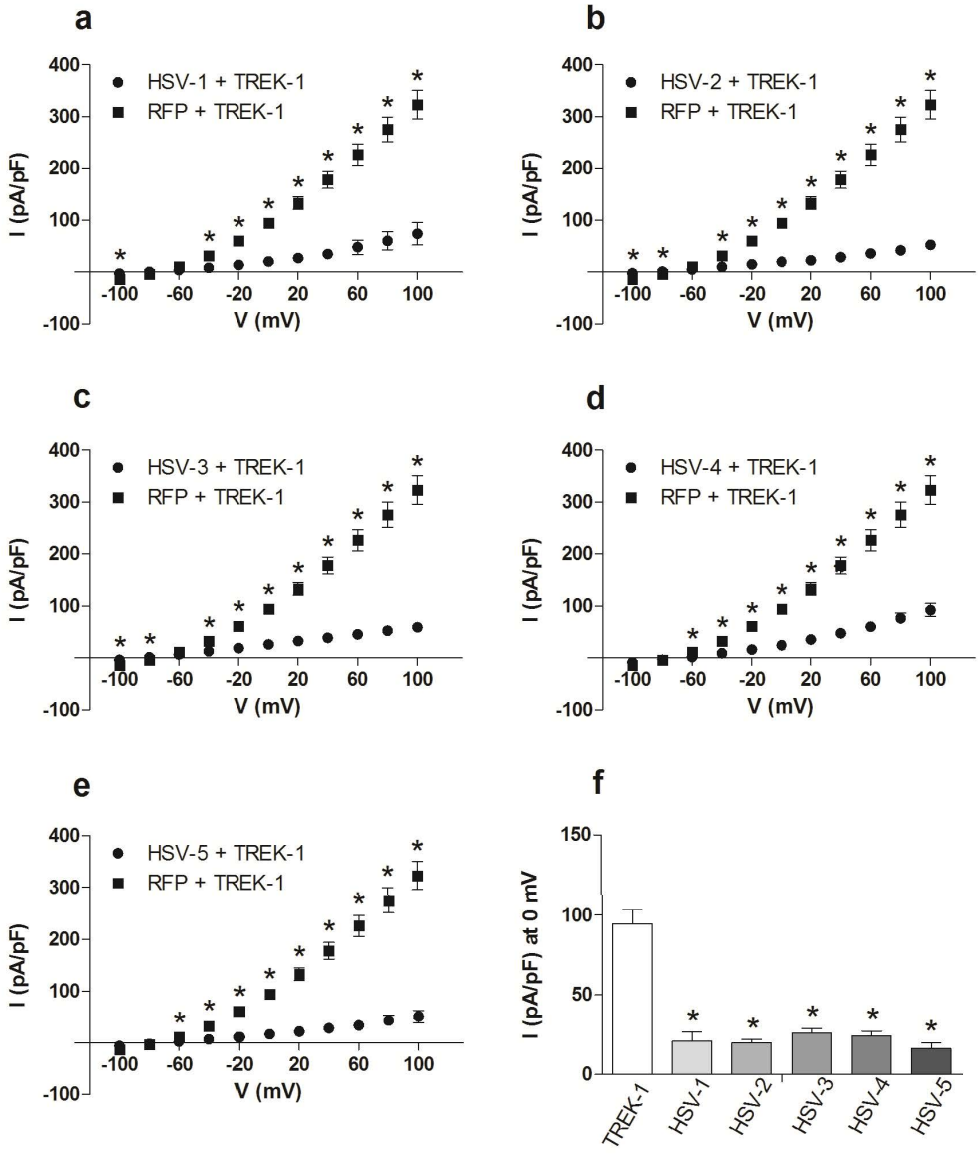

a
HSV-1 + TREK-1
RFP + TREK-1
I (pA/pF)
V (mV)
b
HSV-2 + TREK-1
RFP + TREK-1
c
HSV-3 + TREK-1
RFP + TREK-1
d
HSV-4 + TREK-1
RFP + TREK-1
e
HSV-5 + TREK-1
RFP + TREK-1
f
I (pA/pF) at 0 mV
TREK-1
HSV-1
HSV-2
HSV-3
HSV-4
HSV-5

Figure 11 *Co-expression of TREK-1 splice variants with full-length TREK-1 activated currents:* Co-expression of wt-TREK-1 with TREK-1 splice variants decreases activated currents. HEK293 cells on glass coverslips were transfected (1.0 μg of HA-TREK-1 and 5.0 μg of HSV or RFP) and placed in a recording chamber. Currents were recorded in the whole cell mode of patch clamp. Currents were stabilized in an NaCl bath followed by perfusion of NaHCO3 into the chamber, activated currents were stabilized for 2 min. a-e) Average mean whole cell current densities in response to 20 mV steps from -100 to +100 mV for HA-TREK-1 co-expressing each individual splice variant compared with HA-TREK-1 co-expressing RFP in standard 90 mM NaCl bath solution (TREK-1+RFP, n=10; TREK-1+HSV-1, n=8; TREK- 1+HSV-2, n=10; TREK-1+HSV-9, n=8; TREK-1+HSV-4, n=10; TREK-1+HSV-5, n=8) (*p< 0.05 between average HA-TREK-1+RFP current vs. TREK-1 HSV current). f) Average currents at 0 mV in NaHCO3 bath solution for TREK-1 co-expressing RFP or each individual splice variant *0.05). (Chad L Cowles et al., 2015)

TREK-1 co-expressing RFP. The relatively low basal current density observed was consistent with TREK-1 channel activity, which is reported to require a chemical or mechanical stimulus to open. To test if activated TREK-1 currents were altered in the presence of variants after the basal currents were stabilized, $NaHCO_3$ was perfused into the bath solution. In general, depending on the exact perfusion rate, currents would increase within 3-5 min and would stabilize after 5-7 min. The average currents at 0 mV were 20.7 ± 5.8, 19.7 ± 2.1, 26.0 ± 2.9, 24.4 ± 2.7, and 16.3 ± 3.8 pA/pF for HSV-1, HSV-2, HSV-3, HSV-4 and HSV-5 respectively, compared to the significantly higher activation 94.5 ± 8.9 pA/pF for TREK-1 co-expressing RFP (Figure 11). When applied voltages were -40 mV or greater, there was a significant difference between TREK-1 co-expressing variants compared to co-expression with RFP. The average currents and SEM of TREK-1 co-expressing each of the variants or RFP at every input potential is shown in Supplemental Table S3. These data show that activated TREK-1 currents are decreased in the presence of each of the splice variants. It should be pointed out that compared to variants expressed alone, HSV-2, HSV-3, HSV-4, and HSV-5 co-expressed with TREK-1 demonstrated activation that was significantly greater than variants expressed alone (Chad L Cowles et al., 2015). These activated currents most likely are generated by TREK-1 that is expressed and still functional. Collectively, the functional assessment of TREK-1 and TREK-1 splice variants indicates: (1) variants demonstrate minimal currents and show little if any activation by intracellular acidosis; (2) TREK-1 basal currents are generally decreased when

splice variants are co-expressed (~64% average reduction); (3) Activated TREK-1 currents are more significantly decreased than basal currents when splice variants are co-expressed (~77% average reduction)" (Cowles et al., 2015).

Discussion:

Without a better understanding of the nuances that distinguish the regulation of preterm myometrium from that of term myometrium, as well as the particulars of what divides the myometrium as a whole from other types of smooth muscle, we have little chance of identifying effective therapeutics to treat early onset labor. Two critical mediators of smooth muscle contractile dynamics are cyclic nucleotides, and ion channels that control the resting membrane potential of the cell. Here we have identified a unique role of guanylyl cyclase (GC) in the myometrium, and we have electrophysiologically characterized TREK-1 and its splice variants. Taken together, these two disparate influencers of myometrial quiescence shed light on the unique mechanisms that drive uterine contractile dynamics.

GC and cGMP during pregnancy: Research over the past 20 years, bolstered by our findings here, emphasize the idea that sGC plays an exceedingly limited role in myometrial quiescence. The product of sGC, cGMP, is a prevalent second messenger important to many processes in the cell (Buxton and Brunton, 1983; Fiscus and Murad, 1988; Kuenzli et al., 1996). Despite this, the inhibition of

sGC does not prevent ·NO-mediated relaxation, indicating an alternative function of ·NO, perhaps through protein S-nitrosation (see chapter 5). Furthermore, the activation of sGC by BAY58-2667 does not promote relaxation of uterine smooth muscle in pregnant humans or guinea pigs (figure 3). These data, combined with our previous finding that a global increase in cGMP actions by using its surrogate, 8-Br-cGMP, does not alter contractile dynamics (Iain L O Buxton et al., 2010a), questions the function of sGC and cGMP in the contractile regulation of the myometrium.

sGC is responsible for increasing global cellular levels of cGMP. This is, however, not the only route of cGMP generation in the cell. pGC-C, the membrane bound isoform of GC in the myometrium, promotes relaxation through compartmentalized action of cGMP (figures 1,2). In recent years, compartmentation of second messenger signaling, such as with sGC, PKG, and PKA in caveolae complexes, has been observed in rat aorta (Linder et al., 2005), and the effects of soluble vs. particulate GC-generated in ventricular myocytes has also been seen (Su et al., 2005), further reinforcing the concept that localized cGMP actions are important in muscle signaling.

There are three common ways in which a cyclic nucleotide can be “compartmented”: (1) Physical containment, as with organelles, (2) bound signaling complexes, such as with AKAPs/GKAPs and caveolae, or (3) by localized depletion events, normally mediated by the cyclic nucleotide’s cognate phosphodiesterase (Arora et al., 2013). Additionally, there are temporal aspects in

flux, such as the rate of diffusion of the compartmented cyclic nucleotides (Agarwal et al., 2016), further layering the signal. Much as AKAPs are important scaffolding proteins for PKA compartmentation, we are beginning to learn more about the role GKAPs and PKG, which anchor the N-terminus of PKG-II (Casteel et al., 2010). Based on our findings that pGC-C and CAV-1 are upregulated during pregnancy, and that pGC-C co-localizes with caveolae, while pGC-C mediated relaxation is muted in cholesterol depleted fractions (Iain L O Buxton et al., 2010b), it stands to reason that a localized signaling complex is formed between cGMP and other downstream factors that influence relaxation. Exposure of pregnant myometrium to the pGC-C inhibitors ISTATIN and 2-CIATP prevents uGN-mediated relaxation, while the same tissue relaxes with a modest dose of uGN (10nM) under condition in which sGC was inhibited by ODQ (figure 1C). This observation does not belie our finding that sGC activation fails to relax the myometrium; in fact, it provides a novel explanation for segmented cGMP actions in the myometrium (Figure 2).

TREK-1 and its splice variants: TREK-1 is an important ion channel that regulates the myometrial cell membrane potential by shuttling K^+ ions to the extracellular environment (Goldstein et al., 2001). Maintaining a negative membrane potential in uterine smooth muscle cells preserves a state of quiescence during gestation by keeping the cell below the action potential threshold, thus decreasing the probability of depolarization and Ca^{2+} influx. Splice variants (SVs) also play an important role in protein function by allowing for a much greater diversity of

expressed proteins (Nilsen and Graveley, 2010). As such, some variants allow for subtle shifts in protein function, while other drive disease states (Blencowe, 2006). For instance, in rat heart, TREK-1 splice variants alter the operating mode of the ion channel (Xian et al., 2006). Previous work from our laboratory has shown that: (1) TREK-1 expression increases during pregnancy, plateauing at mid-gestation, in order to maintain that negative cell membrane potential (I L O Buxton et al., 2010c), and that (2) the transcripts of five SVs of TREK-1 exist in preterm myometrial tissue (Wu et al., 2012). The purpose of our exploration of TREK-1 is to detail the electrophysiological channel properties of wtTREK-1 in the myometrium to further elucidate its role in quiescence, and to determine the functional relevance of the co-expression of the TREK-1 SVs found in preterm myometrial tissue.

TREK-1 currents: When identifying the TREK-1 currents in pHUSMC it was of paramount importance to ensure we were observing TREK-1 and not another similar uterine K^+ channel such as TRAAK (TWIK-Related Arachidonic Acid K_+ Channel, KCNK4), which is expressed in the myometrium (Tichenor et al., 2005). With this in mind, we took a three-pronged approach. First, we used HEK293 cells that had been stably transfected with TREK-1 as a means to compare the target signal in pHUSMC cells. Second, we employed a combination of blocking agents that eliminated undesirable secondary currents, such TEA to block voltage-gated K^+ currents, DIDS for Cl^- currents, and $GdCl_3$ for nonselective cation currents. Finally, we utilized activators and inhibitors that would distinguish TREK-1 from

TRAAK, such as intracellular acidification and fluphenazine, respectively. The addition of AA to pHUSMC and HEK293 hTREK-1 cells activated TREK-1 in a similar, and reversible, manner (Figure 7 A,C). We also found that under conditions of isometric K^+, the reversal potential migrated to 0 mV (Figure 7B), a finding consistent with K^+ channels.

TRAAK, which expressed in myometrial tissue, is not activated under conditions of intracellular acidification. To test conditions of intracellular acidification we used a $NaHCO_3$ bath solution as well as a pH 6.0 pipette solution. Introduction of $NaHCO_3$ into the bath solution causes a dissociation of the bicarbonate anion to CO_2. The CO_2 migrates across the cell membrane and causes a decrease in intracellular pH by a process known as 'paradoxical acidification' (Ritter et al., 1990). TREK-1 is the only K^+ channel reported so far to be directly opened by intracellular acidosis (Maingret et al., 1999), and it does so by interaction of the H^+ ion with E306 on the cytosol facing C-terminus (Enyedi and Czirjak, 2010). It has been proposed that TREK-1 is activated in response to intracellular acidification as a means to mitigate ischemic conditions that are generated during intense contractions (Harrison et al., 1994; Larcombe-McDouall et al., 1999); the belief being that the corresponding drop in pH as the cell becomes hypoxic will activate TREK-1, repolarizing the membrane, relaxing the tissue. We report strong activation of the suspected TREK-1 current through intracellular acidification using both $NaHCO_3$ (Figure 5), as well as with a pH 6.0 pipette solution (Figure 6).

Fluphenazine, a piperazine antipsychotic, is a potent dose-pendent and reversible inhibitor of TREK-1, but not TRAAK (Thummler et al., 2007). Fluphenazine almost entirely abolished TREK-1 currents in both pHUSMC and HEK293 hTREK-1 cells that had been activated with pH 6.0 pipette solution (Figure 8), further bolstering evidence that we were in fact activating TREK-1 currents in the pHUSMC cells.

Finally, as a means to confirm TREK-1 activity in cells that were not cultured or telomerized, we utilized freshly isolated primary uterine myocytes from pregnant and non-pregnant women. We verified that TREK-1 activates as predicted with arachidonic acid in these cells (Figure 9). Interestingly, the current in the non-pregnant myocytes, when challenged with arachidonic acid, was significantly smaller (Figure 9), a finding in line with expectations as TREK-1 is downregulated in non-pregnant myometrium.

Electrophysiological data obtained through the activation of TREK-1 by intracellular acidosis and arachidonic acid, as well as deactivation with fluphenazine and a shift in the reversal potential by an increase in extracellular K^+, are all in keeping with what would be expected of this particular channel. The electrophysiological characterization of wtTREK-1 in pHUSMC was an important first step in not only better understanding TREK-1's role in uterine quiescence, but was also important in establishing a baseline for which to compare its splice variants.

TREK-1 splice variants: Dr. Wu of our lab previously identified the transcripts of five TREK-1 SVs in the myometrium of pregnant women (Wu et al., 2012). Each of these SVs are truncated to a varying degree, with SV-1 only missing a single transmembrane domain, and SV-5 lacking exons 3, 4, 5, 6, and 7. It is of intense interest to determine whether expression of these SVs confer a functional significance to TREK-1, as any information garnered may reveal opportunities for therapeutic intervention in preterm labor.

Interestingly, the co-expression of each SV with wtTREK-1 decreased both basal (Figure 10) and $NaHCO_3$ activated currents (Figure 11). It has been shown that the presence of SV transcripts decrease the amount of wtTREK-1 found in the membrane (Wu et al., 2012), and our electrophysiological data corresponds with that data. Our findings are also in line with later experiments conducted by different research groups using TREK-1 SVs. For instance, TREK1 Δ Ex4 splice variant decreases channel activity in neurons (Veale et al., 2010), and in cases for which exon 5 was skipped, a SV which causes a corresponding frame shift in exon 6, resulted in a decrease in TREK-1 current when co-expressed with wtTREK-1, presumably through inhibition of wtTREK-1 vesicular trafficking (Rinné et al., 2014). The precise cause for the decrease in TREK-1 current in our cells is unclear, but may be the result of impaired trafficking, or by competition of the splice variants with TREK-1 for translation factors (Lodish et al., 2000), or by some other mechanism? Recently it has been discovered that TREK-1 can form heterodimers with other member of the KCNK family, such as TRAAK (Blin et al., 2016).

Therefore, it may be possible that in addition to the lowered density of wtTREK-1 found at the membrane, the decrease in TREK-1 activity seen in our cells may be confounded by heterodimeric SV/TREK-1 formation?

To conclude, before we can hope to identify novel tocolytics that will better treat preterm labor, we must first better understand what distinguishes the myometrium from other smooth muscles. It is a well-established fact that phenotypic variations among tissue sub-types alters their response to stimuli, and our findings confirm similar distinctions in the myometrium that affect how it behaves to endogenous and/or exogenous challenges. This research has shed new light on two important and unique aspects of myometrial function. First, we have further reinforced a growing body of evidence promoting the idea that sGC and global cGMP are not primary drivers of relaxation in the myometrium. We believe that ·NO's ability to relax the myometrium must lie outside of the canonical pathway. As will be discussed in chapter 5, we have evidence to support ·NO's ability to alter protein function through the direct actions of protein S-nitrosation. We have also found that the compartmented actions of pGC-C on cGMP signaling in pregnant myometrium relaxes the tissue, opening the door to potential therapeutic options using uGN as a tocolytic, as others have found (US patent document identifier: US 20120220526 A1; www.google.com/patents/US20120220526). These data, combined with the decreased aggregate activity of TREK-1 when co-expressed with it SVs, provide

new and important data that may be used to develop therapeutics to treat preterm labor.

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Chapter 3

The Role of S-nitrosoglutathione Reductase (GSNOR) in Human Disease and Therapy

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Scott D. Barnett and Iain L. O. Buxton
Department of Pharmacology, University of Nevada, Reno School of Medicine
Reno, NV 89557

(permission to reprint in Appendix C)

Abstract

S-nitrosoglutathione reductase (GSNOR), or ADH5, is an enzyme in the alcohol dehydrogenase (ADH) family. It is unique when compared to other ADH enzymes in that primary short-chain alcohols are not its principle substrate. GSNOR metabolizes S-nitrosoglutathione (GSNO), S-hydroxymethylglutathione (the spontaneous adduct of formaldehyde and glutathione), and some alcohols. GSNOR modulates reactive nitric oxide (·NO) availability in the cell by catalyzing the breakdown of GSNO, and indirectly regulates S-nitrosothiols (RSNOs) through GSNO-mediated protein S-nitrosation. The dysregulation of GSNOR can significantly alter cellular homeostasis, leading to disease. GSNOR plays an important regulatory role in smooth muscle relaxation, immune function, inflammation, neuronal development, and cancer progression, among many other processes. In recent years, the therapeutic inhibition of GSNOR has been investigated to treat asthma, cystic fibrosis and interstitial lung disease (ILD). The direct action of ·NO on cellular pathways, as well as the important regulatory role of protein S-nitrosation, are closely tied to GSNOR regulation and define this enzyme as an important therapeutic target.

Introduction

S-nitrosoglutathione reductase (GSNOR) is an important regulator of human health and disease. The modulation of protein S-nitrosation by GSNOR contributes to a host of maladies and can be exacerbated by the dysregulation of GSNOR. In recent years, much effort has been dedicated to identifying a safe and efficacious means to alter GSNOR activity. A myopic investigation of GSNOR would reveal little more than its inherit ability to metabolize S-nitrosoglutathione (GSNO) (Jensen et al., 1998), S-hydroxymethylglutathione (HMGSH) (Hedberg et al., 2000), and a handful of alcohols (Adinolfi et al., 1984b; Jensen et al., 1998). If we look beyond the direct actions of the enzyme itself, it quickly becomes apparent that GSNOR influences several downstream and parallel pathways (Figure 1). One of the most important is GSNOR's regulation of GSNO, and by extension, nitric oxide (·NO) and protein S-nitrosation. ·NO is a reactive nitrogen species (RNS) that is critical to the normal function of most cells type (Beckman and Koppenol, 1996; Moncada et al., 1991; Radi et al., 1991; Salvador Moncada, 1994). It is a powerful smooth muscle relaxing agent (Bradley et al., 1998a; I. L. O. Buxton et al., 2001; Ricciardolo et al., 2004; Tomita et al., 2002), cardiopulmonary regulator (Liu et al., 2004; Tamargo et al., 2010b), neuroeffector (Bredt and Snyder, 1992; Corti et al., 2014) and immune system modulator (MacMicking et al., 1997). ·NO is likely carried as GSNO from endothelium, and other sources, and acts as a stable ·NO reserve (Katarzyna A Broniowska et al., 2013; Smith and Marletta, 2012). GSNO can transfer

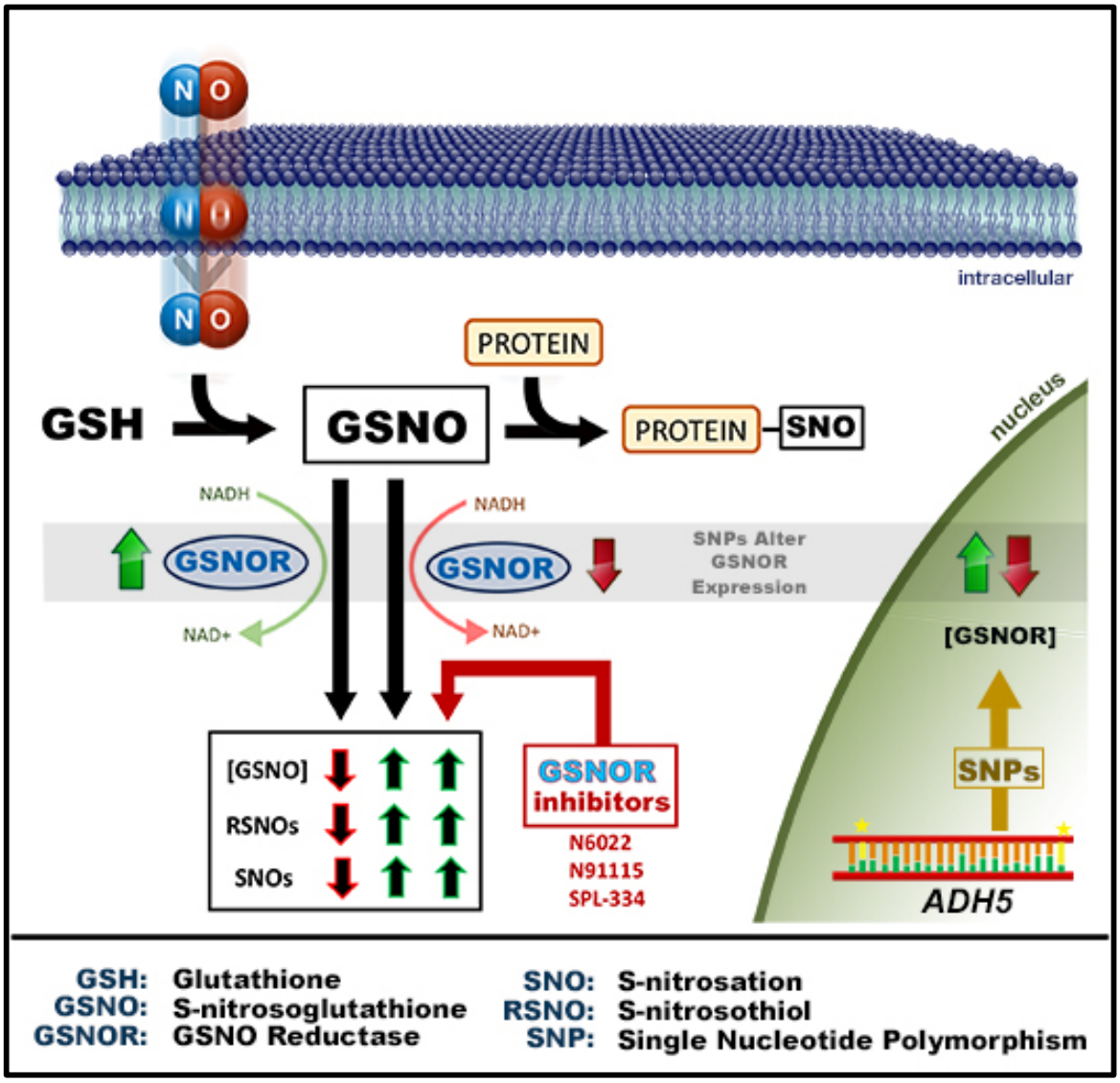

NO
NO
NO
intracellular
PROTEIN
GSH
GSNO
PROTEIN
SNO
nucleus
NADH
NADH
GSNOR
GSNOR
SNPs Alter
GSNOR
Expression
NAD+
NAD+
[GSNOR]
SNPs
[GSNO]
RSNOs
SNOs
GSNOR
inhibitors
N6022
N91115
SPL-334
ADH5
GSH: Glutathione
GSNO: S-nitrosoglutathione
GSNOR: GSNO Reductase
SNO: S-nitrosation
RSNO: S-nitrosothiol
SNP: Single Nucleotide Polymorphism

Figure 1 *Actions of GSNO and GSNOR in the cell*: ·NO enters the cell and reacts with glutathione (GSH) to create S-nitrosoglutathione (GSNO). GSNO can trans-S-nitrosate other proteins with compatible cysteines. GSNOR expression is regulated by single nucleotide polymorphisms (SNPs) in the promoter and 3' untranslated region of the gene, *ADH5*. The amount of GSNO and S-nitrosothiols (RSNOs) in the cells is proportional to GSNOR activity

its ·NO moiety to a cysteine thiol, resulting in the posttranslational modification (PTM) S-nitrosation/S-nitrosylation (Stamler et al., 1992b). S-nitrosation describes a thiol (*e.g.,* cysteine) converted to a S-nitrosothiol (RSNO) by a one-electron oxidation from the ·NO radical (Smith and Marletta, 2012). The term nitrosylation describes addition of an ·NO group to a metal centered protein such as guanylyl cyclase (Martínez-Ruiz and Lamas, 2004). Researchers have used both terms to describe ·NO addition to a protein thiol. We employ S-nitrosation to refer to protein modifications on cysteine residues. Protein S-nitrosations are also referred to in the literature in a fashion that takes into account protein and non-protein nitrosations (*e.g.,* RSNO). We employ the term RSNO as it appears in the literature.

Alcohol dehydrogenase family overview

The alcohol dehydrogenase (ADH) family of enzymes have been investigated for well over a century (Battelli, F and Stern, 1910; Daniel, 1909; Lutwak-Mann, 1938). They are evolutionarily conserved from bacteria to man (Gonzàlez-Duarte and Albalat, 2005; Liu et al., 2001a) and are categorized into five distinct classes that contain seven known isoforms (Table 1). ADH enzymes perform several important functions in human cells. The most well studied of these is the metabolism of short chain alcohols. Ethanol, being of significant cultural relevance due to its widespread consumption and abuse (Oscar-Berman and Marinkovic, 2003), has

led to an extensive investigation of the entire ADH family. Most ADH enzymes have some affinity for ethanol. In hepatocytes, ADH1A (formerly ADH1), ADH1B (formerly ADH2), and ADH1C (formerly ADH3) , are responsible for the oxidative catabolism of ethanol to acetaldehyde before further processing in the Krebs cycle, or elimination (Cederbaum, 2013). ADH4, a class II ADH (Svensson et al., 2001) whose sequence is 70% homologous to ADH1, catalyzes the oxidation of retinol (Vitamin A), and bolsters ethanol metabolism in the liver (Ramchandani et al., 2001). Numerous single nucleotide polymorphisms (SNPs) in the genes encoding the ADH family affect the rate of ethanol metabolism. These SNPs have been linked to some forms of alcoholism and cancer (Edenberg and Ph, 2007; Hurley and Edenberg, 2012). Other ADH SNPs have been correlated with schizophrenia, Parkinson's disease, asthma, and autism in certain populations (Bowers et al., 2011; Buervenich et al., 2000; Wu et al., 2007; Zuo et al., 2013). GSNOR (ADH5), the focus of this review, is differentiated from other ADH enzymes in that primary short chain alcohols, in particular ethanol, are not its principal substrate. ADH6 has been identified in both fetal and adult livers, but its function remains unclear as this enzyme has yet to be isolated for biochemical analysis (Edenberg and Ph, 2007; Östberg et al., 2016). A recent examination of ADH6 has provided evidence that it may act as an S-nitroso-CoA reductase (Anand et al., 2014). Similarly, ADH7's function remains elusive. Available data suggests ADH7 may serve a role in seemingly disparate cellular functions and diseases, such as: first pass gastric metabolism of ethanol (Lee et al., 2006), retinol metabolism

Gene Name	Principle Substrate	Uniprot Identifier	Subunits	Enzyme Class
ADH1A	Ethanol	P07327	α	I
ADH1B - (formerly ADH2)	Ethanol	P00325	β	I
ADH1C - (formerly ADH3)	Ethanol	P00326	γ	I
*ADH1(D-H)**	Unkown	-	-	-
ADH4	Ethanol/Retinol	P08319	π	II
ADH5	GSNO/HMGSH	P11766		III
ADH6	Ethanol/S-nitroso-CoA **	P28332	μ/σ†	V
ADH7	Retinol	P40394	σ	IV
*ADH(8-14)**	Unkown/Retinol**	-	-	-

*non-human **limited evidence †cDNA data

SOURCE: Modified from Edenberg et al., 2007

Table 1 *Alcohol dehydrogenase variants*: ADHs are most commonly known as highly effective metabolizers of ethanol. ADH5 varies from class I ADH isozymes in that GSNO and S-(hydroxymethyl)glutathione (HMGSH), the spontaneous adduct of formaldehyde and GSH, as its primary substrates. The function of all known ADHs has yet to be fully determined.

(Jennifer R. Chase, Mark G. Poolman, 2009), Parkinson's disease (Buervenich et al., 2000), and even personality traits in some individuals with substance dependence (Xingguang Luo, Henry R. Kranzler, Lingjun Zuo, Huiping Zhang, 2008). Clearly, the ADH family of enzymes perform a diverse and important role in the cellular metabolism of endogenous and exogenous chemicals. Here we focus on the function, significance and therapeutic potential of modulating GSNOR activity.

Nomenclature of alcohol dehydrogenases

The ADH family of enzymes has had several overlapping naming schemes in the past (Holmquist and Vallee, 1991; C. A. Staab et al., 2008). This has led to ambiguity in the literature and is due in part to the fact that naming assessments have historically been guided by substrate specificity, phylogenic classification, and publication date. GSNOR was not disambiguated from glutathione-dependent formaldehyde dehydrogenase until 1989 when it was found that these two proteins were in fact the same enzyme (Koivusalo et al., 1989). A formal attempt to reconcile the nomenclature began in 1999 when it was proposed that ADH proteins use numeric Arabic designators to identify each class of enzyme (Duester et al., 1999). In recent years the research community has generally adopted the gene naming guidelines put forth by the Human Genome Organization's Gene

Nomenclature Committee (Wain et al., 2002). Of all the ADH enzymes, GSNOR naming is particularly convoluted in this respect. While this protein is still sometimes referred to in the literature by its non-standard name, ADH3 (as in class III ADH), the official gene designator is now *ADH5*, and the protein is S-nitrosoglutathione reductase, ADH5, or alcohol dehydrogenase 5 (class III) χ-polypeptide. It can also be found in the literature under several other monikers: Formaldehyde dehydrogenase (FDH or FALDH); alcohol dehydrogenase X (ADHX); alcohol dehydrogenase class-3 (ADH-3); $\chi\chi$-ADH (homodimeric *chi* ADH); alcohol dehydrogenase 5; glutathione-dependent formaldehyde dehydrogenase (GSH-FDH); and S-(hydroxymethyl) glutathione dehydrogenase (EC 1.1.1.284). For purposes of clarity this review will address the gene as *ADH5*, and the protein as ADH5 or GSNOR.

ADH5: Structure/Localization

ADH5, the gene that encodes GSNOR, is located on the reverse strand of chromosome 4's (4q23 - chr4:99993567- 10000985) (Smith, 1986). *ADH5* is tandemly aligned in the same orientation as the other genes that encode for the entire family of ADH enzymes. Phylogenic analysis of the *ADH5* locus revealed that GSNOR evolved independently from class I & II ADH (Adinolfi et al., 1984a), and it is highly conserved across most vertebrate species (Foglio and Duester, 1996). GSNOR has a molecular weight of 39,724 Daltons and is translated to a

374 amino acid enzyme (UniProtKB identifier: P11766) *via* 9 exons (Hur & Edenberg 1992). Glu-67 and Arg-368 are highly conserved essential amino acids important to the catalytic mechanism of this enzyme (Sanghani et al., 2006). Splice variants of *ADH5* exist and result in the production of truncated proteins; however, their functional relevance has not been documented (Höög et al., 2001).

GSNOR functions as a homodimer (Figure 2) (Yang et al., 1997) and is localized to the nucleus and cytoplasm (Fernández et al., 2003). Amino acid substitutions in the subunit interacting portions of the coenzyme-binding domain prevent heterodimeric variants from being generated with other ADH enzymes (Julià et al., 1988). Each subunit binds a catalytic and structural Zn^{2+} cofactor (Kaiser et al., 1988; Östberg et al., 2016), for a total of four Zn^{2+} ions per functional enzyme. In addition to Zn^{2+}, GSNOR also requires a coenzyme that can vary based upon the substrate. These include: nicotinamide adenine dinucleotide (NAD+), its reduced form NADH, NADPH + H^{+}, or NAD(P)+ (Gupta, Kapuganti Jagadis, 2015; Hedberg et al., 2003; Jensen et al., 1998; Sanghani et al., 2000).

In general, ADH enzymes are highly expressed in the liver, the upper digestive tract and the kidneys (Zuo et al., 2013). *ADH5* RNA has been recognized in all major human tissue types with protein expression highest in smooth muscle, liver, epididymis, kidney and testis (Giri et al., 1989). GSNOR is an important negative regulator of neuronal differentiation during development (Wu et al., 2014) and is

the only known ADH enzyme present in the brain (Beisswenger et al. 1985; Galter et al. 2003). Conversely, GSNOR protein expression is negligible or non-existent in skeletal muscle, lymph nodes, spleen, bone marrow, cerebellum and the lateral ventricle (If and Wb, 2009).

Substrates

S-nitrosoglutathione*/formaldehyde*: As with most enzymes, GSNOR has a varying degree of affinity for several substrates. The two primary targets of GSNOR are GSNO, and HMGSH, the spontaneous adduct of formaldehyde and glutathione. HMGSH binds at the zinc active site and interacts with the highly conserved residues Arg114/115, Asp55, Glu57, and Thr46 (Engeland et al., 1993; Sanghani et al., 2002). That being said, the rate of substrate conversion (K_{cat}) is about 20-fold higher for GSNO over HMGSH (Green et al., 2012a; Hedberg et al., 2003; Salisbury and Bronas, 2015; Sanghani et al., 2000; Claudia A Staab et al., 2008a). Both reactions are dependent on an abundant source glutathione (GSH) in the cell. GSH is the major thiol in mammalian cells and while concentrations can reach as high as 10 mM (Bateman et al., 2008), they are typically 1 mM. Under stress conditions the concentration can fluctuate dramatically and drive GSNO towards atypical reactions (Figure 3) (Salisbury & Bronas 2015; Staab et al. 2009). The enzymatic activity of human recombinant GSNOR for GSNO exhibits a K_m of approximately 27 μM and a k_{cat} value of between 2,400 and 12,000 min^{-1}

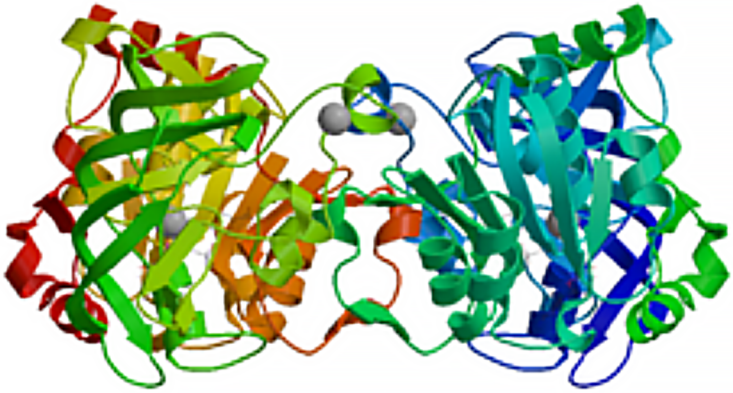


Figure 2 *GSNOR Quaternary structure model*: derived from X-ray diffraction (2.7 Å) and displayed as a functional χχ homodimer with (2) Zn+ ions and (1) NADH co-enzyme per subunit. (CC) swissmodel.expasy.org SMTL id 1teh.1

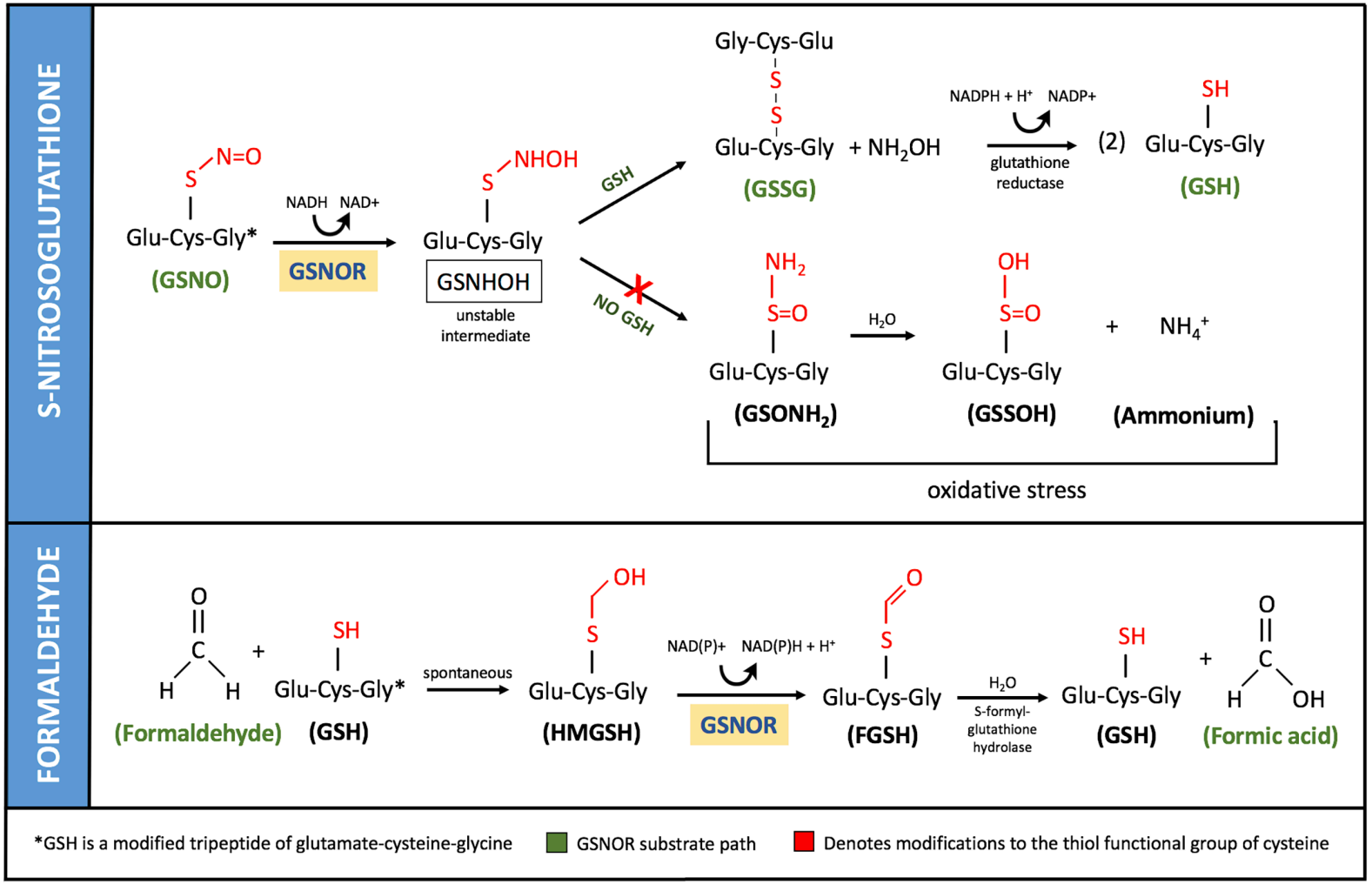

S-NITROSOGLUTATHIONE
Glu-Cys-Gly*
(GSNO)
NADH NAD+
GSNOR
Glu-Cys-Gly
GSNHOH
unstable intermediate
GSH
NO GSH
Gly-Cys-Glu
Glu-Cys-Gly
(GSSG)
+ NH2OH
NADPH + H+ NADP+
glutathione reductase
(2)
Glu-Cys-Gly
(GSH)
Glu-Cys-Gly
(GSONH2)
H2O
Glu-Cys-Gly
(GSSOH)
+ NH4+
(Ammonium)
oxidative stress
FORMALDEHYDE
(Formaldehyde)
+
Glu-Cys-Gly*
(GSH)
spontaneous
Glu-Cys-Gly
(HMGSH)
NAD(P)+ NAD(P)H + H+
GSNOR
Glu-Cys-Gly
(FGSH)
H2O
S-formyl-glutathione hydrolase
Glu-Cys-Gly
(GSH)
+
(Formic acid)
*GSH is a modified tripeptide of glutamate-cysteine-glycine
GSNOR substrate path
Denotes modifications to the thiol functional group of cysteine

Figure 3 *GSNOR metabolizes multiple substrates*: S-nitrosoglutathione (GSNO), one of the primary substrates for GSNOR, is first enzymatically degraded to an unstable intermediate, N-hydroxysulfinamide (GSNHOH). If glutathione (GSH) is present GSNHOH will be converted to glutathione disulfide (GSSG). Under certain condition, such as high levels of oxidative stress, GSH will not be sufficiently available, and other products, such as glutathione sulphinamide (GSONH2) and glutathione sulfinic acid (GSSOH) will be formed. Furthermore, GSNOR can oxidize the spontaneous adduct of formaldehyde and GSH, S-(hydroxymethyl)glutathione (HMGSH), to S-formylglutathione (FGSH).

(Fernández et al., 2003; Hedberg et al., 2003).

Alcohols: GSNOR more readily acts upon alcohols of greater chain length than class I ADH enzymes (Figure 4). This is due in part to a longer span between the binding and active site of the enzyme (Salisbury and Bronas, 2015), as well as amino acid substitutions that affect binding affinity (Julià et al., 1988; Östberg et al., 2016). As a result of these evolutionary divergences, GSNOR is not optimized for metabolizing short-chain alcohols. Consequently, it is not a misnomer to identify GSNOR as an alcohol dehydrogenase. GSNOR metabolizes both ethanol and medium/long change alcohols (preferring a double-bond in the beta position). The active site of GSNOR cannot be saturated by ethanol (Beisswenger et al., 1985), and the high activity of class I ADH enzymes toward ethanol minimizes the functional role of ethanol metabolism by GSNOR. Several K_m values for EtOH (all >2M) (Lee et al., 2003; Sharma C.P., Fox E.A., Holmquist B., Jornvall H., 1989) have been reported in the literature, with a k_{cat} of 33 ± 3 min^{-1} (Beisswenger et al., 1985; Lee et al., 2003). GSNOR's ability to metabolize EtOH is far surpassed by those of class I ADH enzymes whose K_m values range from 0.05 to 40 mM. As such, medium and long chain alcohols (> 4 carbons) (Holmquist and Vallee, 1991; Salisbury and Bronas, 2015; Theorell et al., 1969; Wagner et al., 1984) are more freely oxidized by GSNOR (Staab et al., 2009).

Other substrates: As with most enzymes, the entire cohort of ADH5 substrates is not fully known. Additional classes of molecules such as ω-hydroxy fatty acids (Achkor et al., 2003; Boleda et al., 1993; Moulis et al., 1991) exhibit a limited affinity for the enzyme. The ability of GSNOR to metabolize retinol remains in question. ADH7 (a class IV ADH) is the primary ADH accountable for retinol metabolism (Cederbaum, 2013), but there is evidence to support GSNOR's contribution in the retinoid-signaling pathway. Studies have shown that *ADH5*$^{-/-}$ null mice exhibit reduced retinoic acid production (Molotkov et al., 2002), and the presence of *ADH5* transcript in human fetal lungs correlates with a decrease in the presence of retinol (Coste and Labbe, 2011). Ultimately, the exact nature of relationship between GSNOR and retinol is still under investigation (Boleda et al., 1993; Cañestro et al., 2010; Gonzàlez-Duarte and Albalat, 2005).

GSNOR: Health & Disease

GSNOR is integral to the modulation of ·NO in the cell. ·NO is produced enzymatically in many cell types (Schmidt and Walter, 1994). Free ·NO is a highly reactive uncharged radical with a half-life of ~1-5 second *in vivo* (Kelm and Schrader, 1990), and will often establish a stable RSNO equilibrium with GSH in the form of GSNO (Wink and Mitchell, 1998). ·NO, and by extension, GSNO, plays a critical role in smooth muscle relaxation (Bradley *et al.*, 1998; Tomita *et al.*, 2002; Buxton, 2004 Ricciardolo *et al.*, 2004; Liu *et al.*, 2016) cardiopulmonary regulation

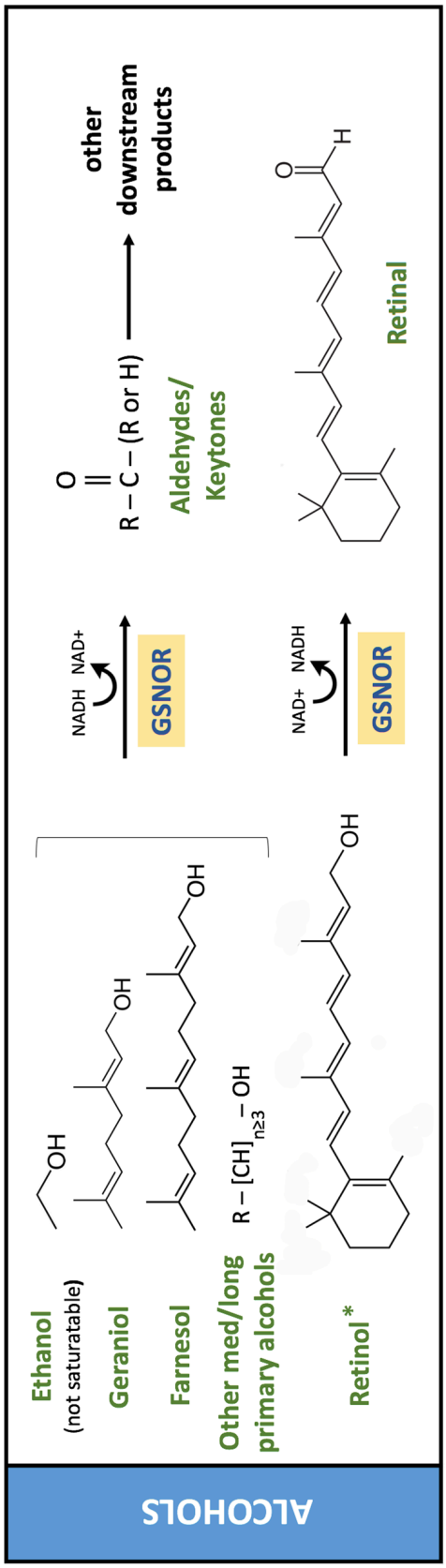

ALCOHOLS
Ethanol
(not saturatable)
Geraniol
Farnesol
Other med/long
primary alcohols
Retinol*
R – [CH]n≥3 – OH
NADH NAD+
GSNOR
O
R – C – (R or H)
Aldehydes/
Keytones
other
downstream
products
NAD+ NADH
GSNOR
Retinal

Figure 4 *GSNOR metabolism of alcohols*: GSNOR can metabolize medium and long chain primary alcohols to aldehydes and/or keytones before being further processed by other enzymes. GSNOR preferentially metabolizes alcohols with a double-bond on the beta carbon; however, despite having a poor affinity for ethanol, GSNOR is quite adept at metabolizing this molecule. *Evidence supporting GSNOR's ability to metabolize retinol to retinoic acid is limited.

(Rastaldo et al., 2007; Sears et al., 2004; Tamargo et al., 2010a) neuronal signaling (Shahani and Sawa, 2011), as well as dozens of other intra/extracellular functions (Pa´ L Pacher, Josehph S. Beckman, 1995; Salvador Moncada, 1994). The dysregulation of ·NO production and metabolism can lead to drastic changes in protein S-nitrosation (Foster et al., 2009a, 2003), an important posttranslational modification, and can have numerous other downstream consequences.

Oxidative/Nitrosative Stress: The dysregulation of GSNO through aberrant GSNOR modulation, when combined with oxidative stress, can further exacerbate disease. During conditions of cellular stress reactive nitrogen species, such as peroxynitrite (ONOO-), are formed when ·NO reacts with superoxide ($O_2^{\cdot}$-) (Squadrito and Pryor, 1998). Not only does oxidative stress commandeer available ·NO and GSH (Rahman and MacNee, 2000), but peroxynitrite can cross the cell membrane and react directly with protein thiols (Alvarez and Radi, 2003) , which may prevent S-nitrosation. RNS also induce S-glutathionylation of protein thiols (Dalle-Donne et al., 2009), further depleting the GSH pool (Klatt and Lamas, 2000). Decades of research have left little question as to detrimental effects of oxidative/nitrosative stress (Dalle-Donne et al., 2006; Guzik et al., 2002; Münzel et al., 1997), and the mechanistic underpinnings of this process have been thoroughly investigated (Apel and Hirt, 2004; Valko et al., 2007). For the purpose of this review it should be noted that this process can alter the levels of ·NO and GSH in the cell, which in turn can affect NO/GSNO signaling.

GSNO & S-nitrosation: Any investigation into the modulation/activity of GSNOR would not be complete without mention of S-nitrosation. The study of these PTMs and their influence on normal cell-signaling and disease has significantly impacted research and medicine for over 25 years (Broniowska and Hogg, 2012a; Foster et al., 2009a; Stamler et al., 1992a).

The detection and quantitation of RSNOs in biological systems is inherently challenging. The biotin switch technique (Jaffrey and Snyder, 2001), in which S-nitrosated cysteines are reduced and biotinylated, provides a simple and elegant method for the qualitative detection of S-nitrosated proteins. An analysis of a wide variety of RSNO measurement techniques, including the biotin switch, has established that artifacts are common when measuring RSNOs and it is not always possible to identify which thiols have been S-nitrosated (Giustarini et al., 2003). Newer techniques have become available in recent years (Chen et al., 2013; Devarie-Baez et al., 2013), such as tandem mass spectrometry (MS/MS) of S-nitrosated protein thiols (Murray et al., 2012; Ulrich et al., 2013b), that are highly quantitative. Beyond the problem of quantitation, it has been proposed that other thiol modifications such as dithiol/disulfide exchange, S-glutathionylation, and oxidation, may affect signaling more readily than do RSNOs (Lancaster, 2008), and should be investigated along with S-nitrosation.

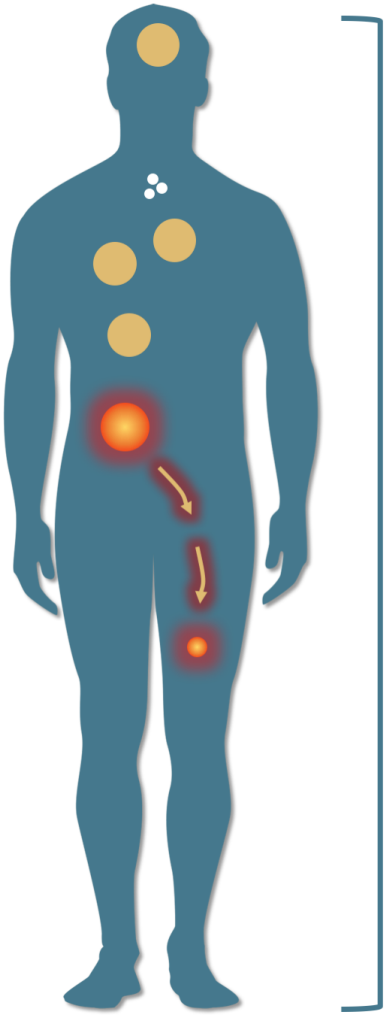
As with phosphorylation, S-nitrosation regulates cellular mechanisms and affects protein-protein interactions. The intracellular availability of nitric oxide and its functional derivatives, like GSNO, affect protein S-nitrosation (Broniowska and Hogg, 2012a; Hess et al., 2005a; Thomas and Jourd'heuil, 2012a). GSNOR is a potent negative regulator of GSNO in smooth muscle (Que et al., 2009). The aberrant expression of ADH5, as with many ADH subclasses, is associated with disease (Jelski et al., 2009; Jelski and Szmitkowski, 2008; Laniewska-Dunaj et al., 2013). In fact, the deletion of the *ADH5* gene increases both the levels of GSNO and total protein S-nitrosation in vivo (Liu et al., 2001a). Protein S-nitrosation is of intense interest to researchers and clinicians as the hypo/hyper-S-nitrosation of a diverse set of proteins, spanning nearly every tissue types, can have a drastic effects in disease (Foster et al., 2009a). Some of these include: Type 2 diabetes (Akt et al., 2005), sickle cell anemia (Bonaventura et al., 2002, 1999), ventricular arrhythmia in individuals with Duchenne muscular dystrophy (Fauconnier et al., 2010), cell death and survival pathways (Anand Krishnan V. Iyera, Yon Rojansakulb, 2011), post-infarct cardio-protection (Methner et al., 2014), pregnancy/parturition (Ulrich et al., 2013b), and many others. Interestingly, GSNOR itself is a cysteine rich protein that is S-nitrosated by GSNO, which in turn initiates a feedback loop that affects GSNOR expression (Guerra et al., 2016) and activity (Brown-Steinke et al., 2010)(Barnett et al., 2017). Although it is beyond the scope of this review, it should be noted that GSNOR dysregulation in plants can

result in significant biotic and abiotic nitrosative events that affect growth, development, and survival (Leterrier et al., 2011; Shi et al., 2015; Yun et al., 2016).

GSNOR dysregulation

GSNOR dysregulation has been implicated in numerous disease sates (Figure 5). The use of models and *ADH5*$^{-/-}$ knockout animals has uncovered surprising and valuable data related to GSNOR function. RSNO levels, as well as canonical NO-mediated pathways, are severely altered when GSNOR activity is modulated.

Cardiovascular health: One of the major organs affected by GSNOR is the heart and surrounding vascularity. It has long been known that ·NO and S-nitrosation protect the body from cardiovascular disease. Following myocardial infarction *ADH5*$^{-/-}$ mice exhibit enhanced cardiac regenerative capabilities as a result of increased cardiac stem cell turnover (Hatzistergos et al., 2015), as well as a reduction in myocardial infarct size and higher coronary vascular density (Lima et al., 2009). Moreover, de-S-nitrosation of cardiac ryanodine receptor 2 (RyR2) in *ADH5*$^{-/-}$ mice results in decreased peripheral vascular tone due to calcium “leak” (Beigi et al., 2012). In skeletal muscle only about 1 in 50 cysteines on the ryanodine receptor are S-nitrosated, indicating that this PTM, even when conservatively distributed, can drastically alter protein function (Sun et al., 2001). Taken together this data suggests that RyR2 S-nitrosation modulates calcium storage in the



ORGAN	GSNOR REGULATION	DISEASE
Brain	↓ ↑	• Parkinson's Disease • Neuronal Development • Hypoxia
Immune System	↓	• Decrease CD4 Single-positive Thymocytes • Increases Lymphocytic Apoptosis
Lungs	↑	• Asthma • Cystic Fibrosis • SNPs
Heart	↓ ↑	• Myocardial Infarction • Dilative Cardiomyopathy • Vascular Tone
Liver	↓	• Human Hepatocellular Carcinoma (HCC)
Cancer	↓	• Breast Cancer (HER2) • DNA Repair (AGT) • iNOS Regulation • Formaldehyde Metabolism

Figure 5 *Diseases associated with GSNOR dysregulation*: The dysregulation of GSNOR can initiate or exacerbate many disease states. This is due in part to GSNOR's indirect function as a S-nitrosothiol modulator, as well as ability to mediate canonical NO cell signaling though GSNO metabolism. GSNOR inhibitors are being actively investigated to treat certain disorders in which increased NO availability would be beneficial.

sarcoplasmic reticulum. There is clearly a complex relationship between the correlative observation of an increase in S-nitrosation and GSNOR dysregulation.

Immune system: GSNOR performs an important protective role in the immune system's development of lymphocytes. *ADH5*$^{-/-}$ KO mice show increased RSNO production that decreases CD4 single-positive thymocyte development, and increases lymphocytic apoptosis (Yang et al., 2010). Damage to immune cells from nitrosative stress in *ADH5*$^{-/-}$ mice results in a significant increase in the animal's susceptibility to pulmonary infection by *K. pneumoniae* as well as multi-fold increases of the bacteria in the spleen and blood, resulting in increased inflammation (Tang et al., 2013a). Enhanced nitric oxide synthase (NOS) 2 activity in monocytes and macrophages increases ·NO production and elicits a cytostatic or cytotoxic response against bacteria, viruses and other intruders, but also increases inflammation (MacMicking et al., 1997). The bronchoalveolar lavage fluid of asthmatics consists of high macrophage levels as well as a significant increases GSNOR activity (Que et al., 2009). Inhibiting GSNOR in these patients increases total RSNOs and restores inflammatory markers to near baseline levels while limiting ova-induced NFκB activation (Blonder et al., 2014). Ultimately, the balance between GSNOR activation and inhibition is critical in maintaining balance in the immune system.

Brain development and function: GSNOR regulation in the brain affects a broad swath of cellular functions ranging from neural development and maturation to

other neurodegenerative diseases more typically associated with adult and geriatric populations. These disease states are often the result of aberrant protein S-nitrosation caused by the dysregulation of GSNO. For instance, in developing and adult mouse brains the overexpression of GSNOR results in decreased neuronal differentiation in part due to de-S-nitrosation of histone deacetylase 2 (HDAC2) (Wu et al., 2014). Conversely, *ADH5*$^{-/-}$ mice exhibit neuromuscular atrophy as a result of a decrease in muscle mass, while also presenting with neuropathic behavior (Montagna et al., 2014). In *Drosophila*, GSNOR overexpression results in visual pattern memory defects which can be rescued by co-expression of cyclic-GMP dependent protein kinase G (PKG) (Hou et al., 2011). This occurs independently from neuronal development and implies an adjacent regulatory role for GSNOR the PKG phosphorylation pathway. Neuronal homeostasis is also affected by GSNOR. In a Parkinson's disease model using neuronal (SH-SY5Y) cells a decrease in GSNOR availability results in activation of nuclear factor Nrf2 ((erythroid-derived 2)-like 2), which regulates the expression of antioxidant proteins (Rizza et al., 2015). Interestingly, GSNOR may also affect the phosphorylated state of platelet-derived growth factor receptor-β (Palmer et al., 2015) in the brainstems of mice during hypoxic exposure. When these data are considered as a whole, it is apparent that deviating GSNOR activity and expression from baseline can have drastic consequences in both the developing and mature brain.

Cancer: The link between GSNOR dysregulation and cancer is not well understood. GSNOR deficiency has been known to affect the rate of genomic mutations in mice by increasing the frequency of A:T to T:A transposition (Leung et al., 2013). This may be the result of a GSNOR-mediated reduction in activity of the DNA repair protein O6-alkylguanine-DNA alkyl transferase which can lead to an increase in the rate of human hepatocellular carcinoma (HCC) (Tang et al., 2012; Wei et al., 2011, 2010). Pharmacologic inhibition of inducible NOS (iNOS) when GSNOR is down-regulated shows strong potential as a therapeutic for those patients with HCC (Chi-Hui Tang, Wei Wei, Martha A. Hanes, 2013). As with HCC, some types of breast cancer are linked to a decrease in GSNOR expression. Specifically, high levels of human epidermal growth factor receptor 2 (HER2) expression in breast tumors is associated with low GSNOR expression and an increase in apoptosis-related protein S-nitrosation (Cañas et al., 2016). This study also determined that an increase in GSNOR expression in *HER2* tumors correlates with higher patient survival and begs the question as to whether or not NOS inhibition would also serve this population well. These examples are of course complicated by the fact that ·NO is a pleiotropic regulator of gene function and the modulation of GSNO by GSNOR can have both cytostatic and cytotoxic effects on tumor survival (Xu et al., 2002). To this point, GSNOR is effective at removing formaldehyde, a known carcinogen, from the cell; however, *ADH5* polymorphisms do not significantly affect an individual's capacity to protect against DNA damage when exposed to formaldehyde (Xie et al., 2010). Furthermore, $ADH5^{-/-}$ mice are

known to generate DNA damage when formaldehyde forms and adduct with guanine to create N2-hydroxymethyl-*d*G which can result dysfunction of hepatocytes and nephrons (Pontel et al., 2015).

Asthma & Single nucleotide polymorphisms (SNPs): SNPs can alter the transcriptional output of a gene as well as the structure/function of proteins they encode. Several SNPs in the promoter and 3' UTR of the *ADH5* gene can result in the aberrant expression of GSNOR (Choudhry et al., 2010a). Of particular interest is the observation that airway hyperesponsivity in wild-type mice correlates with increased expression of GSNOR and decreased RSNO production, while *ADH5*$^{-/-}$ mice are protected from airway hyperresponsiveness and maintain higher total RSNO levels (Que et al., 2005). In humans GSNOR upregulation can lead to changes in airway smooth muscle tone in asthmatics (Henderson and Gaston, 2005a; Wu et al., 2007). A study involving Mexican children with asthma who possess SNPs in the promoter region of *ADH5* at suspected NF-κB binding sites (rs2602899 and rs2851301), were found to exhibit a decreased relative risk of asthma due to suppressed GSNOR production (Wu et al., 2007). Interestingly, alternative SNPs (rs1154404 and rs28730619) were associated with an increase in childhood asthma risk, although the mechanism behind this correlation has not been determined (Wu et al., 2007). Another study in African American children found that SNPs in *ADH5* and the β2 adrenergic receptor gene are associated with acute response to asthma-specific therapy (Moore et al., 2009a).

Looking beyond GSNO-mediated relaxation of airway smooth muscle we may also consider GSNOR's ability to metabolize formaldehyde, a chemical known to induce bronchoconstriction after long term exposure at low concentrations (Leikauf, 1992). It has been suggested that the presence of formaldehyde in airway smooth muscle may stoichiometrically favor bound NADH/GSNOR, thereby increasing GSNOR metabolism of GSNO, and by extension, promote smooth muscle contraction (Thompson and Grafström, 2007).

Regardless of the mechanism driving GSNOR-mediated consumption of GSNO in airway smooth muscle, it is easy to see why the inhibition of GSNOR has been of particular interest to researchers for its therapeutic potential as a smooth muscle relaxant.

Myoendothelial Junctions: GSNOR plays an interesting role at myoendothelial junctions (MEJ) where it co-localizes with the hemichannel Connexin-43 (Cx43). Cx43 hemichannels form gap junctions between cells by linking to hemichannels in opposing membranes to couple endothelial and vascular smooth muscle cells and when Cx43 is S-nitrosated this pore allows for the free movement of inositol trisphosphate from vascular smooth muscle to endothelial cells. Due to the co-localization of GSNOR and Cx43 at the MEJ, basal ·NO availability at this site is blunted, which in turn increases the likelihood that Cx43 will not be S-nitrosated

(Straub et al., 2011). This decreases channel permeability until Ca^{2+} levels increase as a result of smooth muscle cell stimulation, which in turn activates eNOS and increases the probability of Cx43 S-nitrosation.

Myometrium: ·NO is an important mediator of relaxation in the myometrium. It has been well established that ·NO relaxes vascular and gastrointestinal smooth muscle by activating soluble guanylyl cyclase (sGC), which in turn converts guanosine triphosphate to cyclic guanosine monophosphate (cGMP), activating PKG, which in turn dephosphorylates the regulatory light chain (MYL9) of myosin *via* the amplified phosphatase activity of MYPT1 (pS695) (Grassie et al., 2011; Nakamura et al., 2007; Puetz et al., 2009; Roux et al., 2012). This is not the dominant ·NO-mediated relaxation pathway in uterine smooth muscle however. ·NO can relax the myometrium even when sGC has been inhibited (I L O Buxton et al., 2010b). The pathway through which NO relaxes the myometrium independent of cGMP is unknown, but it is likely that the S-nitrosation of contractile proteins plays a role. It has been determined that the state of labor (full term vs. preterm) can vastly alter the S-nitrosated protein landscape in uterine smooth muscle after exposure to GSNO (Ulrich et al., 2012a). It is also well known that S-nitrosation can vary significantly based upon the cytoplasmic availability GSNOR (Broniowska and Hogg, 2012a; Hess et al., 2005a; Thomas and Jourd'heuil, 2012a). Regardless of the pathway through which ·NO acts to relax uterine smooth muscle, it does beg the question as to whether or not inhibiting GSNOR, and

thereby increasing intracellular availability of GSNO, may serve as an effective tocolytic strategy by promoting uterine quiescence through ·NO-mediated relaxation pathways. This notion is supported by data showing an increased expression of GSNOR in patients delivering spontaneously preterm.

Therapeutic Inhibition of GSNOR

GSNOR is an attractive therapeutic target. GSNOR inhibition increases GSNO availability in the cell and in turn facilitates ·NO-mediated signaling pathways. Dozens of small molecules have been identified that can inhibit GSNOR to varying degrees (Green et al., 2012a; Jiang et al., 2016a; Sanghani et al., 2009; Sun et al., 2012, 2011a, 2011b). Two of these, N6022 (3-(5-(4-(1H-imidazol-1-yl) phenyl)-1-(4-carbamoyl- 2-methylphenyl)-1H-pyrrol-2-yl) propionic acid) and N91115 from Nivalis Pharmaceuticals show promise as potentially safe and effective GSNOR inhibitors that have undergone clinical trial for both the treatment of mild asthma (clinicaltrials.gov - NCT01316315), and cystic fibrosis in individuals who are heterozygous for the cystic fibrosis transmembrane conductance regulator (CFTR) gating mutation CFTRΔF508+ (clinicaltrials.gov – N6022: NCT01746784; N91115: NCT02724527). Endogenous GSNO levels are low in the airways of cystic fibrosis patients (Grasemann et al., 1999) and GSNOR inhibition is an appealing alternative to the direct administration to of GSNO (Snyder et al., 2002; Zaman et al., 2013, 2001). N6022 is well tolerated with minimal side effects, even at high

concentrations, in both animals (Blonder et al., 2014; Colagiovanni et al., 2012a) and humans (clinicaltrials.gov – NCT01147406, NCT01746784). Another GSNOR inhibitor, SPL-334 (4-{[2-[(2-cyanobenzyl) thio]-4-oxothieno[3,2-d]pyrimidin-3(4H)-yl]methyl}benzoic acid) from SAJE Pharmaceuticals (Baltimore, MD), is being tested as a therapeutic to treat allergic asthma and interstitial lung disease (ILD). Using an allergic asthma mouse model, intranasally administered SPL-334 decreased CD4+ Th2 cytokines, eosinophils, and mitigated the lung inflammatory response (Ferrini et al., 2013a). Likewise, in a mouse model of ILD SPL-334 functions as both a prophylactic agent and a therapeutic to attenuate profibrotic cytokines and collagen accumulation in the lungs (Luzina et al., 2015). Unlike N6022 and N91115, SPL-334 is not in human clinical trials.

FDA-approved drugs are also being tested as potential GSNOR inhibitors. Nebivolol, a $ß_1$-adrenergic receptor antagonist used for the management of hypertension, has been shown to increase total RSNO levels in animal and cell models (Jiang et al., 2016a). Our own investigation of GSNOR fails to confirm Nebivolol as an inhibitor of GSNOR in an enzyme activity assay. Since there are no FDA-approved GSNOR inhibitors, the repurposing of existing therapeutic agents that inhibit GSNOR and/or modulate GSNO and RSNOs is of interest.

When considering GSNOR inhibitors as therapeutic agents, it should be taken into consideration that enzymes other than GSNOR modulate ·NO availability in the

cell. ·NO is critical to the normal function of most cells types, and as is often the case, there are multiple concurrent and complementary mechanisms to regulate ·NO and RSNOs (Benhar et al., 2009; Liu et al., 2001b). Two of the most well-known are thioredoxin-1 (Sengupta and Holmgren, 2012a, 2012b) and carbonyl reductase (Bateman et al., 2008). NOS, the predominate source of ·NO in the body, can also be dysregulated in certain disease states, as can its substrate, L-arginine (Ckless et al., 2007). For instance, after stimulation of the cavernous nerve in *ADH5* $^{-/-}$ mice, eNOS phosphorylation did not increase as predicted (Musicki et al., 2016). Modulating GSNOR activity may insufficiently control, or even aggravate some conditions if these alternate •NO-regulators are the source of the disorder. Unfortunately, direct application of endogenous •NO-donors, such as GSNO, Cys-NO, or SNO-albumin, as well as some exogenous donors, are of limited clinical value because they either degrade rapidly, cause intolerable side effects, or lead to a toxic systemic build up nitrates (H.H. Al-Sa'doni, 2005).

The therapeutic inhibition of GSNOR to treat ·NO-mediated disorders should be weighed carefully against potential contraindications. For example, the inhibition of GSNOR may increase a patient's susceptibility to bacterial or viral infection. The inhibition of GSNOR will also increase total RSNO levels and this can have adverse effects in the body, especially if the drug is administered systemically and not targeted to a specific tissue type through means such as liposomal delivery. GSNOR regulation varies widely in different cancer types (Cañas et al., 2016; Chi-

Hui Tang, Wei Wei, Martha A. Hanes, 2013). Inhibiting GSNOR may lead to a further increase in GSNO at the tumor site which can favor angiogenesis (Prudente et al., 2017). Conversely, with disorders such as asthma and hypertension, GSNOR inhibition results in the desired relaxation of the smooth muscle.

Conclusion

·NO, and by extension ·NO-donors, have been investigated intensely for over a century as therapeutics (Schmidt and Walter, 1994). ·NO modulation not only affects traditional pathways connected to this highly reactive molecule, but it also drastically alters S-nitrosation levels in the cell. GSNOR is unique among the ADH family of enzymes in that it targets GSNO and varies the body's response to endogenously generated NO carried as GSNO. *ADH5*$^{-/-}$ animal and cell models have provided a unique window into the importance of GSNOR in nearly every tissue type. The up/down regulation of GSNOR in humans has also provided invaluable data to the medical and research communities concerning its role in disease states. There are currently no FDA-approved modulators of GSNOR; however, several drugs are being investigated, and some are in clinical trial. Indeed, our understanding of the dysregulation of GSNOR and its effect on protein S-nitrosation and other glutathione/·NO-mediated events is in its infancy. Further investigations into the role of GSNOR in health and disease are needed to reveal the most effective therapeutic options.

Disclosure Statement

The authors declare that they have no conflict of interest.

Chapter 4

S-nitrosoglutathione Reductase and Spontaneous Preterm Labor

Abstract:

The underlying mechanism(s) of spontaneous preterm labor (sPTL) are unknown. Currently available therapeutics do not reliably delay preterm birth beyond 48-hours after the onset of labor. More effective tocolytics will require a better understanding of the pathophysiology of sPTL. Here we show that sPTL myometrium exhibits a blunted relaxation response to ·NO, a finding that corresponds to our discovery that S-nitrosoglutathione reductase (GSNOR), an enzyme that regulates ·NO, is upregulated sPTL myometrium as well. We investigate GSNOR inhibitors to establish if they serve as effective tocolytics. We find that N6022, a known GSNOR inhibitor, and nebivolol, which does not inhibit GSNOR, attenuate uterine contractions, although through differing mechanisms, revealing a novel class of tocolytics for the treatment of sPTL.

INTRODUCTION

Approximately 15 million preterm births occur annually worldwide (Blencowe et al., 2012). Preterm infants that survive are at risk for learning disabilities, cerebral palsy, vision and hearing loss, respiratory and digestive problems (Ray and Lorch, 2013). In 2012, more than 11% of US births were premature (Hamilton et al., 2013). The etiology of spontaneous preterm labor (sPTL) is almost certainly a complex amalgam of disparate medical, environmental, and genetic risk factors thought to converge on effector pathways in the myometrium to influence contractility and birth timing in women (Romero et al., 2014). Tocolytics used to prevent or halt sPTL, in an effort to prevent preterm birth, are not FDA approved for this purpose, and on average are said to delay labor for only 48 hours (Elvira et al., 2014), a window for antenatal steroid (Roberts et al., 2017), but hardly a solution to the problem. Microbial infection might initiate preterm labor (PTL) in some cases, but antibiotic treatment, prophylactic or otherwise, does not prevent preterm birth (Prince et al., 2014; Vinturache et al., 2016). If we are to advance our understanding of preterm labor in order to delay or prevent preterm birth, we posit that understanding the biochemical mechanisms of relaxation of the uterus is paramount. This point is bolstered when we consider that employing tools such as terbutaline, used to relax airway smooth muscle, or nifedipine, used to relax vascular smooth muscle, in an effort to prevent preterm labor are borrowed pharmacology. Even Atosiban, a selective oxytocin–vasopressin receptor

antagonist designed specifically to mitigate contractions, is not approved for use in the United States and does not reduce the risk of preterm birth or improve neonatal outcome (Papatsonis D, Flenady V, Cole S, 2005). It is not unreasonable to conclude that myometrial relaxation signaling is unique, and a detailed understanding of myometrial relaxation signaling is urgently needed.

Unlike most tocolytics, few drugs target the relaxation pathway in an attempt to mitigate contractile force and frequency. Nitric oxide (·NO), a powerful endogenous smooth muscle relaxing agent, is an interesting therapeutic target. In pregnant women, the administration of nitric oxide or NO-donors, such as with nitroglycerine transdermal patches, show little (Smith et al., 2007) to no (Nankali et al., 2014) clinical efficacy; and as we will show here, ·NO fails to quiesce sPTL myometrium. This is not to dismiss the role of ·NO in the myometrium. ·NO functions as an important endogenous mediator of relaxation in myometrium, and we offer evidence that ·NO metabolism is dysregulated in sPTL uterine smooth muscle.

·NO availability in the myometrium is regulated by enzymes such as thioredoxin (Trx) and its cognate reductase (TrxR) (Sahlin et al., 2000), carbonyl reductase (Khan et al., 2010), and the class-III alcohol dehydrogenase, S-nitrosoglutathione reductase (GSNOR or ADH5). GSNOR utilizes the co-enzyme NADH to carry out a 2^{e-} reduction of GSNO to generate glutathione sulfinamide (Claudia A Staab et al., 2008b) before it is further reduced back to glutathione by glutathione reductase (Figure 1). A drug that could effectively mitigate ·NO

metabolism, while minimizing adverse or off-target effects — a common problem with these classes of drugs — could function well as a tocolytic. Here we look closer at GSNOR and its function in myometrial quiescence.

The dysregulation of GSNOR is linked to many diseases throughout the body (Scott D. Barnett and Buxton, 2017b). The possibility that the dysfunctional expression of GSNOR may contribute to sPTL has not been previously investigated; however, there is precedence to merit a more detailed exploration of GSNOR's role in pregnancy and parturition. What initially lead our laboratory to investigate GSNOR is its known dysregulation in airway smooth muscle (Wu et al., 2007), which shares many of the same biochemical pathways as the myometrium, including its responsiveness to ·NO. In humans, GSNOR upregulation heightens airway smooth muscle tone in asthmatics (Henderson and Gaston, 2005a; Wu et al., 2007). Conversely, studies involving *ADH5*$^{(-/-)}$ knockout mice show that these animals are protected from airway hyperresponsiveness (Que et al., 2005), presumably due to the increased availability of ·NO, as well as an increase in s-nitrosothiols (SNOs), a topic that will be covered in chapter 5. While correlative and not necessarily causal, it has also been reported that asthma is a positive predictor of preterm labor with a relative risk estimate of 2.33 (Doucette and Bracken, 1993). Here we investigate the relationship between GSNOR and the sPTL, and we examine the usefulness of GSNOR inhibition as a tocolytic to mitigate contractions in the myometrium.

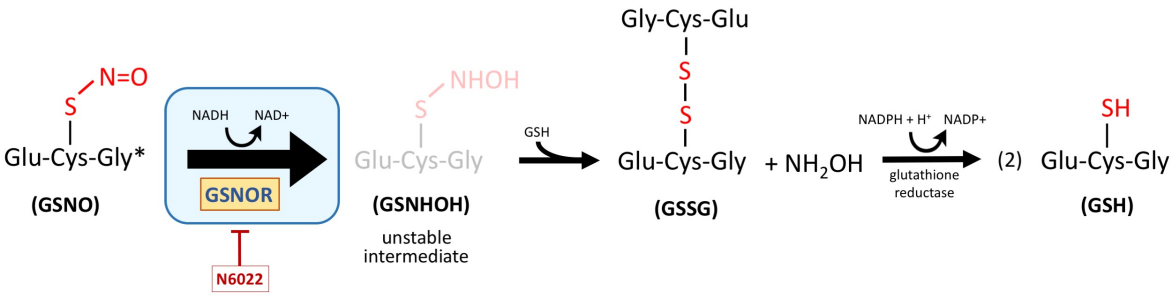

N=O
S
Glu-Cys-Gly*
(GSNO)
NADH
NAD+
GSNOR
N6022
NHOH
S
Glu-Cys-Gly
(GSNHOH)
unstable
intermediate
GSH
Gly-Cys-Glu
S
S
Glu-Cys-Gly
(GSSG)
+ NH2OH
NADPH + H+
NADP+
glutathione
reductase
(2)
SH
Glu-Cys-Gly
(GSH)

Figure 1: *GSNO Metabolism by GSNOR* **-** GSNOR metabolizes multiple substrates, but has the highest affinity for S-nitrosoglutathione (GSNO). GSNOR requires Zn^{2+} and NADH (co-factor/co-enzyme) for activation, and can be inhibited by a number of drugs, to include N6022. GSNO is first enzymatically degraded to an unstable intermediate, N-hydroxysulfinamide (GSNHOH). If sufficient glutathione (GSH) is available, GSNHOH will be spontaneously converted to glutathione disulfide (GSSG). Glutathione reductase returns GSSG back to GSH, completing the cycle (not shown).

Material and Methods:

Tissue collection. Modified from (Ulrich et al., 2013c). Human tissue collection and research was approved by the University of Nevada Biomedical Review Committee for the protection of human subjects. Human uterine myometrial biopsies were obtained with written informed-consent from mothers undergoing Cesarean section, and did not have infection or rupture of membranes. Tissues were stored in cold Krebs buffer containing: NaCl (118mM), KCl (4.75mM), $CaCl_2$ (2.5mM), KH_2PO_4 (1.2mM), $NaHCO_3$ (25mM), $MgCl_2$ (1.2mM), dextrose (20mM), and adjusted to pH 7.4. Tissues were microdissected under magnification to isolate smooth muscle, employed in contractile experiments or snap frozen in liquid nitrogen, and stored at -150°C. The average age for patients in the pregnant laboring group was 28.9 ± 5.6 yr and in the preterm laboring group 34.1 ± 2.5 yr. Pregnant laboring patients ranged from 39 to 41 wk gestation, with the mean at 39 wk. Preterm laboring patients without evidence of infection, PROM or preeclampsia ranged from 29.2 to 36 wk of gestation.

Animal studies were approved by the University Institutional Animal Care and Use Committee. Dunkin-Hartley Guinea pigs (Elm Hill, Chelmsford, MA) were purchased as either virgin juveniles (300-350g) and bred on site, or as timed-pregnancies (30-35d). Non-pregnant guinea pigs were estrogen primed (3mg/kg β-estradiol) 48-hours prior to tissue collection to ensure alignment of estrous cycles. Virgin female guinea pigs, and timed-pregnant animals, were sacrificed

under isoflurane anesthesia. Uterine tissue was dissected and used immediately as previously described (Iain L O Buxton et al., 2010a) or flash frozen and stored at -80°C.

Contractile studies. Strips of myometrium (~0.5x15mM) were clip-mounted by silk thread, attached to a force transducer, and isometrically stretched to an initial tension of 1.2x tissue length in an organ bath (WPI, Sarasota, FL) containing Krebs buffer. Tissues were maintained at 37°C and gently bubbled with balanced oxygen (95% O_2, 5% CO_2). Tissues were then challenged with KCl (60mM replacing NaCl) for 3 min, followed by washout, then allowed to equilibrate for 1 hr, during which time regular spontaneous contractions were seen. Only tissues that responded to KCl-challenge were employed in experiments. Under some conditions, tissues were further challenged with oxytocin (10nM), followed by washout. Both Cysteine-NO (100μM) or GSNO (300μM) were made daily. Data were analyzed with LabScribe (version 3.015800, Mac OS 10.11, iWorx systems inc., Dover, NH). Aorta was collected from 3-month-old Sprague-Dawley rats, cut into 2mm rings, and hung by stainless steel triangles that were passed through the lumen of each ring.

Wes Protein Assay. Each sample was ground to a powder under liquid nitrogen and reconstituted in RIPA buffer (0.8 mg/ml final): Tris pH 7.5 (20mM), NaCl (150mM), EDTA (1mM), EGTA (1mM), NP-40 (1%), sodium deoxycholate (1%), and protease inhibitors (cat.78430: Thermo Fisher Scientific Inc., Waltham, MA). Wes was run according to manufacturer protocols (SM-W004 -

ProteinSimple, San Jose, CA) using a 12-230 kDa Wes Separation Module coupled to a 25-capillary cartridge. GSNOR was labeled with rabbit anti-ADH5 primary antibody (1:100 dilution, ab59134: Abcam, Cambridge, MA) and mouse anti-GAPDH (1:100 dilution, sc-47724: Santa Cruz Biotechnology, Inc., Dallas, TX). Linearity of ADH5 1° Ab was tested (Supp. Figure 1A/B), as well as specificity of the antibody in guinea pig tissue lysates (Supp. Figure 1C). GSNOR and GAPDH were not multiplexed due to insufficient separation of bands as a result of similar molecular weights. Protein identification and quantification was determined using Compass software (version: 2.7.1, Mac OS 10.11: ProteinSimple, San Jose, CA), followed by Prism (version 7.0c for Mac OS 10.11, GraphPad Software, La Jolla California USA) when necessary. Power analysis for each cohort was set to alpha=0.05 and an 90% power level (G*power, Düsseldorf, Germany). A sample size of n=8 for PTL and TL groups was calculated.

Enzyme Activity Assay. The Enzyme Activity Assay was performed as previously described (Liu et al., 2001a) using total protein lysate from human uterine smooth muscle tissue taken from the superior portion of the incision. The lysate was prepared to a final concentration of 1 mg/ml in oxygen-purged experimental buffer containing: Tris-HCl pH 8.0 (20mM), EDTA (0.5mM), NP-40 (0.1%) and 1mM phenylmethylsulphonyl fluoride (PMSF). Lysate was equilibrated at r.t. for 10 min in the presence of NADH (300μM) prior to addition of GSNO (200μM), and tested against appropriate controls (Supp. Figure 2). Absorbance at 340nm (A340) (SmartSpec^{TM} Plus: Bio-Rad Laboratories, Inc., Hercules, CA) was recorded at t =

0, 5, 10 min to ensure stability of the NADH pool prior to the addition of GSNOR and/or inhibitors. Following equilibration, GSNO was added to the lysate mixture and A340 recording were collected at t = 0, 5, 10 min. N6022 (8nM) (S77589: Selleck Chemicals, Houston, TX), a GSNOR inhibitor, was used to verify negligible NADH conversion to NAD^+ in the presence of GSNO. N6022 was added concurrently with NADH to the protein lysate and equilibrated for 10 min prior to the addition of GSNO.

SC-TR Activity Assay. The selenocystine-thioredoxin reductase (SC-TR) assay was performed as previously described (Cunniff et al., 2013) with the exception that substituted selenocystine with selenocystamine due to poor aqueous solubility of selenocystine. The SC-TR assay measures the reduction of diselenide-bridged amino acids (substrate) by TR through monitoring the consumption of NADPH (co-enzyme) at 340 nm (a 365nm filter was used but showed adequate Abs at this wavelength, Supp. Figure 8). HEK293 cells were grown to 90% confluency and lysed with ice-cold TE-buffer (pH 7.5) containing 1mM EDTA, 1% NP-40, 1 μg/ml leupeptin, 1mM NaF, and protease inhibitors (cat.78430: Thermo Fisher Scientific Inc., Waltham, MA). Lysed cells were sonicated for on ice for 30 seconds with 2 second pulses. Lysates were centrifuged at 10,000xg for 10 minutes and protein concentrations were determined by EZQ Protein Quantification (Invitrogen, Carlsbad, CA). 100μl reaction volumes (final volume) were used in costar 3396 96-well polystyrene plates and read on a Hidex Plate Chameleon (model 425-106, MikroWin software ver. 4.43). Reaction mixtures consisted of: 500μM NADPH

(sigma N1630), 800μM selenocystamine (sigma S0520), and 25μg total protein lysate from HEK293 cells, with or without 1 or 10 nM auranofin (sigma A6733), in a final volume of 100μl in lysis buffer. Protein lysates and inhibitors were incubated at r.t. for 5 minutes prior to the start of the assay to allow for equilibrium to occur. Samples were read at 30 second intervals for 20 minutes at 365 nm.

Confocal Microscopy. Myometrial tissues were sliced into 10µm sections using a cryostat at -30°C and placed on coated slides (Surgipath, Buffalo Grove, Illinois). Samples were fixed in 4% paraformaldehyde then permeabilized with 0.5% Triton X-100. GSNOR protein was labeled with goat anti-rabbit ADH5 (1:250) 1° antibody (ab59134: Abcam, Cambridge, MA), followed by either TRITC-donkey anti-rabbit 2° (Santa Cruz Biotech) for tissue sections, or FITC- donkey anti-rabbit 2° (Santa Cruz Biotech) for cultured cells, then mounted in Vectashield plus DAPI (VectorLabs, Burlingame, California). Negative control images were obtained through omission of ADH5 1° Ab. Images were acquired on an Olympus IX81 Fluoview confocal microscope system at 40x magnification and analyzed with bundled software FV10-ASW (version 04.02, Windows 7 professional, Olympus America, Inc., Melville, NY). Brightness and contrast were adjusted globally using identical values for each image (brightness +256, contrast +53, Photoshop CC 2017.1.0, Adobe Systems Inc., San Jose, CA).

Analysis. All data analysis was conducted using Prism (version 7.0c for Mac OS 10.11, GraphPad Software, La Jolla California USA). Significance is defined as $P < 0.05$ using an unpaired, two-tailed, student's t-test, unless otherwise stated.

Results:

NO-Mediated Relaxation is Blunted in Preterm Human Myometrium. Patients who enter labor spontaneously preterm without infection have a blunted relaxation response to the ·NO donor, Cys-NO (Figure 2), suggesting that the mechanism of ·NO action may be involved in the pathophysiology of preterm labor. Addition of ·NO donor in increasing concentrations (1nM – 100μM) to term tissues (TL) relaxed both spontaneously active and oxytocin-treated tissues. 100μM Cys-NO relaxed TL tissue by 84% and 90% over basline, in sponateous-contracting and oxytocin-treated tissues, respectively ($P<0.05$). In tissues from sPTL myometrium, Cys-NO relaxation was insignificant compared to baseline in oxytocin-treated samples ($p=0.6$), and severely blunted (26%) in spontaneous samples as compared to TL tissue (84%). Treatment of sPTL tissues in an identical fashion revealed that Cys-NO could not relax OT-treated tissues and the relaxation of spontaneous contractions was blunted. This is the first study to measure the ability of ·NO to relax spontaneous preterm *vs.* term pregnant human myometrium.

GSNOR is upregulated in spontaneous preterm laboring myometrium. One possibility to explain the blunted relaxation of sPTL myometrial tissue to ·NO is that ·NO's availability in sPTL tissue may be limited. Glutathione (GSH) is the major thiol in mammalian cells and it is expressed in the mM range (Bateman et al., 2008; Leeuwenburgh et al., 1994). S-nitrosoglutathione (GSNO), a modified

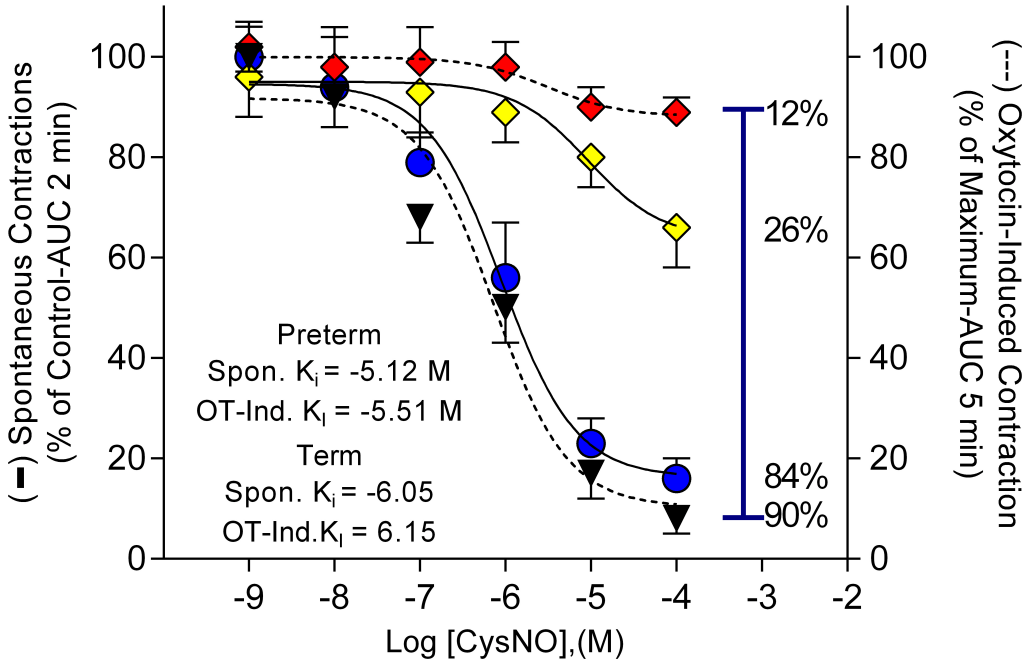

(▮) Spontaneous Contractions
(% of Control-AUC 2 min)
(---) Oxytocin-Induced Contraction
(% of Maximum-AUC 5 min)
Log [CysNO],(M)
Preterm
Spon. Ki = -5.12 M
OT-Ind. Kl = -5.51 M
Term
Spon. Ki = -6.05
OT-Ind.Kl = 6.15
12%
26%
84%
90%

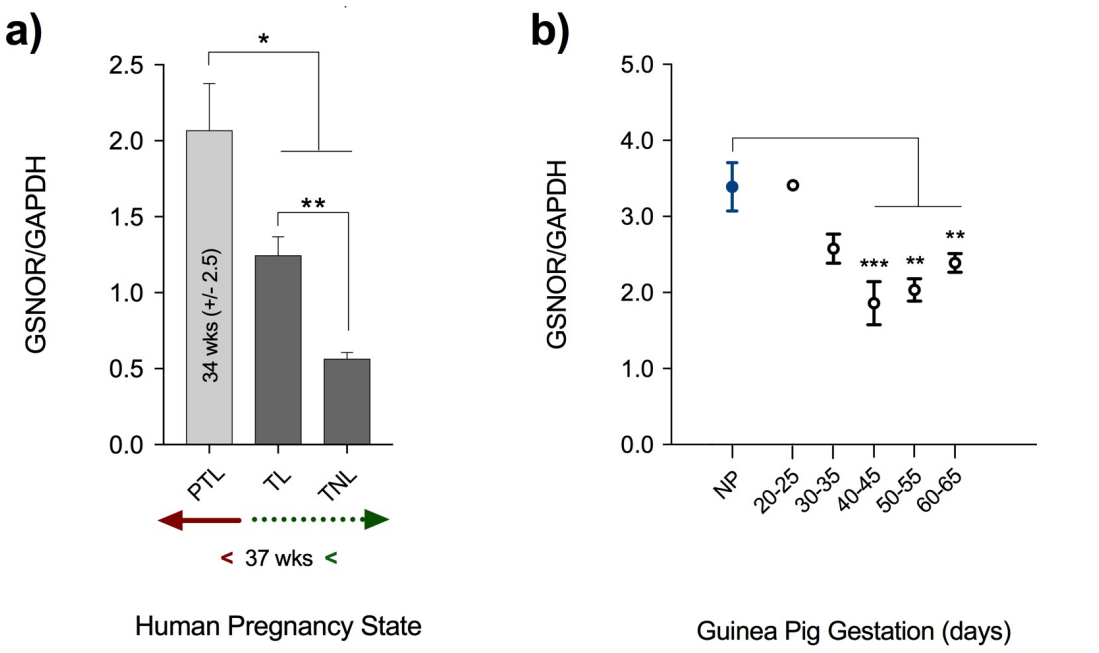
Figure 2: *The myometrium exhibits a blunted response to nitric oxide*: Myometrial strips from patients in labor at term or preterm were hung in tissue baths and allowed to contract spontaneously. Strips served as their own control (compared to baseline). (a) In tissues from women in labor at term Cys-NO relaxed oxytocin (100nM) induced contractions (▼). (b) In tissues from women in labor spontaneously preterm Cys-NO relaxation was insignificant (◆) compared to relaxation in baseline measurements (p=0.6). Cys-NO relaxed term laboring myometrium contracting spontaneously (●) while the relaxation seen in tissues from women in labor spontaneously preterm was blunted (◇, 26% *vs.* 84% relaxation) and the apparent IC_{50} was right-shifted 10-fold. Data are mean ± SEM of triplicate determinations in 5 patients in each pregnancy state.

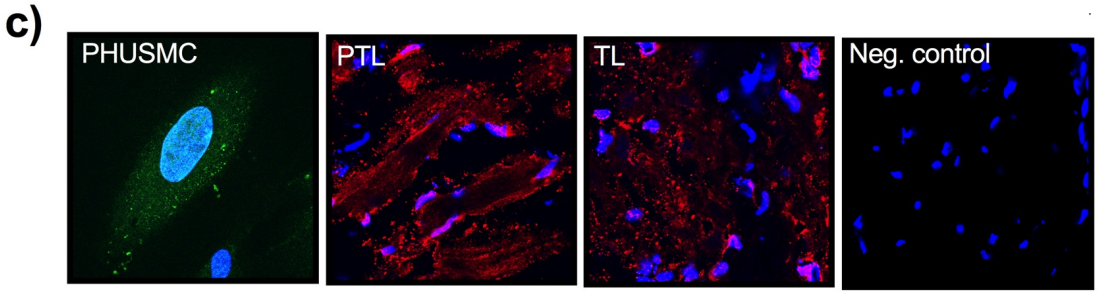
form of GSH in which an NO-moiety has been added to its sole cysteine, is the likely form in which ·NO resides in the cell to trans-nitrosate proteins (Gaston et al., 1993). GSNO is efficiently metabolized by GSNOR, making this enzyme an interesting and appropriate target to investigate. Using a Wes® Simple Western™ assay we determined that GSNOR protein expression increased in sPTL myometrium (sPTL, N=9) compared to term tissues (TL, N=8) p=0.03, as well as in term laboring (TL) myometrium compared to term non-laboring (TNL) tissue (TNL, N=3, p=0.0089), as determined by a two-way unpaired student's t-test (Figure 3A). Women of African descent are known to be disproportionately affected by sPTL. We also compared GSNOR expression in Caucasian women to those of women of African descent, both in term-laboring and term non-laboring myometrial samples, but did not find a significant difference in GSNOR expression (TNL p=0.7541, n=3; TL p=0.4138, n=3) between African American vs. Caucasian samples. (Supp. Figure 3A). Due to a limited "n" in this cohort there is insufficient data to draw a definitive conclusion, one way or the other, as to whether or not GSNOR expression varies between race in sPTL myometrium.

In order to better understand the GSNOR protein expression profile in the myometrium over the entire length of the pregnancy, something not possible in humans, we utilized a guinea pig model. Here we address the possibility that the observed differences in sPTL GSNOR expression are the result of gestational length, rather than a pathological feature of sPTL. GSNOR expression in guinea is pigs biphasic (Figure 3B), dropping until mid-gestation, then trending towards

pre-pregnancy levels at term, and was significantly lower than the non-pregnant (NP) guinea pig by gestational days 40-45 (p=0.0005), 50-55 (p=0.002), and 60-65 (p=0.005), as determined by one-way ANOVA (F (5, 21) = 9.382" P<0.0001) followed by Dunnett's multiple comparisons test. Decreased expression of GSNOR increases SNOs (Liu et al., 2004), which promotes critical S-nitrosations that are consistent with quiescence (Ulrich et al., 2012b), while increased expression of GSNOR, as with sPTL, would serve to lower the availability of ·NO and thus lower critical S-nitrosations that may govern uterine quiescence, a topic that will be covered in chapter 5.

GSNOR Enzyme activity in Pregnant Myometrium. To insure that the increased protein expression of GSNOR correlates to a functional increase in enzymatic activity, we adapted an enzyme activity assay that follows NADH consumption, NADH being an obligate co-enzyme of GSNOR (Figure 1), in protein lysates of TL and sPTL myometrial protein lysates (Liu et al., 2001b) (Figure 4). N6022 (3-(5-(4-(1H-imidazol-1-yl) phenyl)-1-(4-carbamoyl- 2-methylphenyl)-1H-pyrrol-2-yl) propionic acid) from Nivalis Pharmaceuticals is known to be a potent and specific inhibitor of GSNOR (Green et al., 2012b), and was used to verify specificity of the assay. The enzyme activity assay uses a total protein lysate from frozen tissue in the presence of NADH and GSNO. Once frozen tissue shows no





Human Myometrial Confocal Microscopy

Figure 3: *GSNOR expression in the myometrium*: (a) Wes® protein assay of normalized GSNOR expression in tissues from women laboring spontaneously preterm (PTL - mean delivery at 34 wks. ± 2.5 wks., n=9), tissues from women in labor at term (TL, n=8), and tissues from women non-laboring at term (TNL, n=3). Statistical comparison by Dunnett's multiple comparisons test; sPTL vs TL p=0.04; PTL vs TNL p=0.02; TL vs TNL p=0.007. (b) A gestational timing control of GSNOR expression was performed in guinea pigs. GSNOR expression at several times during pregnancy was compared to non-pregnant (NP) control. (n=3-6 at each time). Statistical comparison gestational times to NP by ANOVA (c) Confocal images of GSNOR expression in telomerized human uterine smooth muscle cells (PHUSMC 60x magnification, green = GSNOR, blue = nucleus) and whole tissue from TL, and PTL myometrial tissue (40x magnification, red = GSNOR, blue = nucleus). All data presented as mean ± SEM.

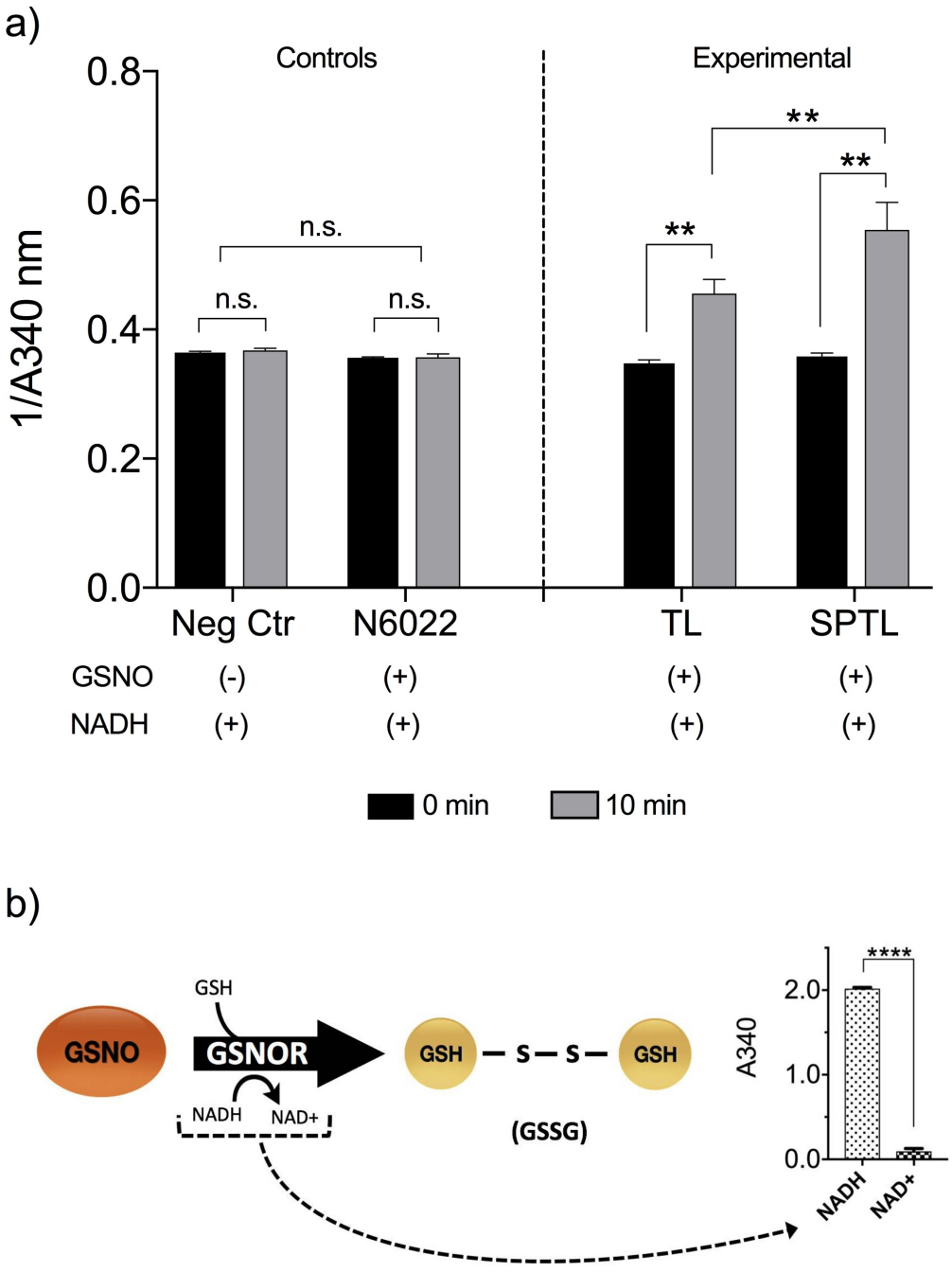

a)
Controls
Experimental
1/A340 nm
0.8
0.6
0.4
0.2
0.0
n.s.
n.s.
n.s.
**
**
**
Neg Ctr
N6022
TL
SPTL
GSNO (-) (+) (+) (+)
NADH (+) (+) (+) (+)
0 min
10 min
b)
GSNO
GSH
GSNOR
NADH
NAD+
GSH — S — S — GSH
(GSSG)
A340
****
2.0
1.0
0.0
NADH
NAD+

Figure 4: *GSNOR enzyme activity*: GSNOR enzyme activity assay measured at time = 0 & 10 min: (a) Total GSNOR activity from myometrial protein lysate (1 mg/ml) is higher in tissues from women laboring spontaneously preterm (sPTL n = 4) than in tissues from women laboring at term (TL n=4) p=0.006. The addition of N6022 (8nM) reduces GSNOR activity to baseline levels. (b) NADH, a required coenzyme for GSNOR activation, absorbs strongly at 340nm. A decrease in A340nm occurs when the enzyme converts NADH to NAD+ and serves as a measure of GSNOR enzyme activity during the conversion of GSNO to glutathione disulfide (GSSG). Values (1/A340 by convention) are reported as mean ± SEM.

depreciable decline in GSNOR enzymatic activity as compared to fresh tissue, although frozen protein lysates are not suitable for this assay (Supp. Figure 2A). NADH is required for the conversion of GSNO to GSSG and absorbs strongly at 340nm. GSNOR converts NADH to NAD^+ which does not absorb at 340nm and can therefore be used as an indirect measurement of GSNOR activity (Liu et al., 2001b). Both term laboring (P= 0.0001, 0-Min Mean ± SEM = 0.448 ± 0.00252; 10-Min Mean ± SEM = 0.45325 ± 0.01091) and preterm laboring (P= 0.0001, 0-Min Mean ± SEM = 0.35850 ± 0.00272; 10-Min Mean ± SEM = 0.55425 ± 0.02141) protein lysate displayed a significant increase in GSNOR activity after a 10-minute incubation with 300μM GSNO. Furthermore, preterm laboring protein lysate exhibited significantly higher enzyme activity than term laboring lysate (P= 0.0063, TL Mean ± SEM = 0.45575 ± 0.01087, PTL Mean ± SEM = 0.55425 ± 0.02141). 8nM N6022 abolished GSNOR enzyme activity. Control absorbance (-GSNO) did not change significantly after a 10-minute incubation in total protein, indicating that NADH is not being metabolized or degraded by other components in the lysate. As with GSNOR protein expression, there was not a significant difference in enzyme activity between the two racial cohorts (TNL p=0.5318; TL p=0.6946; n=3 per group). Other experimental controls, such as the addition of GSNO (no NADH), only NADH (no GSNO), or GSNO in the presence of GSH, did not significantly change absorbance over the 10-minute observation period (Supp. Figure 2). This finding indicates that components in the protein lysate, other than GSNOR, do not contribute significantly to the degradation of NADH in this assay.

GSNOR Inhibition: Nebivolol is the most potent of the 3rd generation β1AR antagonists. It has been proposed that nebivolol also acts as a GSNOR inhibitor (Jiang et al., 2016b) based on two observations: (1) It shares structural similarities with N6022, and (2) it is known to increase total levels of s-nitrosothiols in cells and tissue. In order to determine if nebivolol alters the activity of GSNOR we employed the same enzyme same activity assay as described above, in the presence of N6022, nebivolol, or the β2AR agonists formoterol or terbutaline (Figure 5), all of which share structural similarities to N6022 (except terbutaline, which serves as a control) as identified in the PubChem Substance and Compound database (Supp. Figure 04) [structural identifiers: nebivolol CID=71301, formoterol CID=3083544, N6022 CID=44623946, terbutaline CID=5403] (Database, 2017). It has also been reported that at higher concentrations nebivolol may act as a βAR agonist (Rozec et al., 2009); therefore, we also tested two β2AR agonist for GSNOR inhibition: Formoterol, due to a 2° structural similarities to nebivolol and N6022 (Database, 2017), as well as the fact that it is effective as a tocolytic agent in rats (Shinkai and Takayama, 2000), and terbutaline, a known and effective β2AR agonist with no structural similarities to N6022 or nebivolol, to test whether or not the family of β2AR agonist has an affinity for GSNOR. Data is reported as GSNOR activity within a range between 0 and 1. NADH consumption of the reaction mixture after 10 minutes (GSNO (300μM) + NADH (200μM) + protein lysate (1mg/ml)) was set to a nominal value of “1”, while NADH consumption of the reaction mixture (same as above (-)GSNO) after 10 minutes was set to a nominal

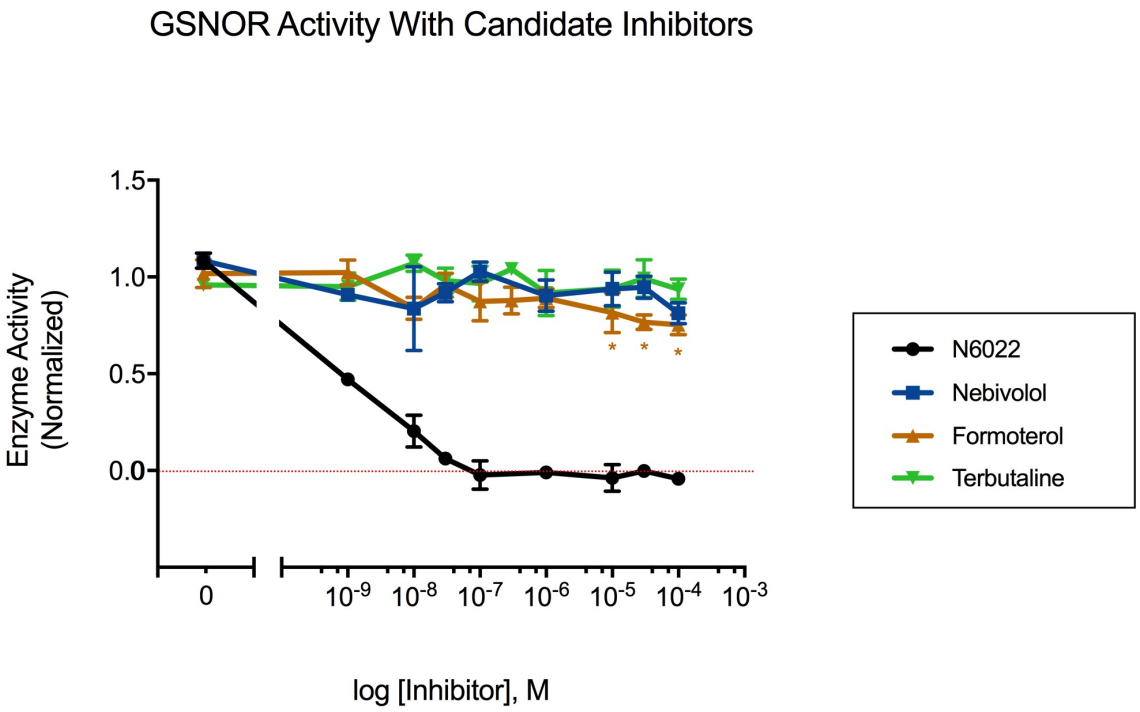

GSNOR Activity With Candidate Inhibitors
Enzyme Activity (Normalized)
1.5
1.0
0.5
0.0
0
10-9
10-8
10-7
10-6
10-5
10-4
10-3
log [Inhibitor], M
N6022
Nebivolol
Formoterol
Terbutaline

Figure 5: *GSNOR enzyme activity in presence of candidate inhibitors*: GSNOR activity measured over 10 minutes in the presence of the β2AR agonists. GSNOR activity from myometrial protein lysate (1 mg/ml) is not significantly inhibited by nebivolol ($p=0.1129$) or terbutaline ($p=0.3323$), at all concentrations from 1nM to 100μM, but it is inhibited by formoterol ($p=0.0007$) at concentrations of 10μM and greater. N6022, a known inhibitor of GSNOR (K_i = 2.5 nM), significantly inhibited GSNOR across the entire dosing range ($p<0.0001$).

value of “0”. The K_i of the inhibitors for their target receptors are as follows: N6022 for GSNOR, K_i = 2.5 nM (S77589: Selleck Chemicals, Houston, TX), Nebivolol for β1AR, K_i = 0.9 nM (Leysen et al., 1991), formoterol and terbutaline for β2AR, K_i = of 0.4 nM and 53 nM, respectively (Shinkai and Takayama, 2000). Drug concentrations between 1 nM and 100μM were tested (drugs tested well above their K_i, as their affinity for GSNOR is unknown). One-way ANOVA analysis for each drug’s actions on GSNOR are as follows: N6022 "F (8, 18) = 174.1" P<0.0001, Nebivolol "F (7, 16) = 2.038" P=0.1129, terbutaline "F (9, 20) = 1.229" P=0.3323, and formoterol "F (9, 20) = 5.526" P=0.0007. Of note, while there was a decrease in GSNOR activity in the presence of formoterol, the change was not significantly different from baseline until a concentration of 10μM, a 2,500-fold increase from the K_i of formoterol for its principal target, β1AR.

Blockade of GSNOR Activity Relaxes Term Non-Laboring (TNL) Myometrium. In order to determine if GSNOR actively contributes to myometrial quiescence, we inhibited the enzyme with N6022, a potent and selective inhibitor of GSNOR (Green et al., 2012a). *Ex vivo* organ bath experiments using oxytocin (OT) primed, TNL human myometrium, revealed that the addition of N6022 relaxes TNL myometrium and results in a reduction in both peak force, 100μM N6022: 93.9% ± 4.67 (p=0.0126, n=4) of baseline, and 300μM N6022: 83.0% ± 4.79 (p=0.005, n=4) over DMSO vehicle control maximal dose, and a decrease in area AUC, 100μM N6022: 90.5% ± 3.29 (p=0.0612 (n.s.), n=4) of baseline, and 300μM

N6022: 82.4% ± 4.40 (p=0.013, n=4) over DMSO vehicle control maximal dose (Figure 6). Contractions per unit time (CPUT) are defined as the average number of contractions per 15-minute dosing period, normalized to the average number of contractions in the vehicle control group during the same period of time, and were not significantly different at either dose (100μM p=0.3144, 300μM p=0.1325). Area under the curve (AUC) and peak force were calculated by normalizing the treatment group to vehicle control during the same dosing period, 15-minutes (treatment/vehicle), then a secondary normalization was applied using the value (AUC or peak force) during 10-mintues prior to initial drug treatment, in order to account for variability between each individual tissue strips. Similar results were found when N6022 cumulative dose-dependent fashion (1μM-300μM) (Figure 7). AUC was significant at 300μM (p=0.0147, n=3), Peak force was significant at all doses other than 1μM (10 μM p=0.0373, 30μM p=0.0363,100μM p=0.0112, 300μM p=0.0022, all n=3), and CPUT was not significant at any concentration. Contractions did not return to baseline after washout (data not shown), possibly because N6022 acts intracellularly, as opposed to drugs that act on extracellular receptors; therefore, a return to baseline would occur at a rate consistent with metabolism of the drug or its passive/active transport from the cell, which was not observed during the course of the experiment. Concentrations were determined based on pharmacokinetic properties (IC50 = 20 nM; Ki = 2.5 nM), and data relating to bioavailability and testing of the drug (~4%) (Sun et al., 2011c) In rats, N6022 was administered safely in rats at circulating levels as high as 0.1 mg/ml

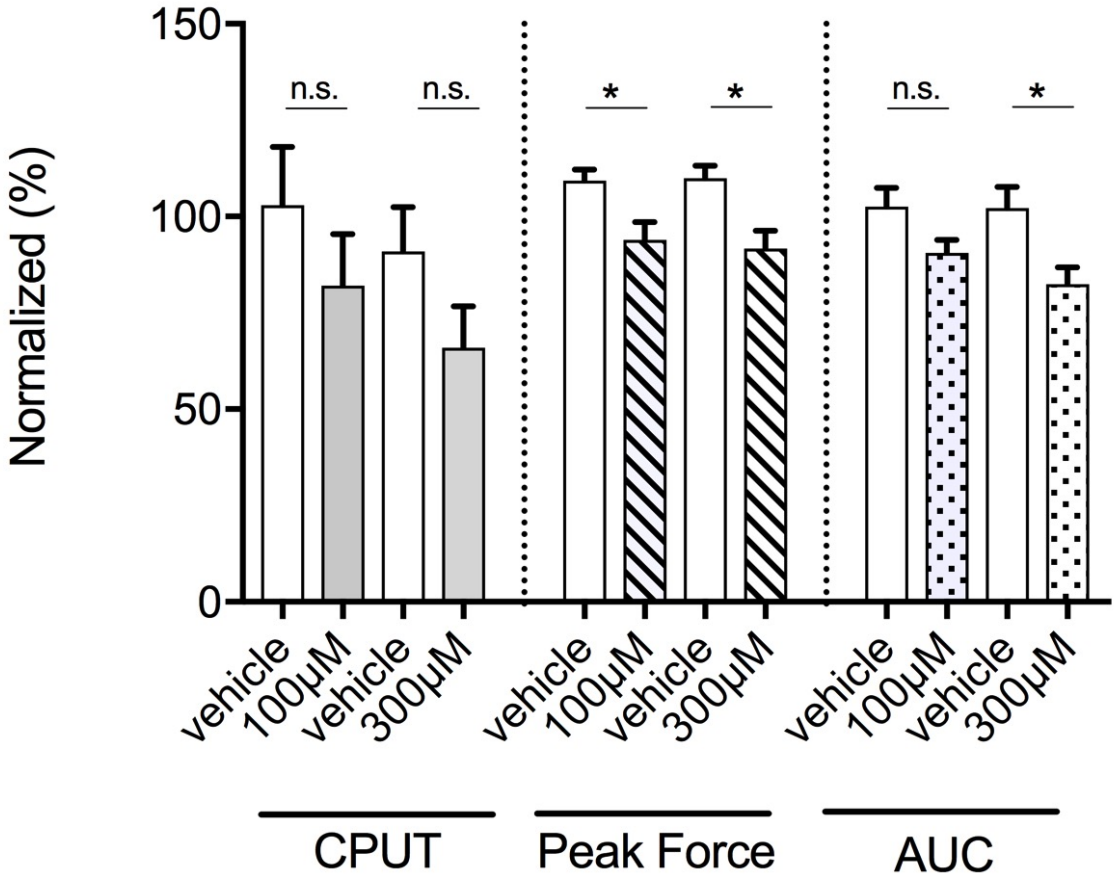
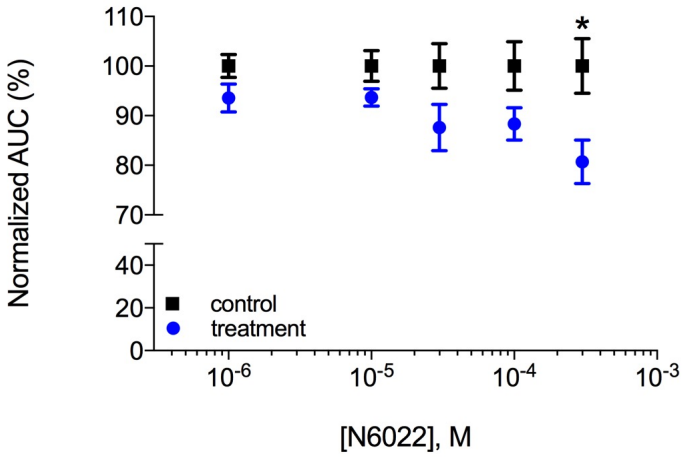
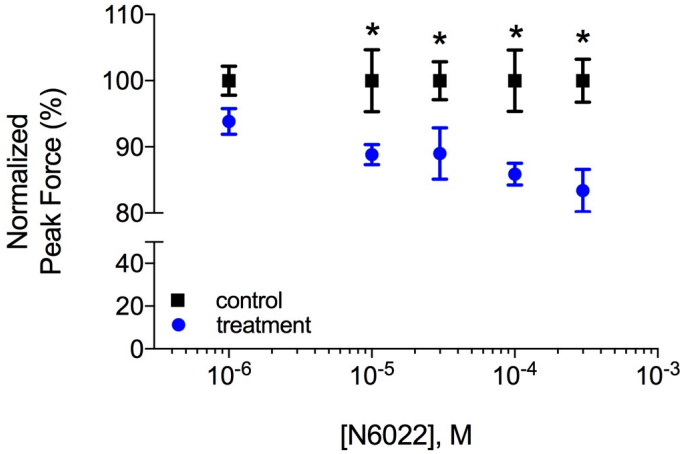

150
100
50
0
Normalized (%)
n.s.
n.s.
*
*
n.s.
*
vehicle
100μM
vehicle
300μM
vehicle
100μM
vehicle
300μM
vehicle
100μM
vehicle
300μM
CPUT
Peak Force
AUC

Figure 6: *The myometrium relaxes in response to N6022*: (a) The GSNOR inhibitor N6022, was applied to human myometrial tissue in an *ex vivo* organ bath at 100 μM and 300 μM (TNL, n=4). Myometrial Response to N6022 inhibition after 30-minute incubation with drug relative to DMSO control. Contractions per unit time (CPUT), an indicator of the relative number of contractions per dosing period, were not significantly different at either concentration of N6022 (100μM p=0.3144, 300μM p=0.1325). Peak force (100μM p=0.0126, 300μM p=0.005), and area under the curve (AUC) (100μM p=0.0612, 300μM p=0. 013) were significant to varying degrees. All data presented as mean ± SEM.

a)


Normalized AUC (%)
110
100
90
80
70
40
20
0
control
treatment
*
10^{-6}
10^{-5}
10^{-4}
10^{-3}
[N6022], M

b)


Normalized Peak Force (%)
110
100
90
80
40
20
0
control
treatment
*
*
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*
10^{-6}
10^{-5}
10^{-4}
10^{-3}
[N6022], M

c)

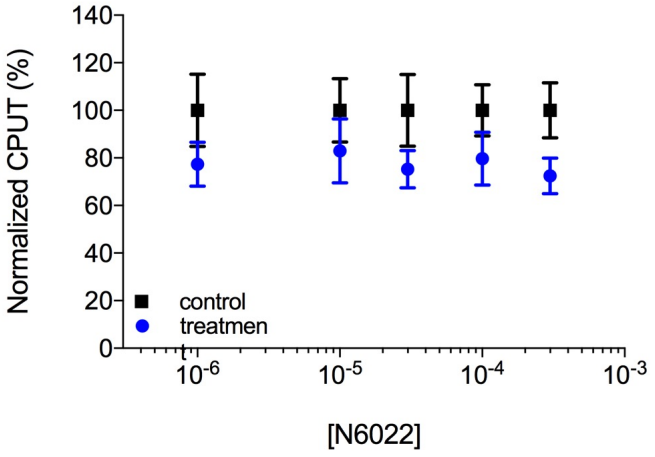

Normalized CPUT (%)
140
120
100
80
60
40
20
0
control
treatmen
10^{-6}
10^{-5}
10^{-4}
10^{-3}
[N6022]

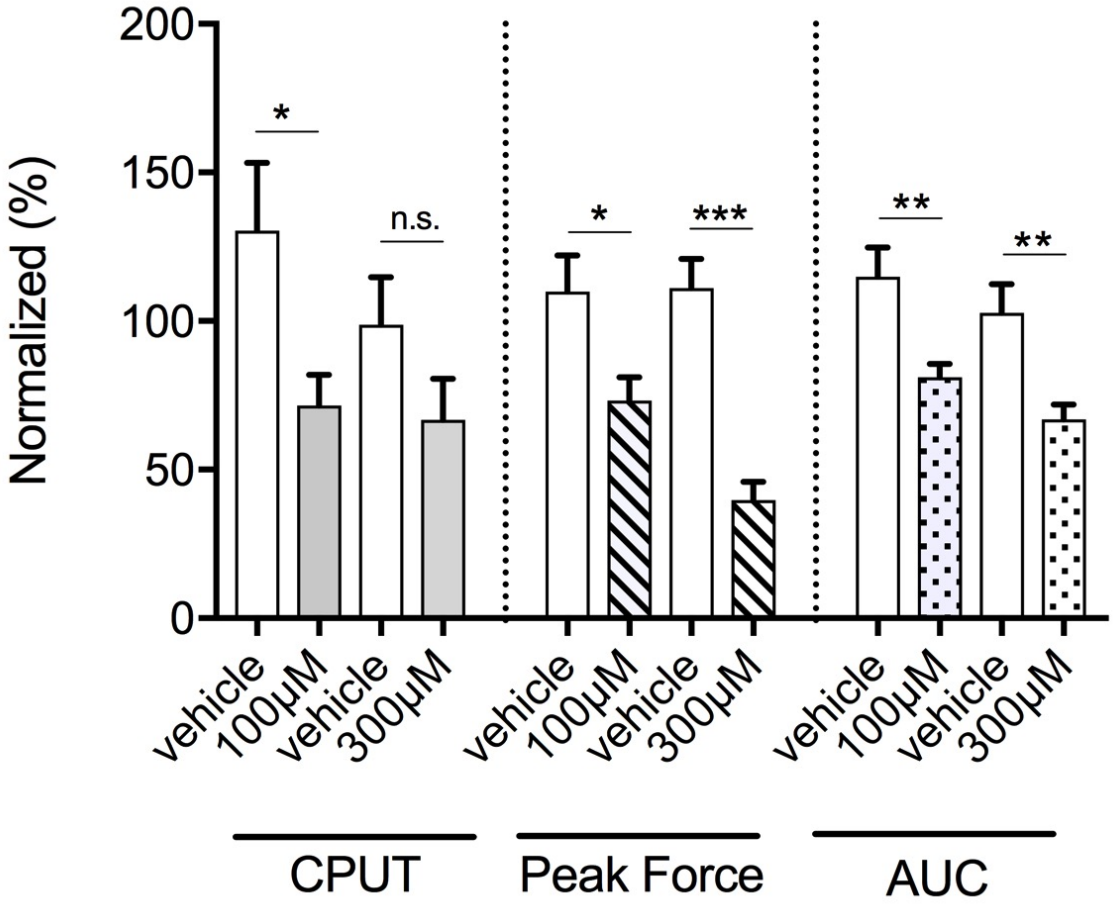
Figure 7: *The myometrium relaxes in a dose-dependent manner to N6022*: (a) An increasing cumulative dose of N6022 (●, 1μM-300μM) was added to a bath with TNL myometrial tissue strips (n=3) at 15 min intervals. (a) Area under the curve (AUC), was significant at 300μM N6022 (p=0.0147, n=3), (b) Peak force was significant at doses from 10μM to 300μM (10 μM p=0.0373, 30μM p=0.0363,100μM p=0.0112, 300μM p=0.0022, all n=3), and (c) the contractions per unit time (CPUT), an indicator of the relative number of contractions per dosing period, was not significantly different at any concentration of N6022. All data presented as mean ± SEM.

(240 μM) (Colagiovanni et al., 2012b), therefore we used a maximal dose of 300μM in our samples.

Nebivolol Exerts Negative Inotropic Effects in the Myometrium: Although nebivolol is not a GSNOR inhibitor, it has been shown to relax smooth muscle. Based upon this information we elected to investigate nebivolol's potential as a tocolytic. Nebivolol was applied at two doses, 100μM and 300μM, to OT-primed TNL human tissues (N=4), in a cumulative dose fashion, to tissues in *ex vivo* organ bath as described above (Figure 8). Nebivolol significantly altered TNL human myometrial dynamics when applied to the organ bath, showing a strong negative inotropic effect. CPUT was significantly decreased at 100μM but not at 300μM (100μM p=0.0319, 300μM p=0.1176, n=4), peak force was significantly decreased at both 100μM and 300μM (100μM p=0.0185, 300μM p=0.0006, n=4), and AUC was significantly decreased at both 100μM and 300μM (100μM p=0.0080, 300μM p=0.0033, n=4). Since nebivolol is known to increase total s-nitrosothiols in both tissue an cells (Jiang et al., 2016b), we next tested whether or not nebivolol was effective at inhibiting another mediator of ·NO in the myometrium, thioredoxin reductase.

Thioredoxin Reductase Assay: The selenocystamine/thioredoxin reductase assay (SC-TR), in a similar manner to the GSNOR assay, can measure thioredoxin reductase (TrxR) activity by observing the change in absorbance at 340 nm as NADPH (NADH is used for the GSNOR assay), a required cofactor of TrxR, is

oxidized to $NADP^+$. The substrate for TrxR in the assay is selenocystamine, and it has been reported that TrxR is the only known enzyme in the cell capable of reducing a diselenide bond (Cunniff et al., 2013). As a result, this assay is continuous, direct, and highly specific to TrxR activity. To confirm the specificity of the assay we employed auranofin, a gold-containing compound which inhibits the enzyme by reacting with the seleno-containing residues of thioredoxin reductase (Gromer et al., 1998; Marzano et al., 2007). Individual reaction mixture components, as well as the protein lysate in combination with either substrate (selenocystamine) or cofactor (NADPH) alone, were tested with no change in Abs over time (Supp. Figure 8). Under experimental conditions, we determined that nebivolol does not inhibit TrxR activity (Figure 9) (n=3 for all conditions), nor was it significantly different from baseline activity at 10μM and 100μM, as analyzed with a one-way ANOVA analysis: "F (2, 117) = 2.694 " P=0.0718. Auranofin, as predicated, inhibited the enzyme in a dose dependent fashion (Figure 9a) ($P<0.0001$ at either dose of auranofin compared with a max dose of nebivolol), whereas nebivolol, at all concentrations tested (0.1μM and 100μM), did not significantly decrease the activity of the enzyme. (Figure 9b).


200
150
100
50
0
Normalized (%)
*
n.s.
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vehicle
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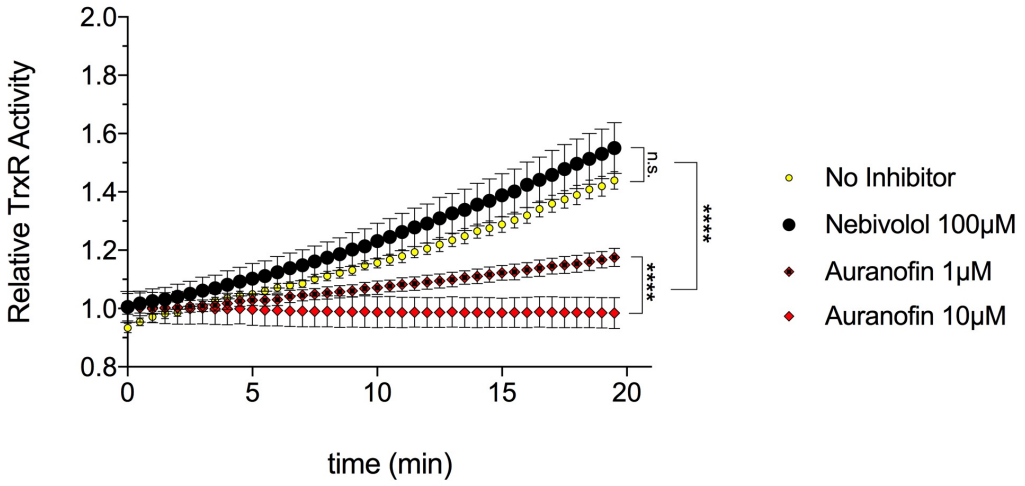
vehicle
300μM
vehicle
100μM
vehicle
300μM
vehicle
100μM
vehicle
300μM
CPUT
Peak Force
AUC

Figure 8: *Myometrium relaxes in response to nebivolol*: (a) The β1AR antagonist, nebivolol, relaxes myometrium at 100 μM and 300 μM (TNL, n=3), after 30-minute incubation with drug, relative to DMSO control tissues. Contractions per unit time (CPUT) were significantly different only at the lower dose of nebivolol (100μM p=0.0319, 300μM p=0.1776). Peak force (100μM p=0.0185, 300μM p=0.0006), and area under the curve (AUC) (100μM p=0.008, 300μM p=0. 0033) were significant at both concentrations. All data presented as mean ± SEM.

a)


Relative TrxR Activity
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0
5
10
15
20
time (min)
n.s.
****
****
No Inhibitor
Nebivolol 100μM
Auranofin 1μM
Auranofin 10μM

b)

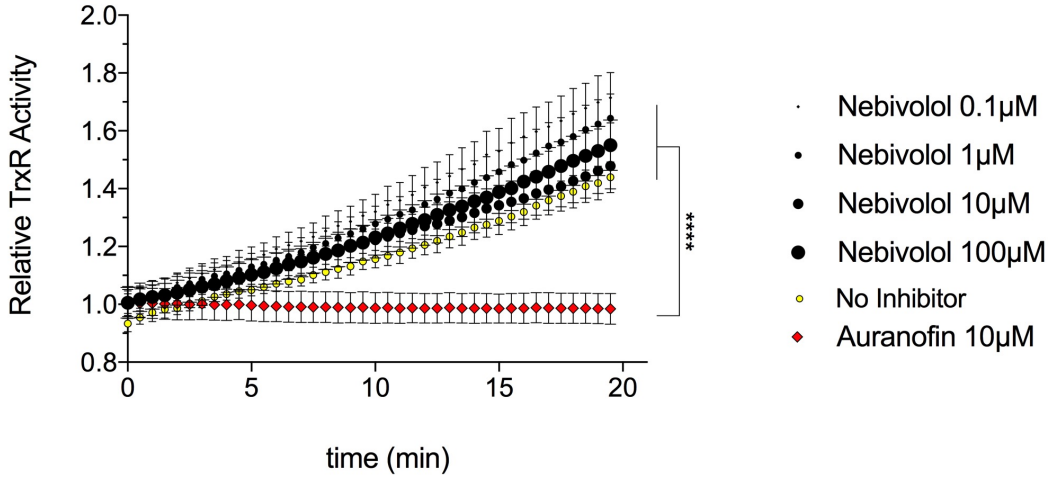

Relative TrxR Activity
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0
5
10
15
20
time (min)
****
Nebivolol 0.1μM
Nebivolol 1μM
Nebivolol 10μM
Nebivolol 100μM
No Inhibitor
Auranofin 10μM

Figure 9: *Nebivolol does not inhibit thioredoxin reductase (TrxR)*: (a) TrxR activity was no different from baseline activity after 20 minutes of activity in the presence 100μM nebivolol (n.s., n=3), whereas 1μM and 10μM auranofin were both significantly decreased from baseline ($P<0.0001$, n=3). (b) Nebivolol at concentrations from 100nM to 100μM failed to decrease TrxR activity below baseline activity ($P<0.0001$, n=3) All data presented as mean ± SEM.

Discussion:

Despite decades of research, tocolytics do not reliably extend pregnancy beyond 48-hours. Approaches to manage sPTL are employed without clear evidence of benefit for acute or maintenance tocolysis (Dodd et al., 2006; Whitworth and Quenby, 2008), and no drug currently in use is without potential adverse effects (Lamont et al., 2016). It is imperative that new approaches to prevent preterm contractions of the uterus be found (Illanes et al., 2014).

When considering tocolytics options for the treatment of sPTL, our findings here provide unique insight. First, is the observation that while ·NO relaxes full-term myometrium, ·NO's actions are severely blunted in sPTL myometrial tissue (Figure 2). This finding reveals a unique, and previously unreported, phenotype of sPTL myometrium. Because ·NO fails to relax sPTL tissue, its use as a tocolytic is not appropriate, beyond the known health risks to mother and child (Duckitt et al., 2014). As a result, we asked the question as to whether or not aberrant ·NO metabolism in sPTL myometrium may explain sPTL's blunted response to ·NO by skewing cellular function and promoting contractions? Interestingly, we found that GSNOR expression (Figure 3) and activity (Figure 4) are increased in sPTL myometrium. The finding that GSNOR dysregulation corresponds to the pathophysiology that is sPTL, prompted a deeper analysis of this enzyme. GSNOR is an important metabolizing agent of GSNO, the stable endogenous form of ·NO in the cell (Figure 1). GSNOR dysregulation has long been known to

regulate many disease states (Scott D. Barnett and Buxton, 2017b). Importantly, this enzyme is also dysregulated in some cohorts with asthma, which leads to enhanced airway smooth muscle constriction, and resistance to bronchodilators (Choudhry et al., 2010b; Henderson and Gaston, 2005b; Wu et al., 2007). This, combined with observations that women with asthma exhibit a higher relative risk of preterm labor and delivery (Doucette and Bracken, 1993), solidified GSNOR as protein of interest.

In order to better understand the normal progression of GSNOR expression in the myometrium over the entire course of gestation, we employed a guinea pig model. Like humans, guinea pigs do not experience a decrease in circulating progesterone prior to the initiation of labor (Nnamani et al., 2013). This makes the guinea pig an attractive model to compare uterine biochemistry at various time-points during gestation. GSNOR expression in guinea pigs is biphasic, dropping during gestation and returning toward pre-pregnancy levels as term nears (Figure 3B). When we consider that GSNOR trends upward from mid-gestation towards term, we would expect that GSNOR expression in sPTL myometrium would be lower than term. However, this is not what we observed (Figure 3A), indicating that GSNOR is dysregulated in sPTL myometrium. A merging of the human and guinea pig GSNOR expression data, while not directly comparable, visually amplifies the disparity between sPTL and TL GSNOR expression (Supp. Figure 5).

Beyond investigating GSONR expression as a function of gestational length, we also acquired a limited data set examining whether nor not GSNOR expression in the myometrium varies between race. This question is particularly germane as sPTL is known to disproportionately skew towards women of African descent (Culhane and Goldenberg, 2011; Hamilton et al., 2015). A study in African American children found that single nucleotide polymorphisms (SNPs) in the *ADH5* gene in airway smooth muscle correlates to severe asthma and resistance to treatment (Moore et al., 2009a). We postulated that GSNOR dysregulation may also be more prevalent in this population, mirroring our generalized sPTL findings. Unfortunately, our preliminary data does not support this hypothesis (Supp. Figure 3). That being said, due to an insufficient number of available samples, we were only able to test a small cohort of TL and TNL individuals. Future work will explore GSNOR expression in sPTL samples of those of African descent, as well as whether the aforementioned SNPs in the *ADH5* gene exists in sPTL samples, as several SNPs in the promoter and 3' UTR of the *ADH5* gene can result in the aberrant expression of GSNOR (Choudhry et al., 2010a).

Perhaps the most intriguing and important outcome of GSNOR dysregulation is that GSNOR now presents as a therapeutic target for which to test novel tocolytics that inhibit this enzyme. N6022, a potent and selective inhibitor of GSNOR, is well tolerated in humans and has already been tested in clinical trials as an airway smooth muscle relaxing agent in asthmatics (clinicaltrials.gov - NCT01316315), and for individuals with cystic fibrosis (clinicaltrials.gov – N6022:

NCT01746784; N91115: NCT02724527(Nivalis Therapeutics, 2014). Here we demonstrate its efficacy in the myometrium, and entertain its use as a tocolytic (Figures 8). Our preliminary data in a small sample of guinea pigs identified N6022 as an unparalleled tocolytic, nearly abolishing all contractile force (Supp. Figure 6). However, after extensive *ex vivo* organ bath experimentation with human uterine smooth muscle, we found N6022's effects on the myometrium, while statistically significant, were very modest (Figure 7). Due to the drug's limited bioavailability (~4%) (Sun et al., 2011c), its potential as a marketable tocolytic remains in question. However, numerous derivatives of N6022 have been identified (Sun et al., 2011a) as well as other compounds targeting GSNOR (Ferrini et al., 2013b), which may prove useful as tocolytic agents. We too have proposed conceptual variants of N6022 that may improve the diffusion of the N6022 across the cell membrane (Appendix 2) through the addition of a promoiety resorption ester (ethyl acetate) to the carboxylic side chain of N6022 (Supp. Figure 7). This promoiety can be hydrolyzed intracellularly by endogenous esterases to return N6022 to its native active state (Jornada et al., 2016).

Aside from known GSNOR inhibitors, nebivolol, a β1AR antagonist, has also been reported as a GSNOR inhibitor due to structural similarities to N6022, as well as its ability to increase s-nitrosothiol (SNO) concentrations in the cell and relax arterial smooth muscle (Jiang et al., 2016b). We, however, were unable to reproduce their finding, and show that nebivolol does not alter GSNOR activity. (Figure 4). What then accounts for the generalized smooth muscle relaxing effects

seen with nebivolol? Some have reported that nebivolol metabolites may serve as ligands to β2ARs (Broeders et al., 2000), and at higher concentrations nebivolol has been shown to act as a β3AR agonist in smooth muscle (Rozec et al., 2009). β3ARs serve as anti-inflammatory mediators in the myometrium, and they have even been proposed as a potential tocolytic (Lirussi et al., 2008). We tested several βAR agonists with varying degrees of tertiary structural similarity to N6022 (Supp. Figure 4), but like nebivolol, they did not inhibit GSNOR (Figure 5).

As nebivolol does not inhibit GSNOR, but is known to increase SNOs, we further investigated nebivolol's affinity for another important mediator of ·NO in the cell, thioredoxin reductase (TrxR). TrxR is another important mediator of GSNO and SNOs in the cell ((Nikitovic and Holmgren, 1996; Sengupta and Holmgren, 2012a). TrxR is found in the placenta and myometrium during pregnancy (Gromer et al., 1998; Sahlin et al., 2000), but its role in myometrial quiescence has not been closely studied. Unfortunately, we found that nebivolol does not decrease TrxR activity (Figure 9). A wide range of concentrations of nebivolol were employed, including concentrations in which cells treated with nebivolol exhibit a strong increase in SNO levels (Jiang et al., 2016b), yet they did not alter TrxR activity.

Interestingly, while nebivolol does not inhibit GSNOR or TrxR, it is, nonetheless, an excellent myometrial relaxing agent. In our *ex vivo* tissue bath experiments nebivolol decreases peak force of contraction by 60% (+/- 6.1), and AUC by 43% (+/- 4.9) (Figure 8). Although the mechanism behind nebivolol's action on smooth muscle relaxation has not been elucidated, it is likely that the

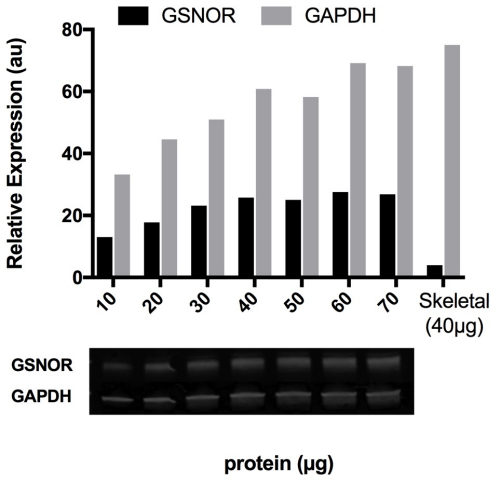
observed negative inotropic effects seen in our experiments are due to nebivolol's activation (Münzel and Gori, 2009; Van Nueten and De Cree, 1998), and/or upregulation (Wang et al., 2017), of endothelial nitric oxide synthase (eNOS), an important generator of ·NO. This eNOS activation and regulation would also explain the increase in SNOs in tissues and cells treated with nebivolol (Jiang et al., 2016b). In Chapter 5, we explain why SNOs may play a crucial role in myometrial function. Beyond nebivolol's actions of eNOS, it has also been reported that nebivolol acts on mechanosensitive ion channels, which would provide an interesting alternate mechanism for nebivolol-mediated uterine smooth muscle relaxation, but this observation has not yet been reported in myocytes of any kind (Kalinowski, 2003).

Ultimately, the underlying cause(s) of sPTL remain unknown. While the complex mechanisms that drive sPTL have not been fully elucidated, our discovery that GSNOR is dysregulated in the myometrium of women who experience sPTL provides novel insight into this disease state. Our findings here afford an opportunity to investigate a new class of drugs in the tocolytics family, GSNOR inhibitors. GSNOR inhibitors not only function to increase endogenous levels of ·NO, but by extension, they also increase total SNOs in the cell. We believe that the cGMP-independent mechanism through which ·NO exerts its influence (chapter 2) is protein S-nitrosation, and GSNOR has been shown to modulate SNO concentration in the cell. In the next chapter we discuss the connection between

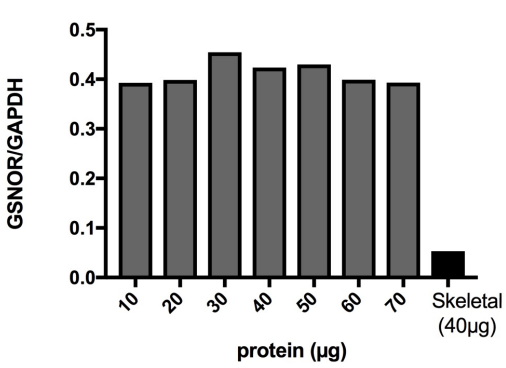
aberrant GSNOR expression in the myometrium and the functional effects of protein S-nitrosation on important proteins associated with uterine contractions.

SUPPLEMENTAL FIGURES

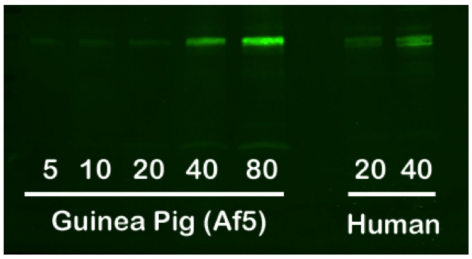
a)


GSNOR - Loading Gradiant
GSNOR
GAPDH
Relative Expression (au)
80
60
40
20
0
10
20
30
40
50
60
70
Skeletal (40µg)
GSNOR
GAPDH
protein (µg)

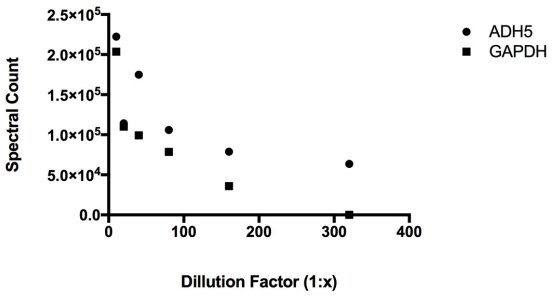
b)


Normalized GSNOR - Loading Gradiant
GSNOR/GAPDH
0.5
0.4
0.3
0.2
0.1
0.0
10
20
30
40
50
60
70
Skeletal (40µg)
protein (µg)

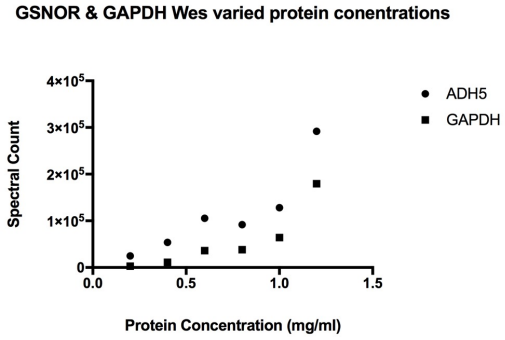
c)


GSNOR Ab specificity for human and guinea pig
5 10 20 40 80
Guinea Pig (Af5)
20 40
Human

d)

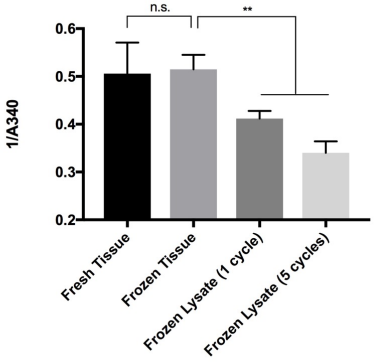

GSNOR & GAPDH Wes Ab dillutions
Spectral Count
2.5×10^5
2.0×10^5
1.5×10^5
1.0×10^5
5.0×10^4
0.0
0
100
200
300
400
Dillution Factor (1:x)
ADH5
GAPDH

e)

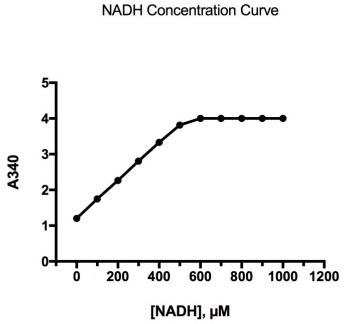

GSNOR & GAPDH Wes varied protein conentrations
Spectral Count
4×10^5
3×10^5
2×10^5
1×10^5
0
0.0
0.5
1.0
1.5
Protein Concentration (mg/ml)
ADH5
GAPDH

Supp. Figure 1: *Linearity and specificity of GSNOR antibody*: Preliminary GSNOR expression data acquired by western blot. (a/b) linear range of antibody (1:100 dilution, ab59134: Abcam, Cambridge, MA), GAPDH (1:00 dilution, sc-47724: Santa Cruz Biotechnology, Inc., Dallas, TX) compared to skeletal muscle control (does not express GSNOR), (c) and verification that GSNOR antibody shows specificity for guinea pig GSNOR (animal Af5). For Wes studies (d) GSNOR and GAPDH antibody dilutions from 1:20-1:320 (protein 0.7mg/ml), and (e) protein concentrations from 0.2mg/ml-1.2mg/ml (antibodies 1:50) to determine optimal protein and antibody combinations.

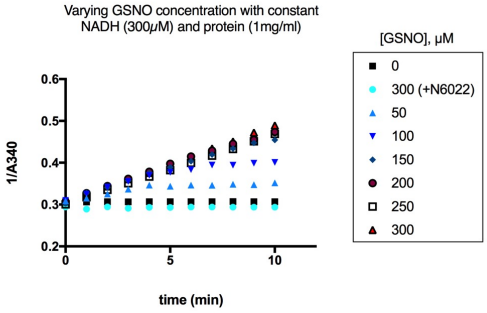
a)


Effect of Freezing on GSNOR Activity
n.s.
**
1/A340
0.6
0.5
0.4
0.3
0.2
Fresh Tissue
Frozen Tissue
Frozen Lysate (1 cycle)
Frozen Lysate (5 cycles)

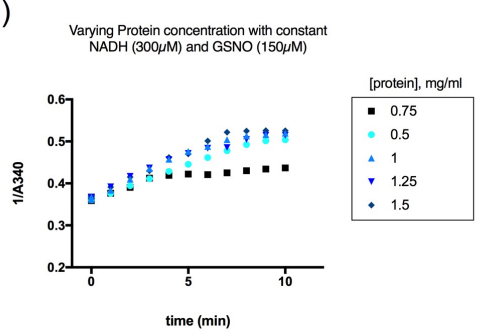
b)


NADH Concentration Curve
A340
5
4
3
2
1
0
0
200
400
600
800
1000
1200
[NADH], µM

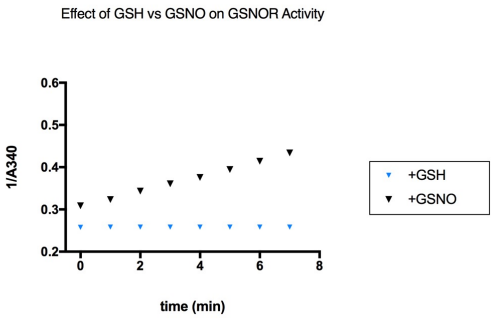
c)


Varying GSNO concentration with constant NADH (300µM) and protein (1mg/ml)
1/A340
0.6
0.5
0.4
0.3
0.2
0
5
10
time (min)
[GSNO], µM
0
300 (+N6022)
50
100
150
200
250
300

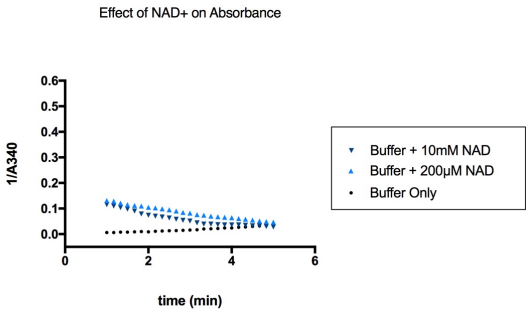
d)


Varying Protein concentration with constant NADH (300µM) and GSNO (150µM)
1/A340
0.6
0.5
0.4
0.3
0.2
0
5
10
time (min)
[protein], mg/ml
0.75
0.5
1
1.25
1.5

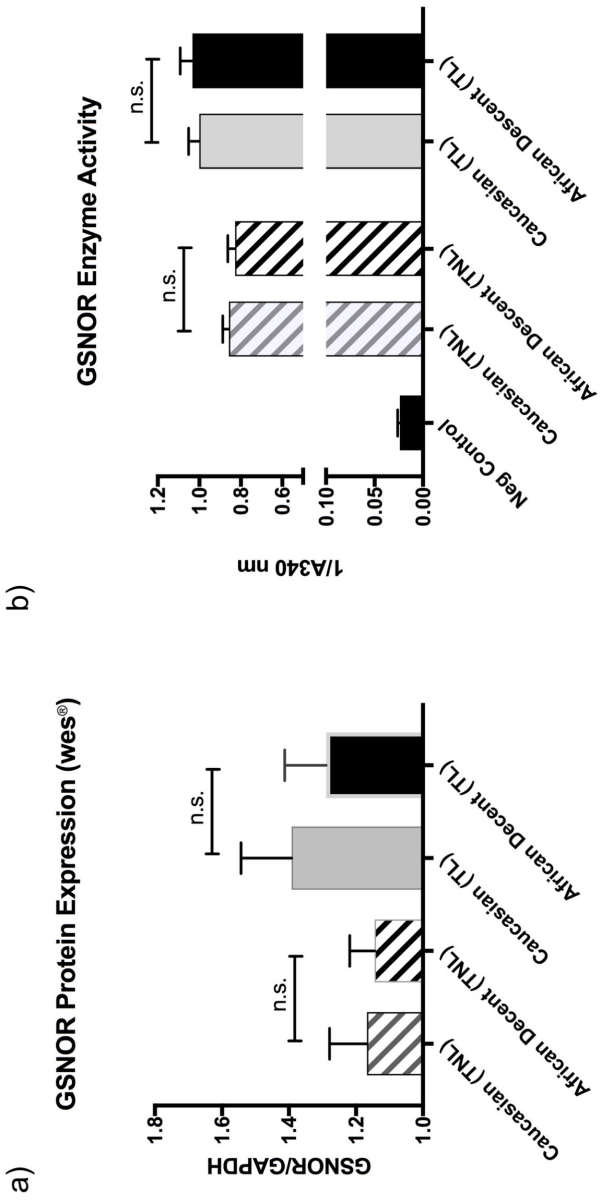
e)


Effect of GSH vs GSNO on GSNOR Activity
1/A340
0.6
0.5
0.4
0.3
0.2
0
2
4
6
8
time (min)
+GSH
+GSNO

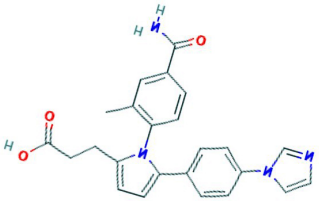
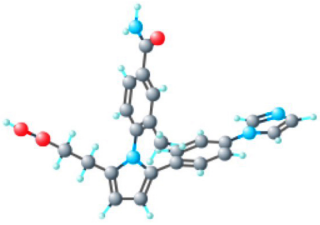
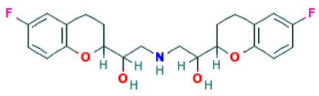
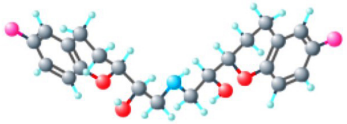
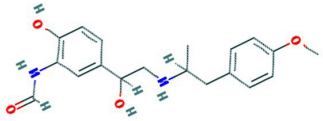
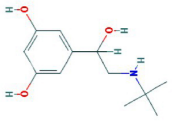
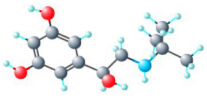
f)


Effect of NAD+ on Absorbance
1/A340
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
2
4
6
time (min)
Buffer + 10mM NAD
Buffer + 200µM NAD
Buffer Only

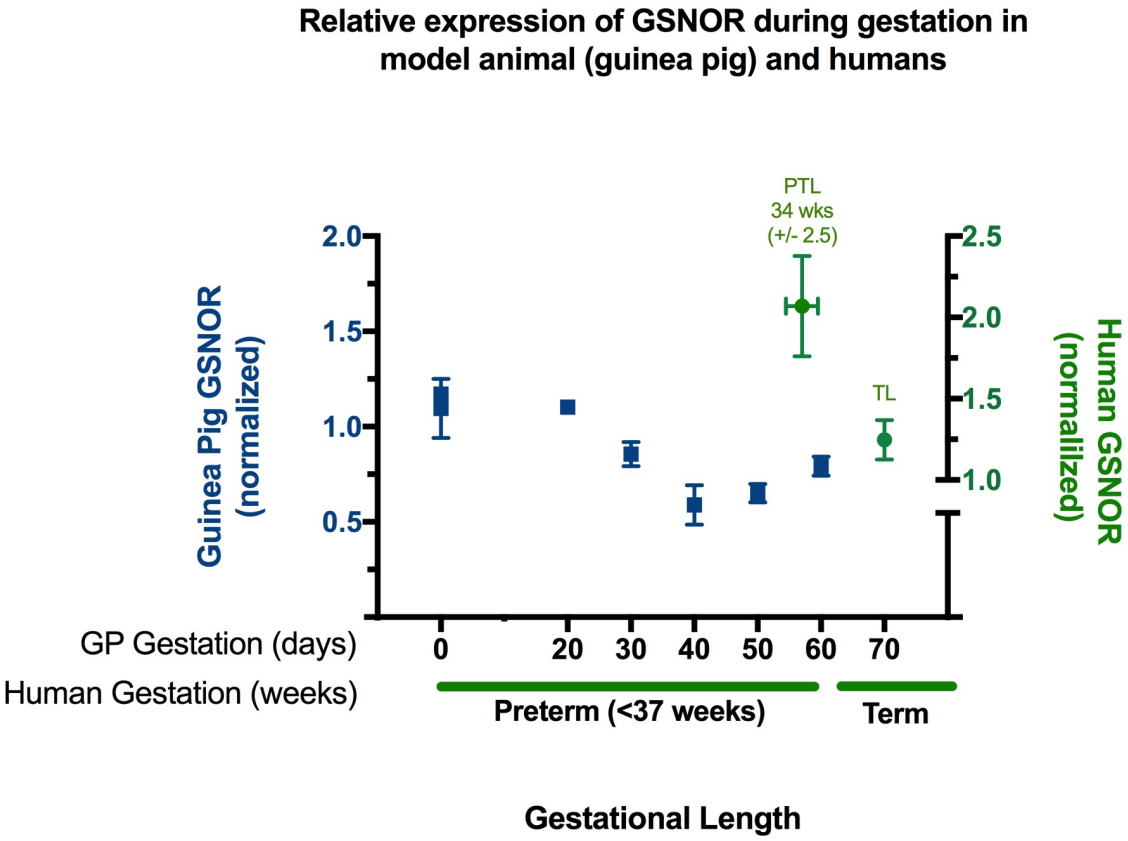
Supp. Figure 2: *Controls for enzyme activity assay*: (a) comparison of GSNOR enzyme activity of 10 minutes with fresh (never frozen) human myometrial tissue (1mg/ml) vs. once frozen intact tissue, and frozen protein lysates between 1 and 5 cycles. Fresh and once frozen tissues show no appreciable difference in enzyme activity. (b) NADH absorbs at 340nm and is the principal detector of GSNOR activity. Assay verified that recommended 200μM NADH was appropriate the range of our sensor. (d) Varied GSNO (substrate) and (e) protein concentrations to determine saturating concentrations. (f) Verified that the reaction products, glutathione and NAD+, do not affect absorbance measurements.


a)
GSNOR Protein Expression (wes®)
GSNOR/GAPDH
1.8
1.6
1.4
1.2
1.0
n.s.
n.s.
Caucasian (TNL)
African Decent (TNL)
Caucasian (TL)
African Decent (TL)
b)
GSNOR Enzyme Activity
1/A340 nm
1.2
1.0
0.8
0.6
0.10
0.05
0.00
n.s.
n.s.
Neg Control
Caucasian (TNL)
African Decent (TNL)
Caucasian (TL)
African Decent (TL)

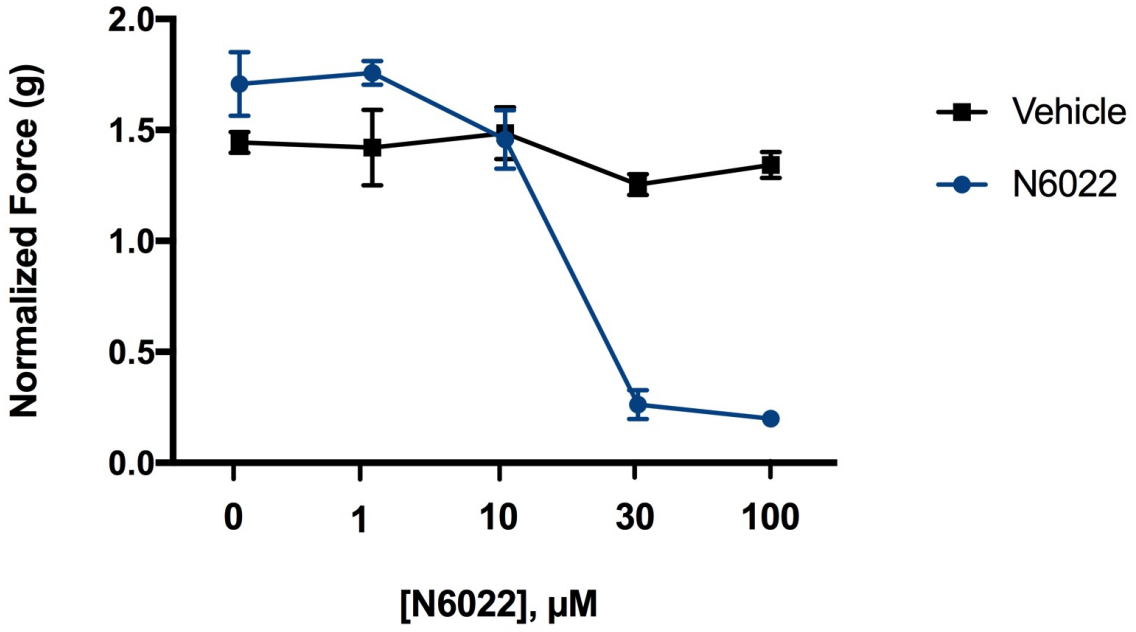
Supp. Figure 3: *GSNOR expression and enzyme activity assay between races*: (a) GSNOR protein expression is not significantly different between racial cohorts (African vs. Caucasian, TNL p=0.7541 n=3, TL p=0.4138 n=3), (b) nor is the GSNOR enzyme activity measured at time = 10 min (African vs. Caucasian, TNL p=0. 5318n=3, TL p=0.6946 n=3). 8nM N6022 was used as a control as it abolishes GSNOR activity.

	2D Structure	3D Structure
N6022		
Nebivolol		
Formoterol		
Terbutaline		

Supp. Figure 4: *2° and 3° structures of candidate GSNOR inhibitors from the National Center for Biotechnology Information*: Structural identifiers: nebivolol CID=71301, formoterol CID=3083544, N6022 CID=44623946, terbutaline CID=5403] (Database, 2017). Images used with permission (https://pubchem.ncbi.nlm.nih.gov/ (accessed Nov. 1, 2017)).

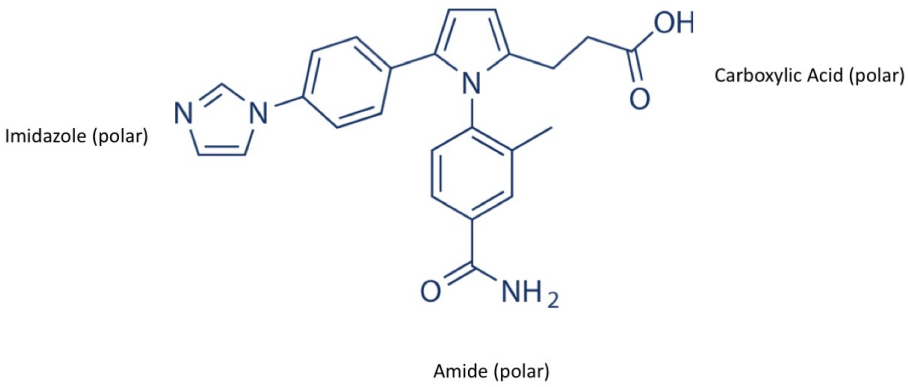

Relative expression of GSNOR during gestation in model animal (guinea pig) and humans
PTL
34 wks
(+/- 2.5)
TL
Guinea Pig GSNOR (normalized)
Human GSNOR (normalized)
2.0
1.5
1.0
0.5
2.5
2.0
1.5
1.0
GP Gestation (days)
Human Gestation (weeks)
0
20
30
40
50
60
70
Preterm (<37 weeks)
Term
Gestational Length

Supp. Figure 5: *Relative GSNOR expression as compared between human and guinea pigs*: Human term laboring (TL) GSNOR expression was set on a scale to match guinea pig GSNOR expression trend at term.

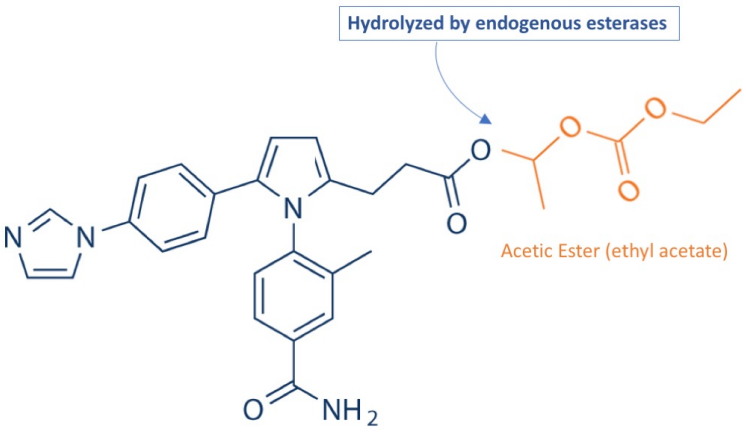

Normalized Force (g)
2.0
1.5
1.0
0.5
0.0
0
1
10
30
100
[N6022], µM
Vehicle
N6022

Supp. Figure 6: *Guinea pig myometrial response to N6022*: Preliminary data indicated that guinea pig myometrium relaxes completely to N6022 in a dose-dependent fashion, with contractions nearly completely abolished at 100μM concentration of N6022. Myometrial Response to N6022 inhibition after 30-minute incubation with drug relative to DMSO control. The experiment was not repeatable to the same extent in human or guinea pig samples.

a) **Native N6022**

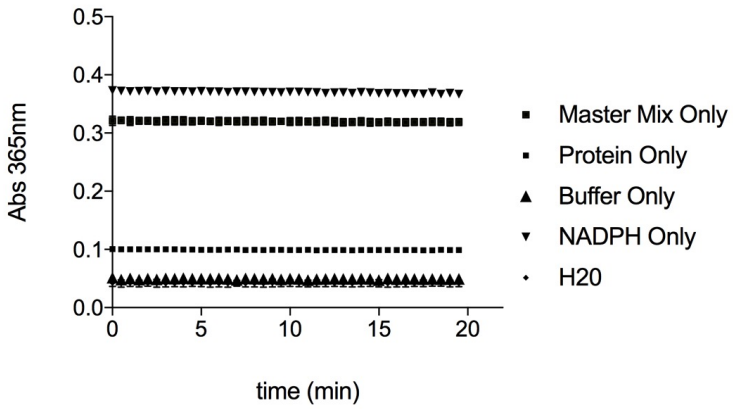


b) **Modified N6022**

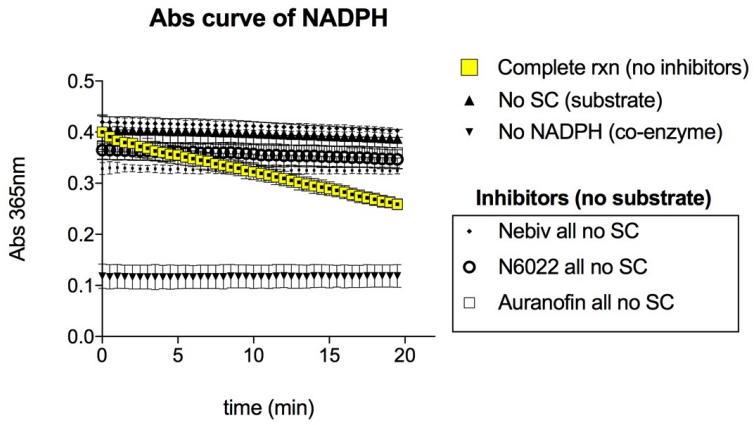


Supp. Figure 7: *Proposed modifications to N6022*: N6022 has a limited bioavailability (~4%). Charged and polar side-chains decrease permeability across the membrane. The addition of ethyl acetate to the charged and polar COO- side change of N6022 may help to neutralize the charge and distribute the electronegativity. Endogenous esterases have been shown to cleave similar bonds and return the drug to its native state once inside the cell.

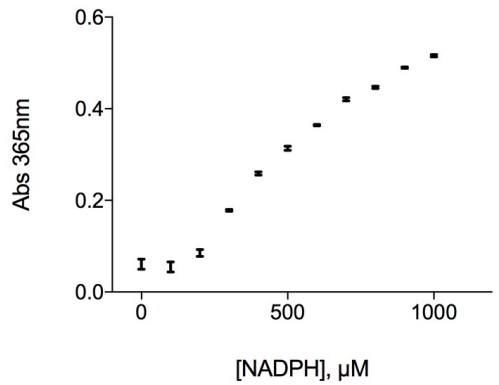
a)


Abs curve of NADPH
Abs 365nm
0.5
0.4
0.3
0.2
0.1
0.0
0
5
10
15
20
time (min)
Master Mix Only
Protein Only
Buffer Only
NADPH Only
H20

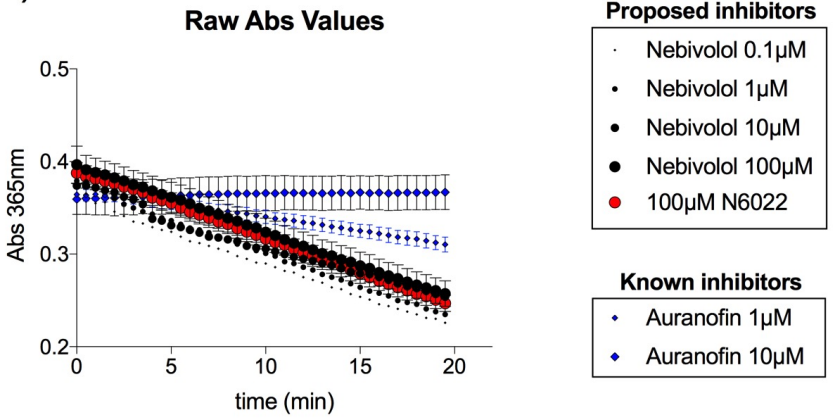
b)


Abs curve of NADPH
Abs 365nm
0.5
0.4
0.3
0.2
0.1
0.0
0
5
10
15
20
time (min)
Complete rxn (no inhibitors)
No SC (substrate)
No NADPH (co-enzyme)
Inhibitors (no substrate)
Nebiv all no SC
N6022 all no SC
Auranofin all no SC

c)


Abs curve of NADPH
Abs 365nm
0.6
0.4
0.2
0.0
0
500
1000
[NADPH], µM

d)


Raw Abs Values
Abs 365nm
0.5
0.4
0.3
0.2
0
5
10
15
20
time (min)
Proposed inhibitors
Nebivolol 0.1µM
Nebivolol 1µM
Nebivolol 10µM
Nebivolol 100µM
100µM N6022
Known inhibitors
Auranofin 1µM
Auranofin 10µM

Supp. Figure 8: *Thioredoxin reductase (TrxR) assay experimental controls*: (a) Determination as to whether or not the master mix (buffer + NADPH + selenocystamine), or its individual components, spontaneously altered absorbance over the 20-minute assay. (b) Determination as to whether or not the complete reaction mixture, minus either the substrate or the co-enzyme, spontaneously altered absorbance over the 20-minute assay. The complete reaction mixture as also tested without substrate, but in the presence of inhibitors. None of these conditions altered absorbance over time. (c) This assay was designed to measure absorbance of NAPDH, which has a peak absorbance of 340nm. Our equipment is only able to determine absorbance at 365nm, therefore, we determined that NADPH will absorb at this wavelength. (d) Relative TrxR activity is measured as the inverse of absorption, with TrxR "initial activity" set to a nominal value of 1 (figure 9). This figure shows the raw data collected from the sensor. Decreasing values seen in this figure represent metabolism of NADPH to NADP+ (which does not absorb at 365nm), which serves as an indirect measurement of TrxR activity.

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Chapter 5

S-nitrosation: Alternative Actions of Nitric Oxide in the Myometrium

Abstract:

The precise mechanism that leads to the onset of spontaneous preterm labor is not fully known; however, our finding that the myometrium relaxes when challenged with nitric oxide (·NO), independent from cGMP accumulation, indicates that ·NO functions through alternative pathways to relax the tissue. ·NO and S-nitrosoglutathione (GSNO) are capable of trans-S-nitrosating critical contractile-associated proteins in the myometrium, such as myosin light chain kinase, and myosin regulatory polypeptide 9. S-nitrosation is an important modulator of cellular function, and its dysregulation is seen in many disease states. Here we find that the increase in GSNO reductase activity in spontaneous preterm laboring myometrium correlates to a decrease in total protein S-nitrosation, and we explore the effects of GSNO on myosin light chain kinase activity, acto-myosin ATP-ase dynamics, and TREK-1 channel activity.

Introduction:

Preterm labor (PTL) and preterm birth (PTB) represent a significant predicament within the global community. The cost, both to the development of the fetus and financially, are substantial. In the United States alone greater than 12% of infants are born prematurely, resulting in 20,000 deaths annually (Martin, Hamilton et al. 2007). Worldwide, the statistics are even more disconcerting. Approximately thirteen million infants are affected by premature birth each year (Martin et al., 2011). Sub-Saharan Africa is of particular concern where as many as 336,000 of the 1.2 million (28%) births each year result in newborn death (Kinney et al., 2010).

A simple, yet persistent realization of pregnancy and parturition is that in order to prevent PTB, we need to also avoid or halt PTL. The myometrium is at the literal and metaphorical core of this problem. In order to identify and develop new tocolytic therapies to treat spontaneous preterm labor (sPTL), it is imperative that we develop a better understanding of how the myometrium differs from other smooth muscles. Our previous work has set the stage for this chapter by revealing two important distinctions of uterine smooth muscle. First, is the fact that the myometrium relaxes independently of global cGMP concentrations when exposed to nitric oxide (·NO), a finding that challenges existing dogma (Furchgott, 1999) established following the classic smooth muscle experiments of Furchgott (Furchgott and Zawadzki, 1980). The currently accepted mechanism of action of ·NO-mediated relaxation of vascular smooth muscle theorizes that ·NO binds to

the haem moiety of soluble guanylyl cyclase in the adjacent smooth muscle to activate the smooth muscle soluble guanylyl cyclase (sGC), resulting in the accumulation of cGMP in the muscle cell. cGMP then activates its cognate kinase, PKG, leading to phosphorylation of critical contraction-associate proteins such as myosin phosphatase, and relaxation of the muscle. In myometrium, however, ·NO relaxes the muscle, but this is largely independent of cyclic GMP elevation (Bradley et al., 1998b; I. L. Buxton, 2004a). This cGMP-independent relaxation indicates that ·NO is acting on alternative pathways to quiesce the tissue, such as through S-nitrosation, and opens a new door for which to investigate new therapies. The second relevant finding from our previous work is that S-nitrosoglutathione reductase (GSNOR), an enzyme responsible for the metabolism of ·NO, is upregulated in sPTL myometrium. This is important because GSNOR's dysregulation affects the availability of ·NO (S D Barnett and Buxton, 2017) and has been shown to alter the amount of S-nitrosothiols (SNOs) in the cell (Liu et al., 2001b). Taken together, these findings provide a base of understanding to better investigate non-canonical functions of ·NO function in myometrium; specifically, through protein S-nitrosation.

S-nitrosation: The posttranslational modification of proteins has long been recognized as a key regulator of cellular function. In recent years, it has been shown that the S-nitrosation acts as an important mediator of disease states (Anand and Stamler 2012). As with phosphorylation, S-nitrosation regulates cellular mechanisms and affects protein-protein interactions, and the emerging

field of S-nitrosation, and its effects on protein function, represents an exciting new branch of research. S-nitrosation cannot occur without an available source of ·NO (Martínez-Ruiz and Lamas, 2004). ·NO is produced enzymatically in many cell types and tissues, and as a low molecular weight molecule ·NO can easily cross the membrane (Bredt and Snyder, 1992). S-nitrosation occurs when an ·NO moiety is covalently added to the thiol side chain of cysteine residues within proteins and peptides (Hess, Matsumoto et al. 2005) by a one-electron oxidation from the ·NO radical (Smith and Marletta, 2012). The term nitrosylation is often used interchangeably with S-nitrosation, though this is incorrect. Both processes produce a S-nitrosothiol, but the manner in which this occurs differs chemically in that nitrosylation requires a metal centered protein such as guanylyl cyclase (Martínez-Ruiz and Lamas, 2004).

An analysis of the myometrial S-nitrosoproteome has revealed that several smooth muscle contractile-associated proteins (CAPS) are differentially S-nitrosated based upon the state of labor in women (Ulrich, Quillici et al. 2012). Included in these S-nitrosated CAP proteins is the regulatory light chain of smooth muscle myosin (SMM), called myosin regulatory light polypeptide 9 (LC20, RLC or MYL9), as well as the telokin domain of myosin light chain kinase (MLCK), an important domain that affects MYL9 phosphorylation (pMYL9) through its binding to SMM (Silver et al., 1997). The phosphorylation of MYL9 by MLCK is at the center of canonical contraction/relaxation pathways in smooth muscle. The finding that these proteins are differentially S-nitrosated in sPTL myometrium when exposed

to S-nitrosoglutathione (GSNO), an endogenous ·NO donor, makes them an interesting target to study. Unfortunately, there is a dearth of published data describing the functional significance of protein S-nitrosation, specifically in regards to its effects on smooth muscle. Here we investigate how GSNO impacts the function of several important CAP systems, to include: Acto-myosin dynamics, the rate of MYL9 phosphorylation by MLCK, and TREK-1 activity.

Material and Methods:

Protein Isolation for S-nitrosation Measurement. Myometrial muscle samples were collected from 12 patients in each pregnancy state and relevant experimentation and data analysis kindly provided by Dr. Craig Ulrich as described here (Ulrich et al., 2013a, 2015): “Tissues were ground to a powder under liquid nitrogen and reconstituted in 20 ml HEN buffer: HEPES-NaOH (25mM), EDTA (1mM), and neocuproine (0.1mM, pH 7.7). Samples were sonicated (10 x 2-sed bursts, 70% duty cycle) and brought to CHAPS (0.4%) (3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate). Samples were centrifuged at 2,000x g for 10 min at 4°C. Protein concentration was determined by the bicinchoninic acid assay and samples diluted to 0.8 mg/ml in HEN buffer. *Biotin Switch and Streptavidin Pulldown*. *Mass spectrometry methods in part from* (Ulrich et al., 2015). For the purposes of total protein S-nitrosation, samples from each patient in each group was independently isolated by biotin switch and

streptavidin pulldown and then pooled for tandem mass spectrometry (MS/MS) analysis (i.e., SPTL1, 4 unique patients; SPTL2, 4 unique patients; SPTL3, 4 unique patients; for a total of 12 unique patients split into 3 biological replicates to help control for human diversity). Protein isolates (1.8 ml 0.8 mg/ml in HEN buffer) were incubated with GSNO (300 μM) for 20 min at r.t. At this concentration, GSNO will produce ~5μM reactive NO over 15–20 min without accumulation (Cleeter et al., 1994). This reactive species concentration matches the IC_{50} concentration for relaxation of isolated myometrium (I. L. O. Buxton et al., 2001). Neither biotin-HPDP nor a maleimide dye lead to false positives because the amines or tyrosines are not labeled even if nitrosated. SDS (0.2 ml of 25% SDS) was added along with 30mM NEM. Samples were incubated at 50°C in the dark for 20 min and proteins precipitated in -20°C acetone for 1 hr and collected by centrifugation at 3,000 g for 10 min. The clear supernatant was aspirated, and the protein pellet was gently washed with 70% acetone (4 x 5 ml). After resuspension in 0.24 ml HEN buffer with 1% SDS (HENS), the material was transferred to a fresh 1.7-ml microfuge tube containing 30 μl biotin-HPDP (2.5 mg/ml). The labeling reaction was initiated by addition of 30 μl of 200mM sodium ascorbate (final 20mM ascorbate) for 1 hr at r.t. in the dark. Four volumes of -20°C acetone were added to the labeled samples and incubated at -20°C for 20 min to remove biotin-HPDP. The samples were centrifuged at 3,000 g for 10 min at 4°C, and the supernatant discarded. The pellet was resuspended in 140 μl of HENS buffer. Neutralization buffer (HEPES (20mM) pH 7.7, NaCl (100mM), EDTA (1mM), and Triton X-100 (0.5%)), was added (280

μl) along with 42 μl of streptavidin-agarose. Proteins were incubated for 1 hr at r.t. and washed five times with 1.5 ml of neutralization buffer with 600mM NaCl. Beads were incubated with 100 μl elution buffer (neutralization buffer with 600mM NaCl plus 100mM β-mercaptoethanol) to recover the bound proteins. This step releases the protein from the streptavidin bead leaving the biotin-HPDP tag bound to the bead as well as natively biotinylated proteins still bound to the bead. Four volumes of -20°C acetone were added to re-precipitate proteins. Samples were centrifuged at 3,000 g for 10 min at 4°C, the supernatant was discarded, and the pellet was washed and dried for proteomic analysis. *Western Blot for SNO-MYL9*: The identification of SNO-MYL9 began with the same biotin switch and streptavidin pulldown as above, but with a single laboring human myometrial sample. Prior to the switch and pulldown, the sample was split into three experimental groups and treated with either 300μM GSNO, 300μM GSH, or the volumetric equivalent of vehicle (H_2O) for 10 minutes. Following the biotin switch and streptavidin pulldown, the S-nitrosated protein lysate was run on a western blot. 20μg of protein lysate were run at 200 V for 45 min on a 4-20% PAGE gel and transferred to nitrocellulose, blocked in Licor® blocking buffer. The Western blot was labeled with rabbit anti-MYL9 polyclonal 1° (1:100, ab64161: Abcam Plc. Cambridge, UK), and 2° 680-donkey anti-rabbit 2° (Santa Cruz Biotech). This is a qualitative visualization of SNO-MYL9 as proteins to normalize the data were not available because only S-nitrosated proteins were in the western lysate.

Mass Spectrometry. “Mass spectrometry sample preparation and analysis were conducted by Dr. Craig Ulrich and the Nevada Proteomics Center as previously described (Ulrich et al., 2015). “Proteins were trypsin digested, followed by liquid chromatography (LC)/MS/MS analysis. Acetone-precipitated pellets were washed twice with 25mM ammonium bicarbonate and 100% acetonitrile, reduced, and alkylated using 10mM dithiothreitol and 100mM iodoacetamide and incubated with 75 ng sequencing grade modified porcine trypsin (Promega, Fitchburg WI) in 25mM ammonium bicarbonate overnight at 37°C. Peptides were first separated by Michrom Paradigm Multi-Dimensional Liquid Chromatography (MDLC) instrument [Magic C18AQ 3μ 200Å (0.2 x 50mM) column (Michrom Bioresources, Auburn, CA) with an Agilent ZORBAX 300SB-C18 5μ (5 x 0.3mM) trap (Agilent Technologies, Santa Clara, CA)] using a 0.1% formic acid/1% formic acid in acetonitrile gradient. Eluted peptides were analyzed using a Thermo Finnigan LTQOrbitrap using Xcalibur v 2.0.7. MS spectra (m/z 300–2,000) were acquired in the positive ion mode with resolution of 60,000 in profile mode. The top 4 data-dependent signals were analyzed by MS/MS with CID activation, minimum signal of 50,000, isolation width of 3.0, and normalized collision energy of 35.0 with a targeted reject list (Ulrich et al., 2013d). Dynamic exclusion settings were used with a repeat count of two, repeat duration of 10 s, exclusion list size of 500, and exclusion duration of 30 s” (Ulrich, et al., 2012).

Criteria for S-Nitrosation Identification. “PROTEOIQ (V2.6, www.nusep.com) was used to validate MS/MS-based peptide and protein identifications. Peptides were

parsed before analysis with a minimum Xcorr value of 1.5 and a minimum length of six amino acids. There were no matches to the concatenated decoy database, and therefore, a false discovery value is not applicable or "0." Peptide identifications were accepted if they could be established at >95.0% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at >95.0% probability and contained at least two identified peptides with five spectra per peptide. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony" (Ulrich, et al., 2012).

Actin Motility Assay: The actin motility assay was performed as previously described (Hooft et al., 2007) with minor modifications to minimize the presence of DTT in the flow chamber. Flow cells were prepared by flushing the following reagents through the flow chamber and incubating at each step for 1 min prior to the next addition: i) myosin added in the appropriate experimental concentration (5,10, 25, 50, 100, 200, 300, 400 μg/ml), ii) BSA (5mg/mL), iii) TRITC-labeled actin (10nM), iv) two washes of DTT-free actin buffer (with or without 300μM GSNO), and v) two washes of DTT-free motility buffer containing 1mM ATP (with or without 300μM GSNO). Note: skeletal muscle actin was used in lieu of smooth muscle actin, but their behaviors in the actin motility assay are functionally identical (Harris and Warshaw, 1993). Motility assays were implemented using a Nikon TE2000

epifluorescence microscope with fluorescent images digitally assimilated with a Roper Cascade 512B (Princeton Instruments, Trenton, NJ) camera. Each flow cell was imaged for 30 seconds from three distinct fields to obtain a single sample (n = 1) and performed in triplicate. Data were analyzed using Simple PCI tracking software (Compix, Sewickley, PA) to obtain actin-sliding velocities. Objects were defined by applying an exclusionary area threshold to minimize background noise. Velocities too slow to be accurately measured by the PCI tracking software were hand-calculated in ImageJ (version 1.50i, Mac OS 10.11) by recording the linear velocity of (3) filaments per recording (9 velocities per n=1). *Phosphorylated smooth muscle myosin (pSMM) preparation*. SMM was isolated (Hong et al., 2009) from frozen chicken gizzard (ID: 43018-2: Pel-Freez Biologicals, Rogers, AR) and phosphorylated (Haldeman et al., 2014) as previously described (Supp. Figure 1). SMM Phosphorylation above 50% minimally affects V_{max} (Warshaw et al., 1990). The resulting SMM product was dialyzed twice for 8 hours each in 2-liters of DTT-free HMM buffer, described below, using a 3ml, 3.5 kDa dialysis cassette (66330: Thermo Fisher Scientific Inc., Waltham, MA). Phosphorylation of the regulatory light chain (MYL9) was also performed as previously described (Ellison et al., 2000)(Supp. Figure 1) in DTT-free HMM buffer: MOPS (10mM) , EGTA (0.2mM), NaCl (50mM), $CaCl_2$ (3mM), $MgCl_2$ (2mM), and ATP (1mM) (#A3377: Sigma-Aldrich, St. Louis, MO). Chicken Gizzards kindly provided by the Cremo laboratory (University of Nevada, Reno, Pharmacology), with additional support by the Baker laboratory (University of Nevada, Reno, Pharmacology).

MLCK Activity Assay: The myosin light chain kinase (MLCK) activity assay was performed as previously reported (Alcala et al., 2016) with minor modifications. Each reaction mixture was prepared on ice and equilibrated in a r.t. water bath for 5 minutes prior to the addition of ATP [γ-32P] (BLU502A001MC: PerkinElmer, Waltham, MA). Reaction mixtures consisted of: buffer [Tris-HCl (pH 7.5, 25 mM), KCl (60 mM), $MgCl_2$ (4 mM), $CaCl_2$ (0.1 mM), 0.1% (v/v) Tween 80], human recombinant MLCK (0.01μM), CaM (2μM), +/- GSNO (300μM), MYL9 at appropriate experimental concentrations (0, 7.5, 10, 15, 20, 50, 75, 100, 150, 200 μM), and ATP [γ-^{32}P] (200μM). The addition of ATP [γ-^{32}P] denotes time = 0 and the reaction mixture was quenched at (5) 1-minute intervals on ion exchange chromatography paper (3698-915: Whatman/GE Life Sciences, Marlborough). Free β-counts from each unquenched hot reaction mixture were measured to account for pipetting variance and ATP [γ-^{32}P] decay between experimental days (repeated twice for each reaction mixture). Quenched reaction papers were allowed to dry completely and were then washed 3 times, 5 minutes each, in 0.5% (v/v) H_3PO_4, followed by a single 5-minute acetone wash and allowed to dry completely. Dried papers were placed into individual scintillation vials filled with scintillation fluid (882470: MP Biomedicals, Santa Ana, CA) and counted in a TriCarb 2900TR Liquid Scintillation Analyzer. Data were analyzed using Prism 7 (version 7.0c, Mac OS 10.11, GraphPad Software, Inc., La Jolla, CA) MLCK and CaM kindly provided by the Cremo laboratory (University of Nevada, Reno, Pharmacology). MYL9 was isolated from purified chicken gizzard SMM as

described by (Facemyer and Cremo, 1992). MYL9-C108-IAM: For blocked C108-MYL9 experiments, preparation was the same as before, but with iodoacetamide (IAM) to block MYl9. To accomplish the blocking of the lone cysteine on MYL9, we incubated the MYL9 with 5mM IAM (+5mM DTT) overnight at 4°C. The resulting proteins were dialyzed using a 3500 MWCO dialysis cassette (Pierce Biotechnology; Rockford, IL). The blocking of C108 was confirmed using a DTNB (5, 5'-dithio-bis(2-nitrobenzoic acid) free thiol detection assay (Anderson and Wetlaufer, 1975; Pouchnik et al., 1996), which gives a spectrophotometric determination of free thiol groups. *MYL9 Isolation*: Isolation of RLC (MYL9) begins with 500mg of total SMM in high salt storage buffer to which $MgCl_2$ (10mM) was added to filament the SMM. Solution is centrifuged at 20k RCF to pellet SMM then resuspended in Tris pH 8.0 at 4°C. Guanidine HCl (5M) is added to further solubilize the SMM. 100% cold EtOH is added until final concentration is 55%, at which time SMM, but not the RLC (MYL9) or ELC, should precipitate out of solution. Pellet out the SMM then continue to add 100% EtOH to 82.5% at which time RLC should precipitate out. Note: Test non-precipitated and precipitate solutions by running contents on Coomassie gel after RLC precipitation to confirm all RLC was isolated. We still had a small amount of ELC contamination, so we used the ratio of ELC to RLC to adjust final concentration of RLC in solution (Supp. Figure 2). Protocol and expertise for the MLCK assay provided by the Cremo laboratory (University of Nevada, Reno, Pharmacology).

Patch Clamp Techniques: Electrophysiology was conducted as previously described (Nathanael S Heyman et al., 2013a), with modifications: "Stably-transfected overexpressing HEK293-TREK1 cells were plated on glass coverslips 4-24 hours before experiments, placed in a chamber for recording mounted on top of an inverted microscope and currents typically were recorded in the standard whole-cell variant of the patch clamp technique using pCLAMP software (V9.2; Axon Instruments/Molecular Devices Inc; Sunnyvale, CA). Currents were amplified with an Axopatch200B amplifier (Axon Instruments/Molecular Devices Inc.; Sunnyvale, CA), digitized using a computer interfaced with a Digidata 1322A acquisition system (Axon Instruments/Molecular Devices Inc.; Sunnyvale, CA), filtered at 1kHz and digitized at 2kHz for whole cell recording. The external bath solution contained (in mM): NaCl (140), KCl (5.4), $CaCl_2$ (1.8), HEPES (10), $MgCl_2$ (1), and TEA (2) adjusted to pH 7.4 with NaOH and osmolarity adjusted to 310 mOsm/L with D-mannitol (measured with Model 3320 Osmometer/ Advanced Instruments; Norwood, MA). The pipette solution contained (in mM): KCl (140), K_2ATP (3), NaGTP (0.2), HEPES (5), and $MgCl_2$ (1), and BAPTA (10; minimize large-conductance Ca^{2+}-activated K^+ currents), adjusted to pH 7.4 with KOH and osmolarity adjusted to 310 mOsm/L with D-mannitol. Solutions were delivered by gravity through a manifold perfusion system. Pipettes were made of borosilicate glass (Sutter Instrument Co; Novato, CA) pulled on a two-stage vertical puller (pp-83; Narishige International USA, Inc.; East Meadow, NY) and had a resistance of 2-4 mΩ when filled with pipette solution. Cell capacitance and series resistance

were measured using the membrane test feature of pCLAMP. Series resistance was then compensated ≈70%. Cell capacitance was later used for normalization of whole cell current to capacitance to yield current density (pA/pF) for each cell." Either 100μM GSNO or GSH, in bath solution, was delivered by gravity through the manifold perfusion system. After a 5-minute lavage with drug whole-cell recording were obtained, followed by washout (data not shown). *hTREK-1 transfected HEK293 cells were generously provided by Dr. Wu (UNSOM, Pharmacology).*

Results:

The Effect of GSNO on Protein S-Nitrosation in Human Myometrium. Our previous findings revealed that GSNOR is upregulated in sPTL myometrium (Chapter 4), therefore, we elected to determine if total protein S-nitrosations in the myometrium are altered in sPTL samples as compared to full-term samples. Total protein S-nitrosations were measured in GSNO treated full-term laboring (TL) and sPTL myometrium using LC/MS/MS. Earlier work from our lab (Ulrich et al., 2013a) was differentially analyzed to identify total protein S-nitrosations of, 110 S-nitrosated proteins that were normalized to spectral counts and AUC measurements of extracted ion chromatographs.

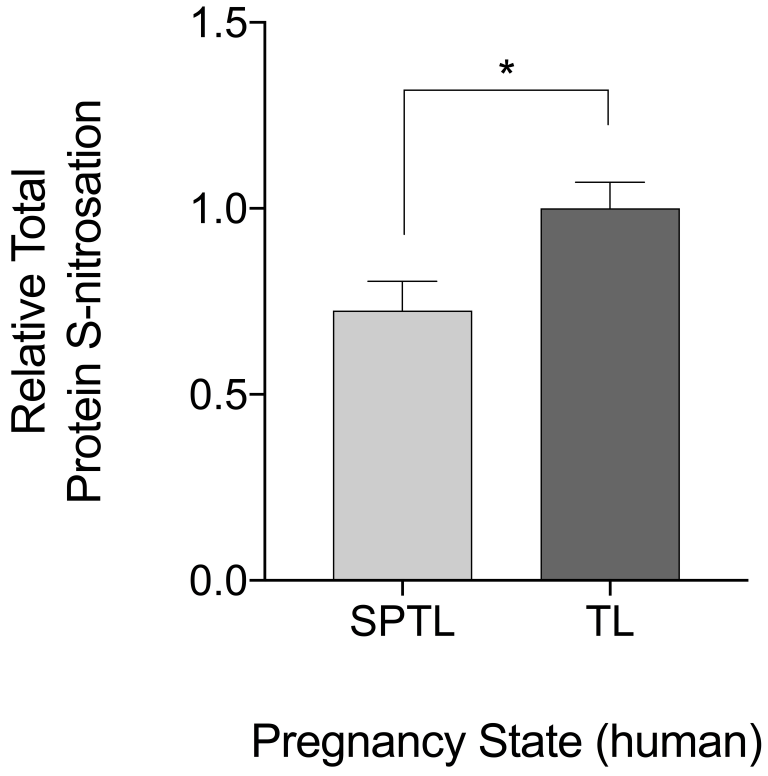
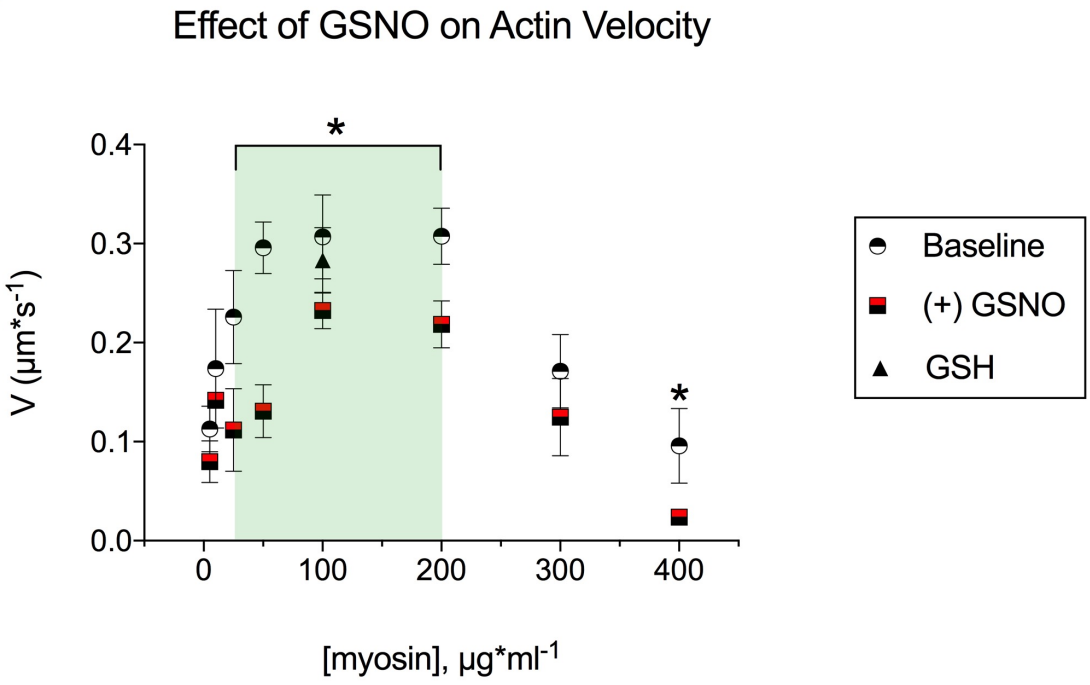

1.5
1.0
0.5
0.0
*
Relative Total
Protein S-nitrosation
SPTL
TL
Pregnancy State (human)

Figure 1: *S-nitrosation differences in TL vs sPTL myometrium*: Relative expression of human uterine smooth muscle S-nitrosated proteins in tissues from sPTL patients (n=12) versus patients in labor at term (n=12) when isolated myometrium is treated with GSNO. Data represent a compilation of total protein S-nitrosations in each tissue state controlled for total protein abundance. S-nitrosations are significantly lower in sPTL tissues ($p < 0.05$). *Data collected by Dr. Craig Ulrich*

a)


Effect of GSNO on Actin Velocity
V (µm*s-1)
0.4
0.3
0.2
0.1
0.0
0
100
200
300
400
*
*
[myosin], µg*ml-1
Baseline
(+) GSNO
GSH

b)

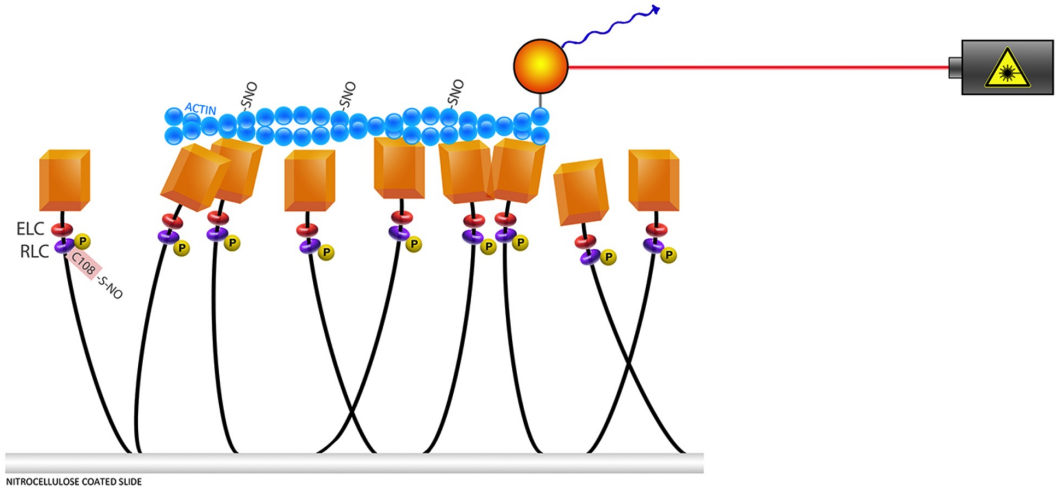

ACTIN
-SNO
-SNO
-SNO
ELC
RLC
C108
-S-NO
P
NITROCELLULOSE COATED SLIDE

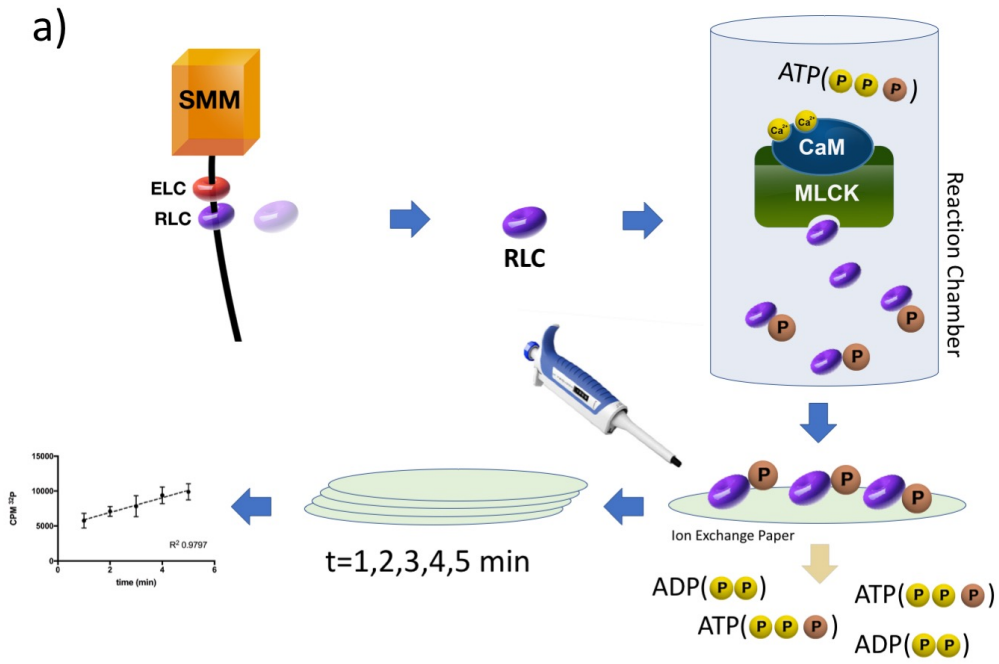
Figure 2: *Actin motility assay*: Actin velocities (± 300μM GSNO) reveal changes in ATPase activity and acto-myosin binding kinetics. Phosphorylated uterine smooth muscle myosin (pSMM) binds to a nitrocellulose-coated cover slip and is inverted onto a glass slide to form a flow cell. TRITC-labeled actin (10nM) and ATP (1mM) ± GSNO are combined in the flow-cell and actin velocities are measured using video imaging. All data presented as mean ± SEM

The number of MS2 events per peptide aligned with the actual peptide count that was determined by area under the curve of the MS1 chromatographs. ANOVA demonstrated that proteins exhibited statistically significant differences between TL and sPTL tissues specified by an F-statistic P value of $P < 0.05$ (Figure 1). These proteins exhibited log2 fold changes in expression of at least ±1 log in preterm laboring patients compared with full-term laboring patients.

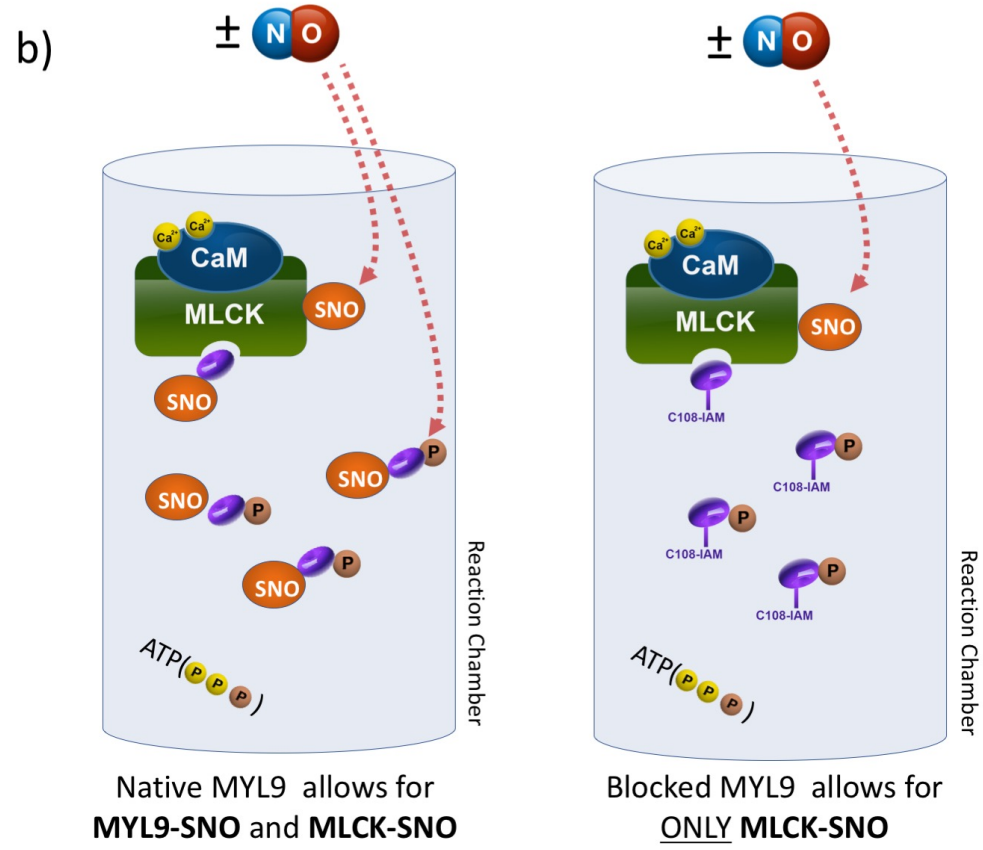
GSNO Alters Actin-Myosin Motility. An important metric of smooth muscle contractile dynamics is the rate at which myosin completes each cross-bridge cycle. The actin motility assay measures myosin-dependent actin dynamics, measured as translocation, or sliding, of actin filaments over myosin bound to a coverslip (Figure 2B). Attachment/detachment limiting kinetics can alter sliding velocities, based on myosin density, and we sought to determine if GSNO affects actin velocity across a large range of myosin densities. Addition of 300μM GSNO to the motility assay resulted in a reduction in velocity consistent with an effect of GSNO to S-nitrosate one or more constituents in the assay, specifically smooth muscle myosin, and/or actin, both of which contain numerous cysteines (Bansbach and Guilford, 2016). At myosin densities of 25, 50, 100, 200, and 400 (μg/ml), the addition of 300μM GSNO significantly decreased actin velocity ($P<0.05$) (Figure 2A), a result is consistent with an effect of GSNO to relax the smooth muscle, and in line with our preliminary data that showed a dose-dependent decrease in velocity in response to GSNO (Supp. Figure 3).

GSNO Increases the Rate of MYL9 Phosphorylation by MLCK. To determine if GSNO alters the rate at which MLCK phosphorylates MYL9, we ran an MLCK activity assay. The assay is a measurement of MLCK activity relative to substrate concentration. The substrate, in this case, is free MYL9, and MLCK action on MYL9 is measured over 5 minutes (t=1,2,3,4,5) to determine the rate of MLCK activity per unit time. The reaction mixture contains only the obligate kinase components: MLCK, MYL9, Calmodulin, Ca^{2+} and ATP [γ-32P]. At each MYL9 concentration (0-250μM) the reaction mixture is quenched at 1 minute intervals for 5 minutes (Supp. Figure 4), and the rate of phosphorylation is determined as a measure of β-decay, P-32 to S-32, per unit time, at each quench point, as read by a scintillation counter. The number of "counts" is proportionate to the amount of phosphorylated MYL9 proteins (n=3 per time point * 5 times points, yielding 15 readings per MYL9 concentration). The assay was run under identical conditions ± 300μM GSNO. The addition of GSNO did not alter the K_m [(-)GSNO: 51.50μM, ± 12.59 SEM. (+)GSNO: 59.31μM, +/- 13.71 SEM]; however, the V_{max} was significantly higher in the presence of GSNO [(-)GSNO: 0.3116 μmol P_i*(min-mg)$^{-1}$, ± 0.0258 SEM. (+)GSNO: 0.582 μmol P_i*(min-mg)$^{-1}$, ± 0.0511 SEM] (Figure 4A) (Supp. Figure 5). Control experiments using 40μM MYL9 in the absence of MLCK showed no detectable activity (data not shown).

MYL9 (C108) is known to be up-S-nitrosated in sPTL myometrium (Ulrich et al., 2013c), while MLCK is down-S-nitrosated (Ulrich et al., 2012b), as compared to term laboring tissue. Because our reaction mixture contained both MLCK and



ATP(PPP) = ATP [γ-32P]

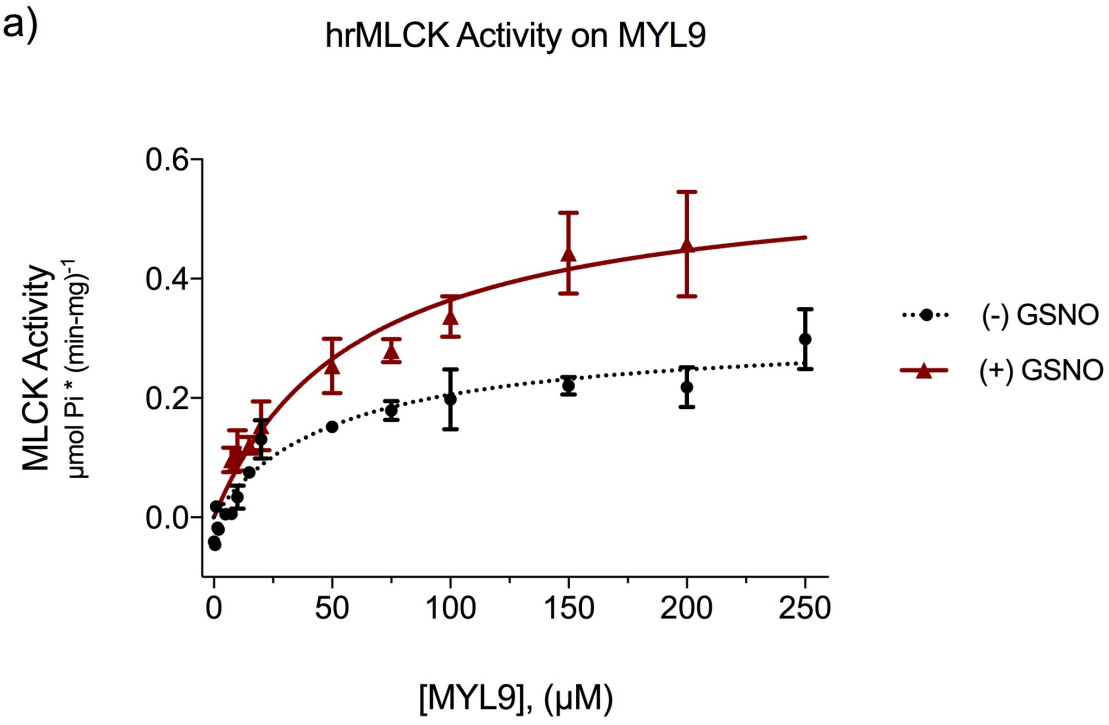


Native MYL9 allows for **MYL9-SNO** and **MLCK-SNO**

Blocked MYL9 allows for ONLY **MLCK-SNO**

Figure 3: *Myosin light chain kinase activity assay*: (a) The MLCK activity assay utilizes radioactive ATP [γ-32P] to determine the rate of phosphorylation of free regulatory light chain (MYL9) by human recombinant MLCK. Beta radiation counts from phosphorylated MYL9 are collected over 5-minutes (at 1-minute intervals) to establish the rate of MLCK activity at each myosin concentration, ± 300μM GSNO. GSNO is a stable endogenous form of nitric oxide, and is capable of the non-enzymatic transfer of the NO-moiety to cysteine thiols, a process called S-nitrosation. We use the MLCK activity assay, ± GSNO, to determine if GSNO affects MLCK activity when (b, left) both MYL9 and MLCK are S-nitrosated, or (b, right) when only MLCK is S-nitrosated.

a)


hrMLCK Activity on MYL9
MLCK Activity
µmol Pi * (min-mg)-1
0.6
0.4
0.2
0.0
0
50
100
150
200
250
[MYL9], (µM)
(-) GSNO
(+) GSNO

b)

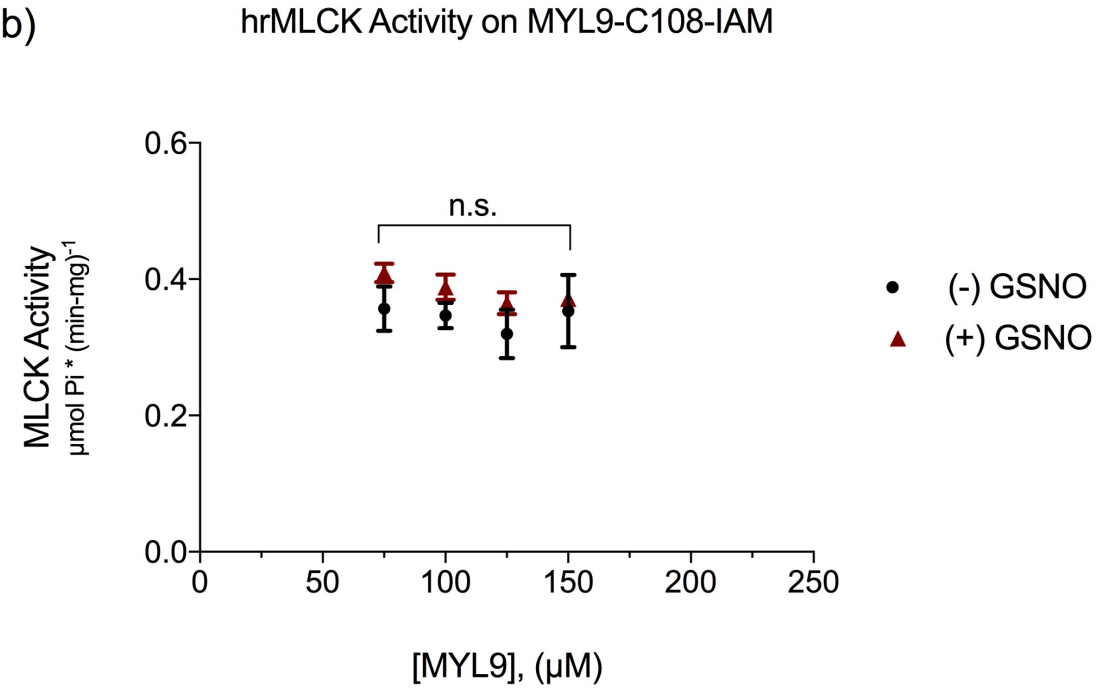

hrMLCK Activity on MYL9-C108-IAM
MLCK Activity
µmol Pi * (min-mg)-1
0.6
0.4
0.2
0.0
n.s.
0
50
100
150
200
250
[MYL9], (µM)
(-) GSNO
(+) GSNO

Figure 4: *GSNO affects the rate of phosphorylation of MYL9 by MLCK:* The MLCK kinase assay utilizes radioactive ATP [γ-32P] to determine the rate of phosphorylation of free regulatory light chain (MYL9) by human recombinant MLCK. Beta radiation counts from phosphorylated MYL9 are collected over 5-minutes (at 1-minute intervals) to establish the rate of MLCK activity at each myosin concentration, ± 300μM GSNO. (a) The K_m was not significantly different between baseline (K_m=49.12μM 95% CI: 29.05 to 86.25) and GSNO (K_m=59.13μM 95% CI: 35.7 to 105) treated samples; however, the V_{max} was significantly higher in the presence of GSNO (1.862x, 95% CI: 1.557 to 2.357) as compared to baseline. (b) Identical experimental conditions, but MYL9 thiols were blocked with iodoacetamide, which prevents S-nitrosation of C108. Assay run at myosin concentrations between 75-150 μM, which allows for a determination of changes in V_{max}. There was not a significant change in V_{max} after the application of GSNO, indicating that MYL9 S-nitrosation is the contributing factor to the changes in V_{max}.

MYL9, the previous experiment is unable to determine whether MYL9-SNO or MLCK-SNO is to account for the change in phosphorylation rates. To address this issue, we blocked the lone cysteine on MYL9 with iodoacetamide (IAM), MYL9-C108-IAM, to prevent its S-nitrosation when exposed to GSNO. The assay was repeated under otherwise identical assay conditions at concentrations of MYL9 (75,100,125,150 μM) that would allow a change in V_{max} to be observed. There was no statistically appreciable difference (p=0.238, n=4) in the phosphorylation rates of MYL9-C108-IAM in the presence or absence of GSNO (Figure 4B). This suggest that MYL9-SNO is the contributing variable to the change in observed phosphorylation rates when exposed to GSNO.

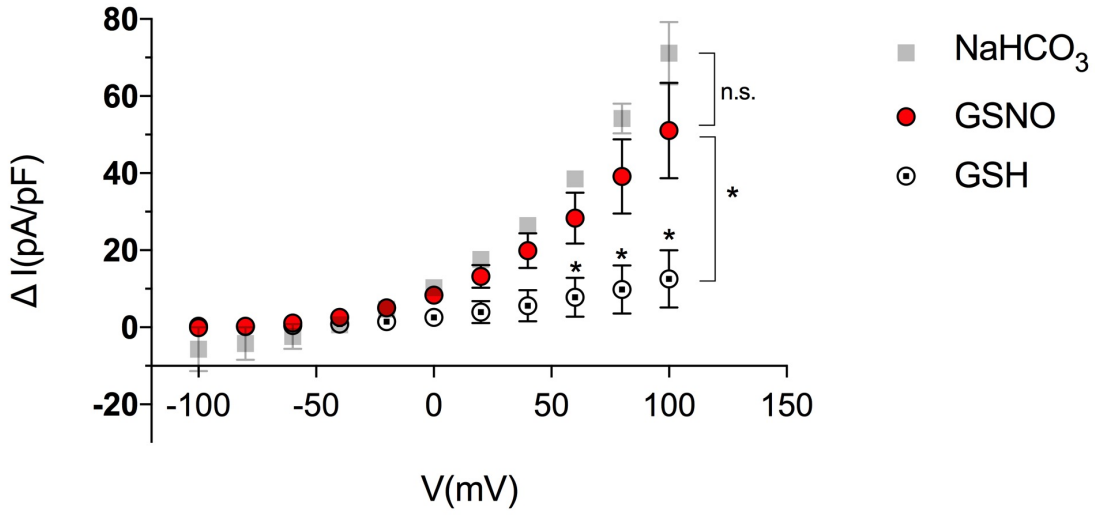
GSNO Increases hTREK-1 Currents. TREK-1 is an outward rectifying K^+ that is important to uterine quiescence during pregnancy by maintaining the negative membrane potential (Nathanael S Heyman et al., 2013b). The presence of TREK-1 splice variants in sPTL myometrium have been shown to impair channel function and membrane localization (Chapter 2) (C. L. Cowles et al., 2015). Beyond splice variants, the C-terminus of TREK-1 is susceptible to several phosphorylative PTMs (Figure 5B) by PKA/PKG/PKC that greatly alter channel function (Enyedi and Czirjak, 2010), and using analytical software (GPS-SNO v1.0) it is predicated that C414 on the cytosolic C-terminus of TREK-1 can be S-nitrosated (Xue et al., 2010). We compared TREK-1 activity in the presence of 300μM GSNO or 300μM glutathione (GSH, which is structurally identical to GSNO but without the ·NO moiety) to that of $NaHCO_3$, a known activator of the channel.

Application of 100μM GSNO for 10 minutes to overexpressing stably transfected HEK293 hTREK-1 resulted in a significant increase in outward current, as compared to GSH control, at applied voltages of +60 to +80mV (+60mV p=0.049, +80mV p=0.043, +100mV p=0.038, n=4 for each) (Figure 5A). At +100mV there was a ~4-fold increase of TREK-1 current over GSH (GSNO 51.1 ± 12.39, GSH 12.62 ± 7.489 pA/pF, n = 4). Conversely, there was no significant difference between GSNO activated currents and $NaHCO_3$ activated current at +100mV (n.s., p=0.3569), or any other voltage. Data is presented as ΔI(pA/pF), indicating a net change in current as compared to baseline for each recording. Taken together, these data demonstrate activation HEK293-hTREK-1 cells with GSNO in a manner similar to those previously reported for TREK-1 channels using known endogenous activators (Caley et al. 2005; Meadows et al. 2000).

Discussion

The myometrium is unique when compared to all other muscles. Of the many distinguishing characteristics of uterine smooth muscle, here we seek to better understand the alternative actions of ·NO in the cell. The ability of ·NO, and its endogenous analogs, such as GSNO, to posttranslationally modify proteins through S-nitrosation indicates a unique pathway in the myometrium to quiesce the tissue. S-nitrosations result in both redox regulation, as well as stable

a) GSNO Activation of TREK-1



b) TREK-1 Translated Sequence

```
        10         20         30         40         50
MLPSASRERP GYRAGVAAPD LLDPKSAAQN SKPRLSFSTK PTVLASRVES
        60         70         80         90        100
DTTINVMKWK TVSTIFLVVV LYLIIGATVF KALEQPHEIS QRTTIVIQKQ
       110        120        130        140        150
TFISQHSCVN STELDELIQQ IVAAINAGII PLGNTSNQIS HWDLGSSFFF
       160        170        180        190        200
AGTVITTIGF GNISPRTEGG KIFCIIYALL GIPLFGFLLA GVGDQLGTIF
       210        220        230        240        250
GKGIAKVEDT FIKWNVSQTK IRIISTIIFI LFGCVLFVAL PAIIFKHIEG
       260        270        280        290        300
WSALDAIYFV VITLTTIGFG DYVAGGSDIE YLDFYKPVVW FWILVGLAYF
       310        320        330        340        350
AAVLSMIGDW LRVISKKTKE EVGEFRAHAA EWTANVTAEF KETRRRLSVE
       360        370        380        390        400
IYDKFQRATS IKRKLAELA GNHNQELTPCR RRTLSVNHLT SERDVLPPLL
       410    *   420
KTESIYLNGL TPHCAGEEIA VIENIK
```

Termini
Helical Domain
Pore Domain
Cysteines

Important C-terminal modifications

S315=PKC
E321=H+
S348=PKA
S360=PKG
C414=SNO*

* C414 - Highest probability S-nitrosation[1]

Figure 5: *GSNO increases TREK-1 currents HEK293-hTREK-1 cells*: (a) Either 100μM GSNO, GSH or $NaHCO_3$ was applied to the bath of HEK293-hTREK-1 cells and incubated for 10 minutes. The change (delta) in mean current density in response to 20-mV voltage steps from 100 to 100 mV before (initial) and after application of GSNO, GSH or $NaHCO_3$ was recorded and GSNO treated cells showed a significantly greater activation over GSH treated cells at voltages of +60 mV to +100 mV. $^*P < 0.05$. (b) The translated sequence of TREK-1 reveals the significance of C-terminus to PTMs. Analytical software (GPS-SNO) indicates that C414 on the C-terminus is likely to be S-nitrosated.

S-nitrosations that alter protein function (Scott D. Barnett and Buxton, 2017a; Rizi et al., 2017; Wolhuter and Eaton, 2017). In recent years, the body of evidence to support protein S-nitrosation as an important regulator of human health and disease has grown dramatically (Foster et al., 2009b). S-nitrosation in the cell is highly dependent on the availability of intracellular GSNO (Smith and Marletta, 2012). As GSNO concentrations increase, so do levels of total protein S-nitrosation. Because $GSNOR^{-/-}$ mice show increased cellular levels of GSNO and SNOs (Liu et al., 2004), it is likely that GSNO, and S-nitrosated proteins, are in equilibrium governed by Cys-to-Cys trans-nitrosation (Dalle-Donne et al., 2000), and GSNOR mediated de-nitrosation (Liu et al., 2001b). The global decrease in total protein S-nitrosations measured between term and preterm myometrium (Figure 1) is consistent with our measured increase in GSNOR expression in sPTL (chapter 4), and highlights the possibility that specific S-nitrosation variability might underlie and contribute to preterm pathology.

We have previously shown that many proteins critical to the uterine contraction/relaxation cycle are differentially S-nitrosated in preterm laboring myometrium, as compared to term laboring, and term non-laboring tissue lysates (Ulrich et al., 2013d). There is a conspicuous paucity of functional data detailing whether or not the S-nitrosation of CAP proteins in smooth muscles are functionally relevant. To further advance this body of knowledge, we investigated how acto-

myosin ATP-ase activity, MLCK activity, and TREK-1 channel activity were altered in the presence of GSNO.

Muscles are unable to contract without active acto-myosin cross-bridge cycling. The actin motility assay measures the translocation of F-actin by myosin (Sellers, 2001), and serves as an indicator of acto-myosin ATP-ase activity, and by extension, cross-bridge cycling. ·NO donors have long been known to impact skeletal muscle systems with a general trend towards decreasing contractile force, sliding velocity, and ATP-ase activity (Bansbach and Guilford, 2016). In fact, S-nitrosation of the heavy chain of skeletal and cardiac myosin causes a decrease in actin velocity (Evangelista et al., 2010a), and S-nitrosation of skeletal myosin affects the catalytic cycle, but does not alter Actomyosin affinity (Nogueira et al., 2009). While the structure and function of smooth muscle myosin differs in several critical ways to skeletal and cardiac myosin, and thus prevent direct comparison of GSNO actions in the two systems, the actin monomers that interact with the myosin head are functionally identical between the muscle classes (Harris and Warshaw, 1993). To this point, relevant studies of skeletal SNO-actin have been shown to affect the rate of f-actin formation (Dalle-Donne et al., 2000), and in both skeletal and smooth muscle, actin can be S-nitrosated at two sites per monomer and this results in a decrease in sliding velocities by ~24% when using skeletal myosin (Bansbach and Guilford, 2016).

When we applied GSNO to the actin motility assay we saw a decrease in actin velocity at intermediate concentrations of myosin (Figure 2), a finding

consistent with ·NO-donor application to skeletal muscle myosin (Evangelista et al., 2010b). While it is common to only investigate actin velocity at a single myosin concentration, typically 100 μg/ml, testing its velocity at a wide range of myosin densities permits one to better understand the nuances of the attachment and detachment kinetics. The velocity of the actin in this assay is defined as V=n*d*v, where n=number of myosin per actin filament, d=distance moved per stroke, and v=rate of ATPase activity (Stewart et al., 2013). At low myosin densities, the velocity of actin is constrained by the lever arm motion at the point of the cycle in which the myosin transitions from a free to actin-bound state, as there are a limited number of myosin heads competing for the actin filament (Baker et al., 2002). This is called the "attachment limit," and our data indicates that the presence of GSNO is affecting this dynamic (Figure 2). As the concentration of myosin increases, it is the myosin transitioning from the bound to unbound state that affects velocity. At high myosin densities, the probability of second myosin head being attached to the actin, while the first myosin is attempting a power stroke, increases. Because of this, at high myosin densities the "detachment limit" dictates the velocity of the actin. The entire ATP-ase cycle takes approximately one second, and as the number of myosin increases, the chances of additional myosin being bound simultaneously increase dramatically, causing the velocity to plateau. At these high myosin densities, we recorded unexpectedly low velocities, both with and without GSNO. While there is a dearth of published data surrounding SMM velocities at our highest velocities (300 and 400 μg/ml), trends suggest that

velocities generally plateau, rather than decrease (Harris and Warshaw, 1993); therefore, this finding is compelling and warrants further investigation. Our experiments lack the specificity to determine which protein S-nitrosations were responsible for this decrease in actin velocity, but future experiments will include S-nitrosating induvial reaction chamber components, such as MYL9 and actin, to pinpoint the relevant protein S-nitrosations.

The actin motility assay just described reports ATP-ase activity, which is an important metric detailing the physical mechanism of force production (Le Clainche and Carlier, 2004). Upstream of the process, however, is the critical phosphorylation of MYL9. Phosphorylation of MYL9 by MLCK is at the crux of all contractile activity in smooth muscle. Beyond MYL9's phosphorylation, which initiates ATP-ase activity and cross-bridge cycling (Word, 1995), MYL9 is also required, from a structural sense, for folding into the 10S conformation (Katoh and Morita, 1996), and pMYL9 interaction with the essential light chain enhances lever action of the myosin head to allow for engagement with actin (Ni et al., 2012). There is little doubt that MYL9 is a fundamentally critical component to SMM structure and function. The obligate phosphorylation of MYL9 for cross-bridge cycling is at S19 (Colburn et al., 1988), but less is known about the relevance of PTMs more distal from the N-terminus where that phosphorylation occurs. Interestingly, through the use of photo-crosslinking, it has been shown that C108, the sole cysteine of MYL9, is unshielded when in the unphosphorylated state (Mazhari et al., 2004), intimating the possibility that C108 may be susceptible to S-

nitrosation when the cell is relaxed. Previous work from our lab has shown that MYL9 in not S-nitrosated when the myometrium is quiescent prior to labor (TNL), and that it achieves the highest state of S-nitrosation during spontaneous preterm labor (Ulrich et al., 2012b). At first glance, it may appear contradictory that GSNO, an agent known to promote relaxation, increases the rate of MYL9 phosphorylation (Figure 4). However, when we consider that sPTL myometrium is functionally a "disease" state, and that MYL9 is highly S-nitrosated during sPTL over TL and TNL, it is easy to entertain the possibility that S-nitrosation of MYL9 is promoting contraction through increased kinase activity. What remains obfuscated is why MYL9 in sPTL fundamentally more permissive to S-nitrosation? This question is further confounded when we consider that total S-nitrosations decrease in sPTL myometrium (Figure 1) as a result of increased GSNOR activity (chapter 4). Whether the cytosolic environment in sPTL myometrium promotes a conformational shift in MYL9, or if it is driven by distinct mutation of the protein, or something else, requires further investigating. These data, however, provide novel insight into the functional relevance of MLCK action on MYL9-SNO.

Phosphorylation of MYL9 cannot occur unless MLCK is activated. MLCK activation requires stimulation by calmodulin, which in turn needs high cytosolic Ca^{2+}. That influx of Ca^{2+}, spurred by CICR, does not occur until the membrane is depolarized. One of the most obvious distinguishing characteristics of the myometrium, when compared to other types of smooth muscle, is that it must remain largely quiescent over the 40 weeks of gestation. The myometrium employs

many tools to achieve this end, such as oxytocin receptor modulation (Takemura et al., 1996), a shift in progesterone receptor ratios to decrease CAP protein production (Tan et al., 2012), among many others, to include an increase in TREK-1 expression (Tichenor et al., 2005), which helps to maintain a negative cell membrane potential, preventing the aforementioned influx of Ca^{2+}. Our laboratory has previously determined that splice variants of TREK-1 affect trafficking of full-length functional TREK-1 in sPTL (Chapter 2) (C. L. Cowles et al., 2015), and computational analysis of TREK-1 using the program GPS-SNO (Xue et al., 2010), suggests a high likelihood that TREK-1 may be S-nitrosated at C414 on the C-terminus, in the same region as other key PTMs that alter TREK-1 function. Our finding that GSNO increases TREK-1 currents (Figure 5), further enhancing membrane hyperpolarization, corroborates the possibility of C414 S-nitrosation. That being said, there are other plausible pathways to TREK-1 activation through GSNO. For example, the HEK293 hTREK-1cells used for these experiments natively express PKG (Protein Atlas: ENSG00000185532), a known activator of TREK-1 through phosphorylation at S360 (previously reported as S351) (Enyedi and Czirjak, 2010). Future experiments will mutate C414 to determine if GSNO still activates the channel.

In conclusion, the underlying cause(s) of sPTL remain unknown. Here we provide a novel mechanism that may provide insight into the unique relaxation pathway in the myometrium through protein S-nitrosation. Our data have revealed that total protein S-nitrosations in sPTL myometrium are decreased relative to term

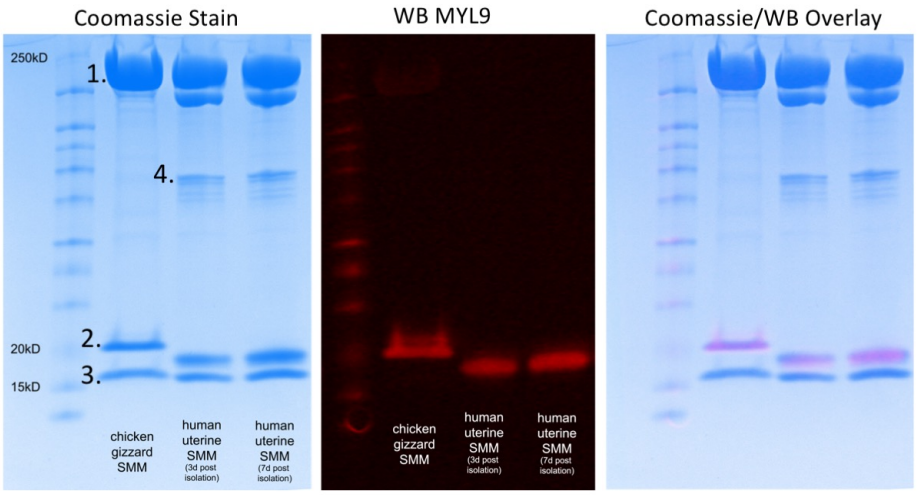
tissue, which serves as an indicator of ·NO availability, and implicates sPTL as a "disease state." In an effort to elucidate the functional implications of protein S-nitrosation, we determined that acto-myosin ATP-ase activity is retarded in the presence of GSNO, MLCK activity is enhanced, and that the outward rectifying K^+ channel, TREK-1, exhibits an increase in current. Our finding that GSNOR, and by extension protein S-nitrosation, are dysregulated in the myometrium of women undergoing sPTL affords an opportunity to investigate a new class of drugs that increase the availability of endogenous ·NO in the cell.

Acknowledgements:

With the highest level of gratitude, I would like to thank the Baker and Cremo laboratory for patience and expertise with the actin motility assay and the MLCK activity assay. This simply would not have been possible without their unreserved assistance. Also, thank you to Craig Ulrich for providing the mass-spectrometry data.

SUPPLEMENTAL FIGURES

a)



1) Heavy Chain 2) MYL9 3) ELC 4) Actin Contamination

b)

MYL9 orthologs between human and chicken

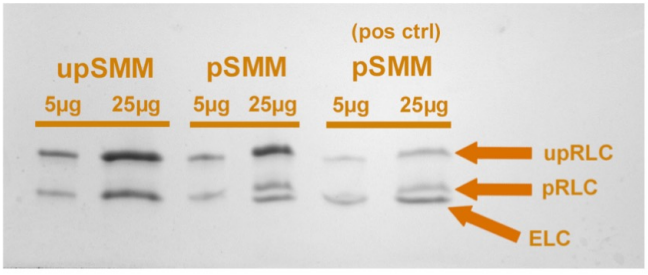
```
1    MSSKRAKAKTTKKRPQRATSNVFAMFDQSQIQEFKEAFNMIDQNRDGFIDKEDLHDMLAS   60  P02612   MLRM_CHICK
1    MSSKRAKAKTTKKRPQRATSNVFAMFDQSQIQEFKEAFNMIDQNRDGFIDKEDLHDMLAS   60  P24844   MYL9_HUMAN
     ************************************************************
     §                                                  †
61   MGKNPTDEYLEGMMSEAPGPINFTMFLTMFGEKLNGTDPEDVIRNAFACFDEEASGFIHE   120 P02612   MLRM_CHICK
61   LGKNPTDEYLEGMMSEAPGPINFTMFLTMFGEKLNGTDPEDVIRNAFACFDEEASGFIHE   120 P24844   MYL9_HUMAN
     :************************************************:***********

121  DHLRELLTTMGDRFTDEEVDEMYREAPIDKKGNFNYVEFTRILKHGAKDKDD  172  P02612   MLRM_CHICK
121  DHLRELLTTMGDRFTDEEVDEMYREAPIDKKGNFNYVEFTRILKHGAKDKDD  172  P24844   MYL9_HUMAN
     **************************************************
```

§ The only amino acid deviation between the two peptides

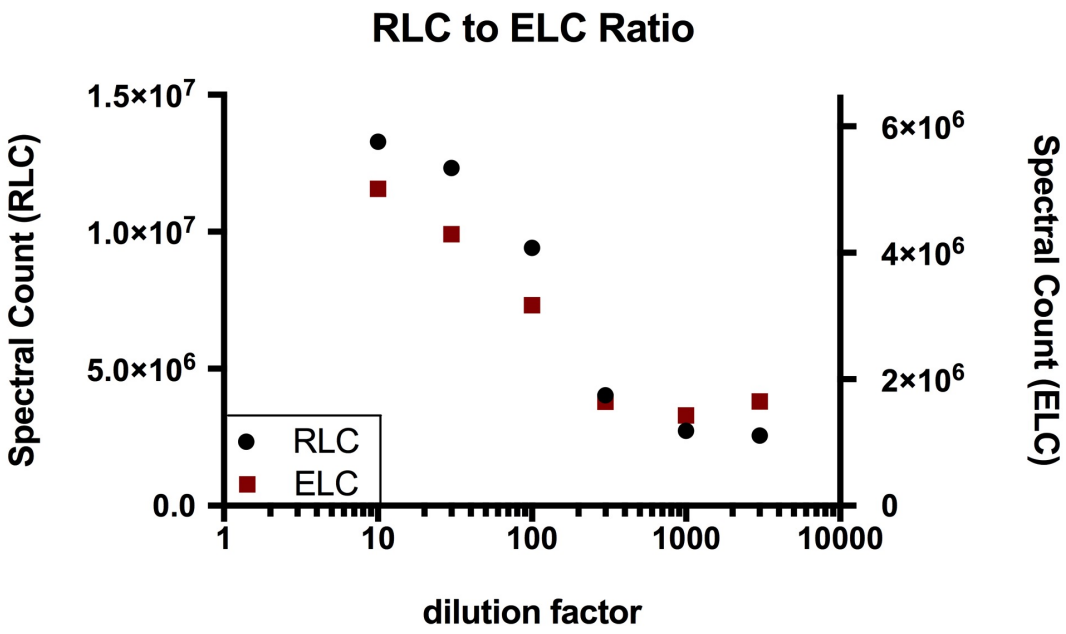
† C108 is the only cysteine in the peptide

c)

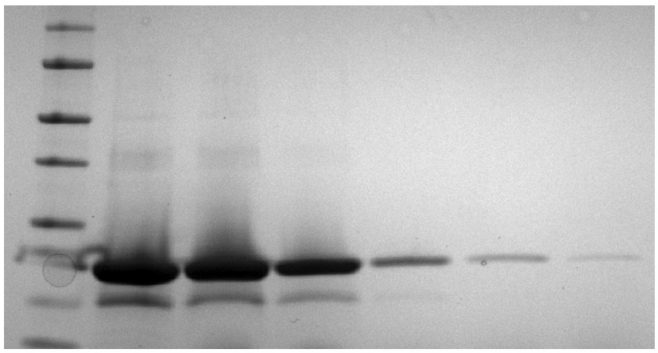


Supp. Figure 1: *SMM isolation, phosphorylation, and MYL9 differentiation*: (a) (*left panel*) A Coomassie stain verifying the purity of SMM isolation in both chicken (*gallus gallus*) gizzard, and human myometrium. Human SMM was tested for degradation after three and seven day. (*center panel*) A western blot using 10*μ*g of SMM to verify specificity of the MYL9 antibody for both human and chicken MYL9. (*right panel*) Overlay of western blot (pink) for MYL9 and Coomassie Blue total protein stain (blue) to verify presence of MYL9 in both gizzard and human myometrial smooth muscle myosin (SMM) isolates. A single 10-well 4-20% polyacrylamide gel with duplicate samples was run. Image was generated using Adobe® Photoshop™ CS5 with a 100% screen overlay. (b) MYL9 differs at only two amino acids in human (uniprot: P24844) and chicken gizzard (uniprot: P02612). Importantly, C108, the only cysteine in the protein, is unaltered. (c) Phosphorylation was assessed using non-denatured, urea prepped samples, on a 10% or 4–20% Tris-glycine gel.

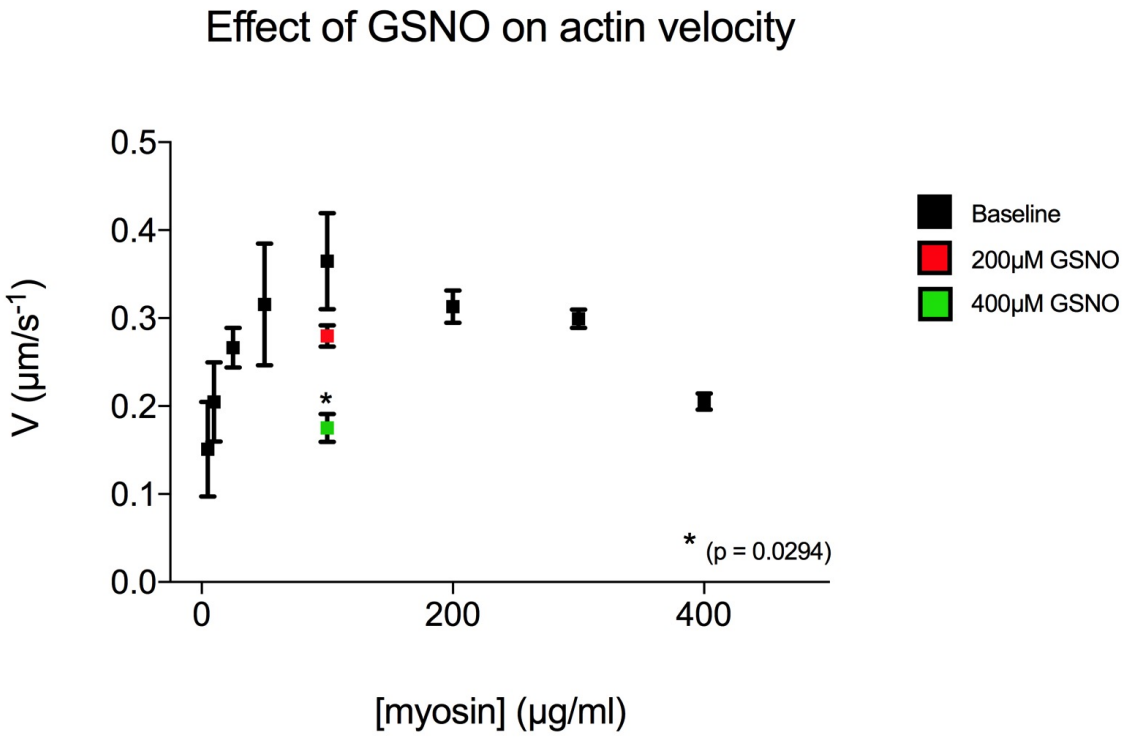
a)


RLC to ELC Ratio
Spectral Count (RLC)
1.5×10^7
1.0×10^7
5.0×10^6
0.0
6×10^6
4×10^6
2×10^6
0
Spectral Count (ELC)
RLC
ELC
1
10
100
1000
10000
dilution factor

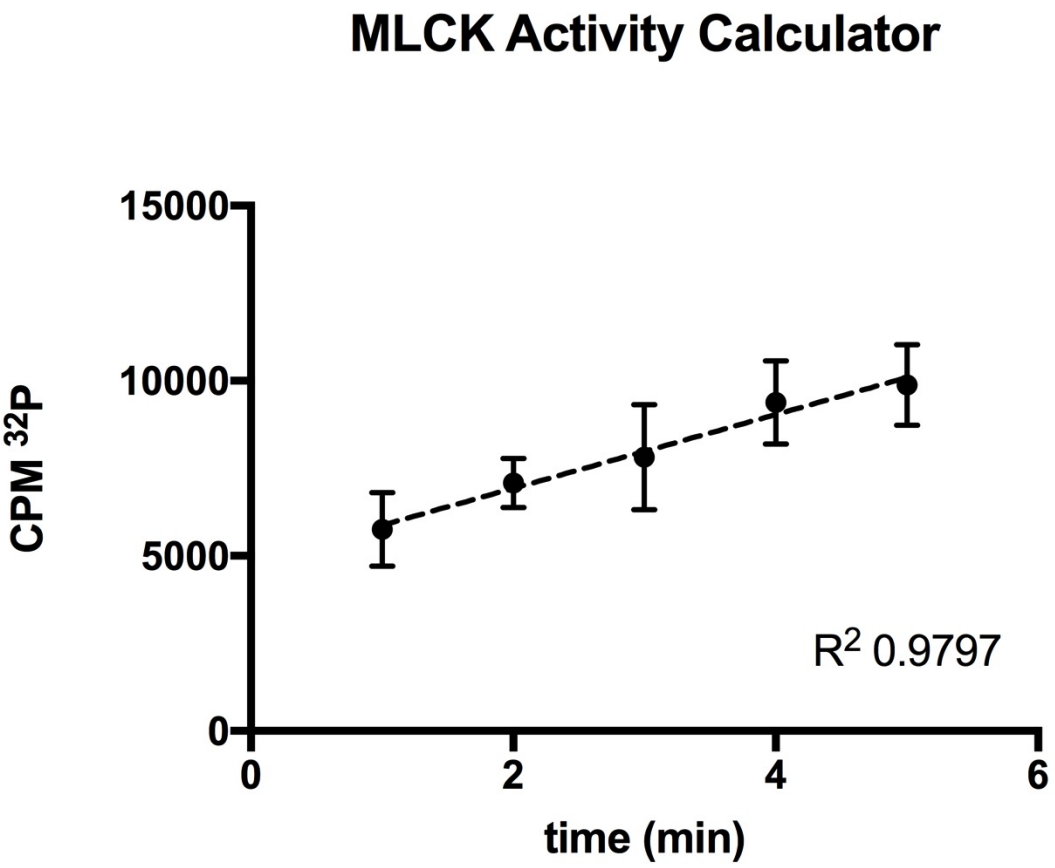
b)



Supp. Figure 2: *MLCK activity assay RLC/ELC ratio*: The regulatory light chain (RLC/MYL9) concentration, for use in the MLCK activity assay, is measured by spectrophotometry. Essential light chain (ELC) does not interact with MLCK, however, there is generally some level of ELC contamination when performing the RLC preparation. Since it is not possible to determine the concentration of only RLC using spectrophotometry, we ran a concentration gradient (1:10 – 1:3000 dilution) of the RLC/ELC isolate (a), and determined the ratio of RLC to ELC by the relative density of the bands run on Coomassie gel. The middle linear range of the bands (1:100 dilution) was used to acquire the ration (b), then the ratio was applied to the total protein concentration to determine an actual RLC concentration in the isolate.

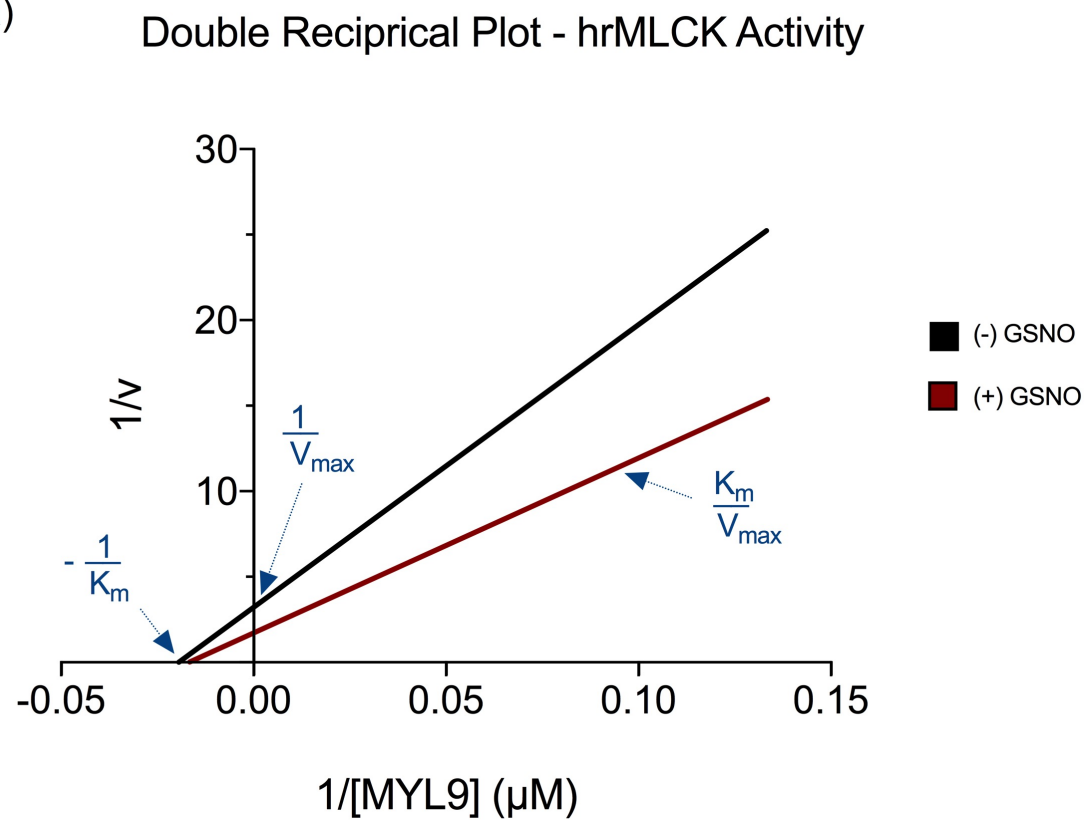

Effect of GSNO on actin velocity
V (μm/s-1)
0.5
0.4
0.3
0.2
0.1
0.0
0
200
400
[myosin] (μg/ml)
*
* (p = 0.0294)
Baseline
200μM GSNO
400μM GSNO

Supp. Figure 3: *Actin motility assay with two concentrations of GSNO*: Preliminary data from the actin motility assay (n=1, with three technical replicates) indicated that at 100μg/ml myosin concertation, actin velocity was affected in dose-dependent manner when exposed to GSNO.

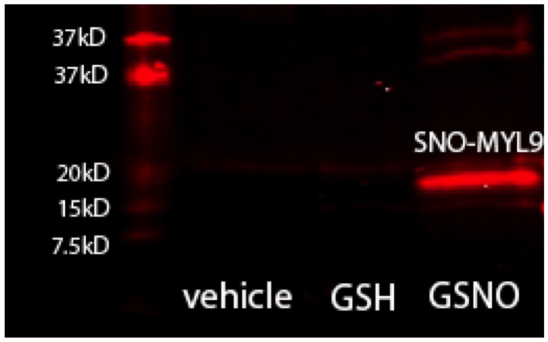

MLCK Activity Calculator
15000
10000
5000
0
CPM 32P
0
2
4
6
time (min)
R2 0.9797

Supp. Figure 4: *MLCK activity determination*: At each myosin concentration, the reaction is quenched at t=1,2,3,4,5 min after the addition of ATP [γ-32P]. From this, the slope can be acquired, which is relative to the counts*min^{-1}. MLCK activity is measured as (slope/[background scint count])*[1/([reaction vol.]*[MLCK concentration]).

a)


Double Reciprical Plot - hrMLCK Activity
30
20
10
1/v
1/Vmax
Km/Vmax
-1/Km
-0.05
0.00
0.05
0.10
0.15
1/[MYL9] (μM)
(-) GSNO
(+) GSNO

b)


37kD
37kD
20kD
15kD
7.5kD
SNO-MYL9
vehicle
GSH
GSNO

Supp. Figure 5: *Double reciprocal plot of MLCK data*: (a) Data from Figure 4A plotted as a double reciprocal (Lineweaver-Burke) indicates that GSNO is enhancing the ability of MLCK to bind to its substrate, MYL9. No change in K_m, but a change in V_{max} can easily be determined from this plot. (b) pHUSMC hTRT cells were incubated in 300μM GSNO for 20 minutes, and a biotin-switch was used to biotinylate and stabilize S-nitrosated proteins. Following this, SNO-proteins were isolate by a streptavidin pull-down, and the resulting proteins were run on a western blot and exposed to MYL9 anti-bodies. This gel indicates that MYL9 can be S-nitrosated when exposed to GSNO, while glutathione (GSH) and vehicle controls are not.

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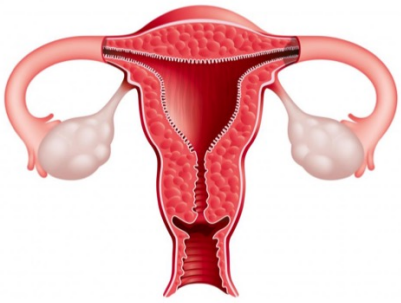
Chapter 6

Conclusions and Future Directions



Unique Myometrial Pathways

·NO
(ODQ) sGC
No relaxation
(BAY58) ⇧sGC
8-Br-cGMP
uGN
pGC-C
TREK-1 Expression & Splice Variants
Relaxation to ·NO Independent Of Global cGMP
Compart-mented cGMP Relaxes Pregnant Myometrium
·NO
MEMBRANE POLARIZATION
·NO
ALTERNATIVE ACTIONS OF NITRIC OXIDE
Protein S-nitrosation
TREK-1
MLCK
MYL9
Acto-Myosin
S-nitrosation Affects Contractile Dynamics

·NO
·NO Regulation in sPTL Myometrium
·NO
GSNOR
GSNOR upregulated
Decrease ·NO
Blunted Response to Nitric Oxide
Protein S-nitrosation
Protein S-nitrosation
Tocolytics
Nebivolol
+
NOS?
Myometrial Relaxation
Increase ·NO
N6022
-
GSNOR Inhibition

Pregnancy and parturition are complex, multi-factored events. As evidence, 80-years of ongoing pharmacological research seeking to halt preterm labor (PTL) have fallen well short. Tocolytics still cannot extend preterm birth (PTB) beyond 48-hours after the onset of labor (Sayres, 2010). While this modest reprieve does provide an important window to deliver antenatal corticosteroids, which bolster fetal lung development when administered from weeks 24-34 of gestation, it does nothing to mitigate the multitude of other complications associated with PTB (Behrman and Butler, 2007). Of the dozens of tocolytics that have been used over the years, nearly all are borrowed pharmacology. That is to say, they were developed to treat other muscle disorders, such as hypertension (Conde-Agudelo et al., 2011), asthma (Neilson et al., 2014b), and heart failure (Guclu et al., 2006), but not PTL. It was posited that these drugs, which work well in other smooth muscles, would have similar effects in the myometrium; they do not. Unfortunately, these drugs are either: (1) completely ineffective (Abramson and Reid, 1955; Duckitt et al., 2014; Romero et al., 2000), (2) must be used in concentrations that are harmful to mother and child (Sayres, 2010), or (3) only extend PTB 48-hours (ACOG, 2016). Why is it that these therapeutics have failed to halt PTL? The answer, at least in part, is that the myometrium is a fundamentally distinct muscle (Figure 1). Because of this, any successful approach to therapeutically moderate this muscle must take into account the nuances of uterine smooth muscle. Here

we have described the unique metabolism and actions of nitric oxide (·NO) in the myometrium, and we employ new drugs that leverage these distinctions.

An important realization that drives much of the research contained in this dissertation is our laboratory's finding that, unlike other smooth muscles, the myometrium relaxes independently of global cGMP accumulation when exposed to ·NO (Chapter 2) (Bradley et al., 1998d). This finding compliments other recent work showing the limited role of cAMP and PKA in myometrial relaxation (Lai et al., 2016). The logical question in response to this observation is what alternative pathways does ·NO act upon to relax the tissue? We believe that the posttranslational modification, S-nitrosation, is a major contributing factor to myometrial quiescence. Earlier work by our laboratory has found that proteins important to contraction in the myometrium can be differentially S-nitrosated based on the state of labor (preterm vs. full-term) when exposed to the endogenous ·NO donor, S-nitrosoglutathione (GSNO) (Ulrich et al., 2013c, 2012b). With this information in hand, we sought to determine what functional significance these protein S-nitrosations impose on contractile dynamics. As reported in Chapter 5, we determined that GSNO inhibits ATP-ase activity, which decreases actin velocities. This can have important consequences in the cell by decreasing the force of contraction (Warshaw et al., 1990; Word, 1995). We also found that the critical regulatory light chain (MYL9) of smooth muscle myosin increases MLCK activity when S-nitrosated, a contradictory finding that is reconciled when we consider that MYL9 S-nitrosation in greatly increased in spontaneous preterm

laboring (sPTL) myometrium over term laboring myometrium, bolstering evidence of sPTL as a disease state. Lastly, we determined that GSNO increases the activity of TREK-1, an outwardly rectifying K^+ channel important to myometrial quiescence by promoting a negative cell membrane potential. Taken together, we have provided novel evidence to support the functional role of protein S-nitrosations on myometrial contractile dynamics.

Beyond its ability to S-nitrosate proteins, the fact remains that ·NO, regardless of its mechanism of action, is a capable and important mediator of myometrial relaxation (I. L. O. Buxton, 2004; Norman, 1996). We have discovered that S-nitrosoglutathione reductase (GSNOR), an enzyme that metabolizes GSNO, is upregulated in sPTL tissue (Chapter 4); ergo, available ·NO and total protein S-nitrosations decrease (Chapter 5). As a result, women who undergo sPTL exhibit a blunted response to ·NO (Chapter 4). Because enzymes are common and effective targets for therapeutics, it is fortuitous, at least pharmacologically speaking, that GSNOR is dysregulated in sPTL myometrium. We found that N6022, an inhibitor of GSNOR, decreases the 'peak force' and 'area under the curve' of contractions in the myometrium (Chapter 4). The discovery of novel tocolytics is uncommon, and by leveraging our newly found knowledge, we may have identified an entirely new class of drugs to treat preterm labor (Appendix B).

Future Directions

Compelling science, when done properly, will generate many more questions than it does answers. While it is not up to me decide whether or not this research contained in these pages is persuasive or important, I can happily report that this work has generated many yet to be explored questions.

One of the most important lingering questions of this research is the mechanism of action of nebivolol. We first investigated nebivolol (Chapter 4) because it was reported to be an inhibitor of GSNOR (Jiang et al., 2016a); it is not. Quizzically, nebivolol is an acutely effective relaxer of uterine smooth muscle. We determined that nebivolol also does not inhibit thioredoxin reductase, which is another GSNO metabolizing agent. Interestingly, and of undoubtable applicability to our research, is the recent finding that nebivolol increases the activity of endothelial nitric oxide synthase (eNOS) (Wang et al., 2017). This is particularly compelling because sPTL myometrium overexpresses GSNOR; therefore, the idea of simultaneously inhibiting GSNOR with N6022, while increasing eNOS activity with nebivolol, presents as a conceivably potent tocolytic combination.

As N6022 has limited bioavailability in the cell, due to its hydrophobic structure, we also proposed to modify N6022 to increase its permeability. We are in talks with the organic chemistry department at UNR to collaborate on the synthesis of a modified form of N6022 that contains an ester-linked side chain, to

increase cellular uptake, that can be cleaved by endogenous esterases, returning the compound to its native state. We have been granted a provisional patent for this concept, and will continue this work moving forward (Appendix B).

Also, the initial data set acquired from the actin motility assay and the MLCK activity assay were compelling and warrant further investigation. With the actin motility assay, we seek to further investigate which protein S-nitrosations are most relevant to ATP-ase activity and actin sliding velocities. Because the 'attachment limit' drives actin velocity at low myosin concentrations, and our data suggest that attachment kinetics may be at play in the presence of GSNO, we will seek to determine if it is myosin S-nitrosation, or actin S-nitrosation, that drives this change in kinetics. Others have determined that in skeletal and cardiac myocytes the myosin heavy chain is S-nitrosated (Evangelista et al., 2010b). We can easily S-nitrosate actin outside of this experimental system, rather than apply GSNO to the entire flow chamber, and we can also perform an MYL9-SNO exchange on SMM to avoid application of GSNO to the entire system as well. Both experiments will allow us to better pinpoint the relevant S-nitrosations.

Lastly, an important future direction for our research should include an investigation of single nucleotide polymorphisms (SNPs). SNPs in the promoter and 3' UTR of *ADH5*, the gene that encodes for GSNOR, have been shown to affect the expression of the gene in some asthmatic cohorts (Henderson and Gaston, 2005b; Moore et al., 2009b; Tang et al., 2013b; Zuo et al., 2013). While our myometrial sample library is inadequate to identify is *ADH5* SNPs pertinent to

sPTL, there are other academic sources of sPTL tissue that may be available to us. Also, commercial enterprises, such as 23andMe®, have begun sharing their vast data sets in an effort to identify genetic anomalies relevant to disease states (Chang et al., 2017), which may be an avenue worth considering. If we were to identify SNPs relevant to sPTL, it would be particularly applicable to the preterm labor research community as we currently do not have a genetic test for sPTL. If *ADH5* SNPs are identified during early pregnancy with a simple blood test, newly identified ·NO modulating drugs, such as N6022 and nebivolol, could be used for maintenance tocolysis, preventing sPTL before it occurs. These notions are, of course, speculative, but they could prove to be important.

Conclusions

Preterm labor is an expensive problem. Each preterm infant incurs an average of $51,000 in additional medical fees, above and beyond uncomplicated normal delivery cost (ACOG, 2016). In the United States alone, we collectively spend approximately $30 billion annually (adjusted) on direct and indirect expenses associated with PTL and PTB. While it also cost billions of dollars to bring a new drug to market (Chit et al., 2015), this number pales in comparison to the financial and emotional burdens experienced by families of PTB infants. Here we offer unambiguous evidence that the myometrium is fundamentally different

from other muscles in the body, and we propose novel mechanisms and therapies to treat a disorder that causes so much grief to families around the world.

The title of this dissertation begins, "**intimations** on the pathophysiology of preterm labor." An intimation is a "hint" or an "indication." I do not possess the hubris to think that the research in this dissertation will "cure" sPTL, but I hope that the evidence put forth will help establish a new beachhead to attack the problem, so that one day sPTL will fade from our collective memories.

improvidus, apto quod victum

Appendix A

Noninvasive determination of pregnancy in Dunkin-Hartley guinea pigs

Our investigation of preterm labor requires a biochemical analysis of the myometrium during pregnancy and parturition. As it is not possible to collect uterine smooth muscle samples from women mid-gestation, an animal model was used. Mice are often the preferred animal for experimental studies due to their relatively low cost, rapid breeding cycle, and the availability of numerous transgenic models. Unfortunately, they are suboptimal for myometrial studies, as endogenous progesterone withdrawal initiates labor in mice through cervical remodeling (Yellon et al., 2009), where it does not humans. Guinea pigs are advantageous in this regard, as they are one of the few animals do not experience progesterone withdrawal (Nnamani et al., 2013), and are therefore more physiologically relevant to our studies.

Dunkin-Hartley (DH) guinea pigs (Elm Hill, Chelmsford, MA) were purchased as either virgin juveniles (300-350g) and bred on site, or as timed-pregnancies (30-35d). All animal studies were approved by the University of Nevada, Reno, Institutional Animal Care and Use Committee. The average length of gestation of a guinea pig is ~70 days, although this can vary slightly based on litter size (Goy et al., 1957). Our research requires the timed-interval collection of guinea pig myometrial tissue as early as 20-days of gestation. While there are numerous methods to determine pregnancy in guinea pigs, we sought to identify a technique that would require minimal handing of the animals to mitigate stress, while still accurately assessing pregnancy state. Studies in mice have shown that the manner in which the animals are handled can have a significant effect on stress

(Hurst and West, 2010), to include less obvious factors, such as the gender of the handler (Sorge et al., 2014), and the presence of unknown individuals (Van Driel and Talling, 2005). Stress in animals can even lead to miscarriage (Xu et al., 2013), a finding particularly applicable to our research.

Approaches to determine pregnancy in DH guinea pigs:

Ultrasonography: Ultrasound is a common, fast, and generally effective method to identify pregnancy in most animals. Unfortunately, this normally requires shaving of the animal, the liberal use of ultrasound gel, anesthetizing agents, and the possibility of the animal being handled by personnel unknown to the guinea pig. Corticosterone has been shown in animals to increase with exposure to isoflurane (Altholtz et al., 2006), with females be more susceptible to repeated isoflurane administration (Hohlbaum et al., 2017). We attempted to identify pregnancy via ultrasound on three guinea pigs, ranging from 10-25 days of gestation, with no success. Two of the three animals were later found to be not pregnant by other methods, yet in none of the cases were we able to conclusively rule out pregnancy through ultrasonography, indicating a high potential for false negatives. For the reasons listed above, we have suspended its use as a tool to identify pregnant guinea pigs.

X-ray Imaging: X-ray imaging is an attractive alternative to ultrasonography, as it only requires anesthetizing the animal for a short period time, and handling of

the animal is kept to a minimum. This approach is limited in that calcification of the fetal skeleton does not begin until day 28 of gestation, and is not complete until day 39 (Graham and Scothorne, 1970). We were able to easily confirm pregnancy in 65d guinea pigs using x-ray imaging (Figure 1); however, by day 65 of gestation there are more obvious indicators of pregnancy, such as weight, abdominal circumference, and the use of palpation. X-ray imaging may prove useful in cases of low-weight gain by mid-gestation, but it is a time-consuming and expensive mean to identify pregnancy.

Copulatory Plug & Vaginal Swab: Like mice, guinea pigs will often develop a copulatory plug after insemination (Martan and Shepherd, 1976). While this is commonly used method of pregnancy detection in mice, it is correlative and does not guarantee impregnation. This technique is further complicated in our experimental setting as the guinea pig cages are lined with chip bedding; therefore, locating the copulatory plug is akin to finding the proverbial needle in a haystack. Despite several attempts, we were unable to find any plugs in the animal bedding, or still attached to an animal. Vaginal swabs, on the other hand, are easily collected and only require minimal handling the animals. Our guinea pigs are co-housed with a breeding male for five-days. Vaginal swabs were collected the morning the 2nd and 4th day of cohabitation with a male. We swabbed the vagina of each female guinea pig with a sterile cotton swab and then immediately smeared onto a slide and used 50μl of Phosphate Buffer Solution (pH 7.4) on a cover slip. Samples were observed under a light microscope to identify spermatozoa via

morphology (Byers et al., 2012) (Figure 2). Alternatively, the vaginal swabs were placed into an Eppendorf tube containing 500uL of PBS immediately after collection and stored on ice for analysis at the laboratory (Humphreys et al., 1982). After 16 swabs, with 4 different animals, only a single positive identification was made. This finding, coupled with the fact that copulation does not always signify pregnancy, lead us to seek alternative methods to identify pregnancy.

Chorionic Gonadotropin: Chorionic gonadotropin (CG) is important to implantation and pregnancy maintenance (Cross et al., 1994). The pituitary gonadotropin hormones, CG, LH, and FSH, share an identical alpha subunit with a unique beta subunit (Themmen and Huhtaniemi, 2000). It has been shown that guinea pig CG is more similar to human CG than other rodents (Bambra et al., 1984), increasing the likelihood that an over the counter (OTC) human pregnancy test would be effective. Human pregnancy test use antibodies to identify the beta-subunit of hCG, however, the precise binding epitope has not been disclosed (Berger and Lapthorn, 2016). CG levels peak in the guinea pig at day 18 (Humphreys et al., 1982), making this a potentially fast and noninvasive method to detect early pregnancy in guinea pigs.

We used two different OTC pregnancy kits (Equate® One Step Pregnancy Test™, and First Response® Digital Pregnancy Test) as a “proof of concept” to determine if they would be effective at determining pregnancy in our guinea pigs. We tested between 250μl-500μl of urine from a 20d and 40d pregnant guinea pig, and neither provided a positive result. This may have been due to insufficient urine

volume, or because the test is incompatible with guinea pig CG, but the sample size was too small to be conclusive.

Palpation: Unlike mice, guinea pigs are born precocious (Sosenko and Frank, 1987). As such, they develop rapidly during the short ~70d gestational period. At day 30 the fetus is only ~2.5g, but weight gain increases exponentially, and the guinea pig will weigh ~250g by day 68 (Engle and Lemons, 1986). The uterus expands to accommodate the fetus and a placental disc forms in concert with fetus development. These factors, combined with the growing volume of the fetus itself, allows for ready identification of pregnancy through palpation. In our experimental setting, palpation is performed weekly on suspected pregnant females during other handling tasks. To accomplish this, the guinea pig is held upright and the abdomen is grasped at the midline. With a gentle squeeze the hand palpates the abdomen of the female while moving outward to the edge of the body until the entire area is tested, and is then repeated bilaterally. Pregnancy was consistently identified by day 20-30, with detection as early as 15 days. Palpation, when done by a handler familiar to the guinea pig can be completed in under a minute and with minimal discomfort (and presumably stress) to the animal. This technique has become our primary means to detect pregnancy under 35 days.

Weight and Abdominal Circumference: As is readily evident, guinea pigs, as with most animals, gain weight during pregnancy. This makes weight tracking an attractive and reliable metric for which to identify pregnancy. Guinea pig fetal weight, and by correlation maternal weight, does not increase linearly during the

pregnancy. At day 30 the fetus is only ~2.5g, approximately 1% of its delivery weight 40 days later (Engle and Lemons, 1986). For this study we tracked 18 pregnancies, for which 39 offspring were birthed. Of these offspring, there were 21 males and 18 females, with an average litter size of 4.3 (SD = 1.5) (Table 1).

Beyond the fact that weight gain will be affected by the number of developing fetuses, using weight a metric to identify pregnancy is further complicated by the fact that guinea pigs are capable of reproduction by a biological age of 60 days, at which point they are still growing themselves, making it more difficult to differentiate maternal growth from weight gain attributed to pregnancy. For this purpose, we bifurcated our gestational groups into juvenile and adult pregnancies. Pregnancy was able to be determined solely by weight gain in our juvenile guinea pig cohort by gestational day 42 ($p < 0.05$) when compared to a control group of identical biological age (Figure 3A). Weight gain per day in the pregnant group (n=4) was 7.142g ± 0.2525 (95% CI 6.493 to 7.791), and 2.907g ± 0.3608 (95% CI 2.054 to 3.76) in the non-pregnant group (n=8) over the course of the pregnancy. Pregnancy in mature adults, on the other hand, was able to be determined by gestational day 35 (Figure 3B) ($p = 0.0357$ d31-40. n=3 to n=7 at each time point, except at day 61-70 for which there was only a single recording, and was only included to show the visual trend). An aggregate of all collected weights shows that a majority of the weight gain occurs from gestational days 30-65 (Figure 4A), as would be expected. Furthermore, when age is not accounted for, the weight of a pregnant guinea pig can be differentiated from a non-pregnant

guinea pig by day 31-40 (P = 0.019), with the average being 34.2d in this cohort (Figure 4B).

Abdominal Circumference: Lastly, we used abdominal circumference as a noninvasive means to detect pregnancy in our guinea pigs. A string measured the animal's circumference once weekly and was compared to non-pregnant controls. Two people were used to ensure accuracy and consistency. One handled the guinea pig while the other took and recorded measurements. To consistently record measurements, the pig is held upright during recordings. The handler placed one hand under the upper appendages of the guinea pig, while providing a stable base for hindquarters with the other hand. Circumference is measured by wrapping a string around the widest portion of the lower abdomen and recorded to the nearest half centimeter. With this methodology alone, we were able to determine pregnancy by gestational day 40 (Figure 5), with a 19.6% (± 5.89%, n=4) increase in abdominal circumference (p= 0.0377). As a comparison, there was no significant difference in abdominal circumference at day 20 (p=0.5311, n=8).

Conclusion

Mitigating stress in experimental animals is not only an ethical impetrative, but it improves the health of laboratory animals (Council, 2008). Of nine methods to identify pregnancy that we employed, palpation proved to be the least invasive

and most reliable technique, and can differentiate guinea pigs by gestational day 15. While we were unsuccessful at using OTC pregnancy tests as a means to identify pregnancy, this technique requires further study, as CG levels peak at gestational day 18, indicating that pregnancy determination may be possible many days earlier. Weight gain is easily trackable with little stress to the animals, and with a sufficient 'n', trends can easily be determined in each cohort to be used a metric to identify pregnancy. That being said, the earliest possible day to determine pregnancy, by weight alone, at least with our adult cohort, was day 35, which has its limitations. Ultimately, there exist multiple, reliable, noninvasive techniques to identify pregnancy in guinea pigs while minimizing stress to the animal.

Acknowledgments:

A special thank you to: Christina Smith for her assistance with our guinea pig breeding program and for helping to research and develop the protocols used in this appendix. Also to Dr. Ben Weigler, DVM, MPH, Ph.D., DACLAM, DACVPM, Animal Resources Director & Attending Veterinarian; to Walter Mandeville, DVM, MS; and to Savannah Maienschein, for their expertise and dedicated service to the well-being of all laboratory research animals at UNR.

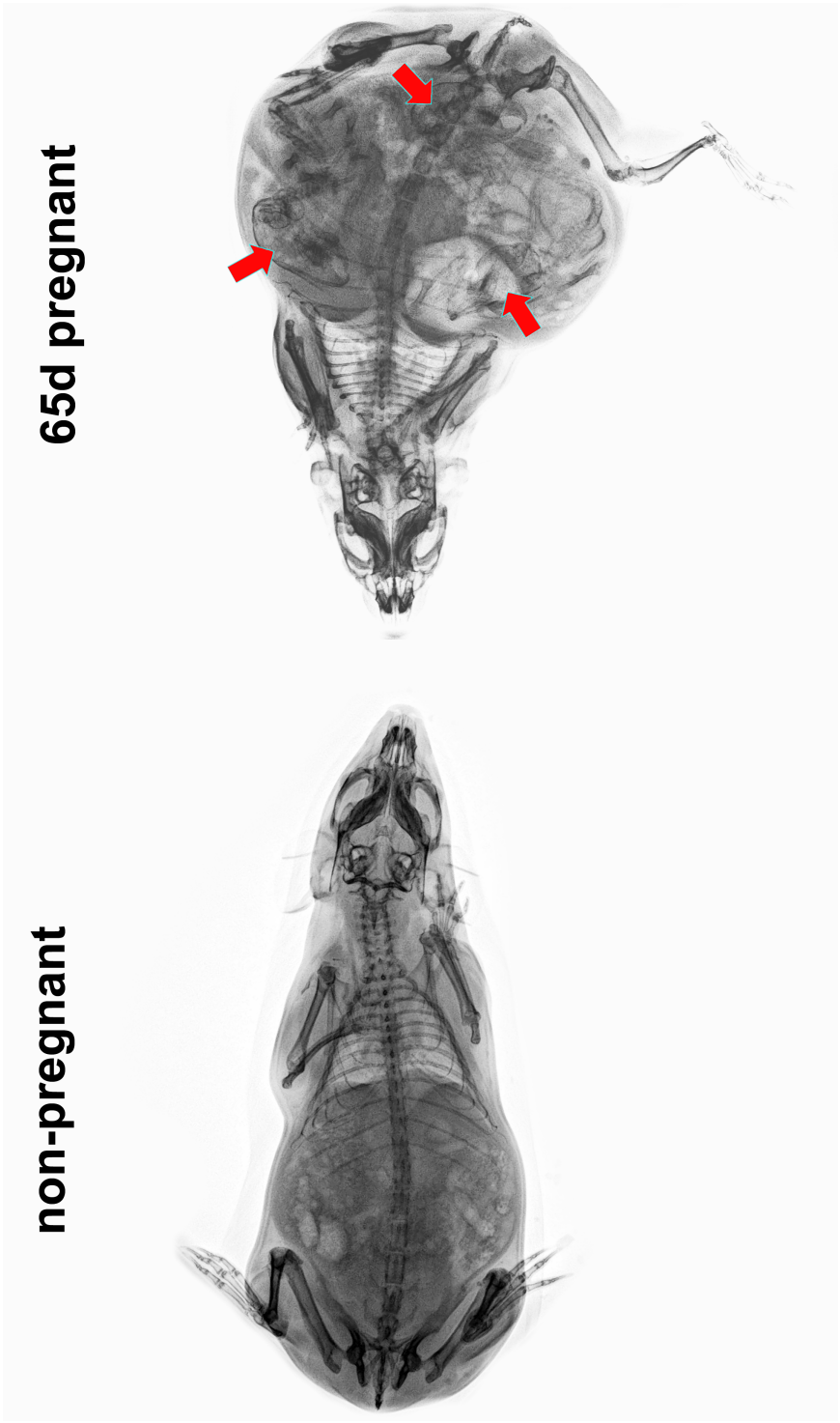
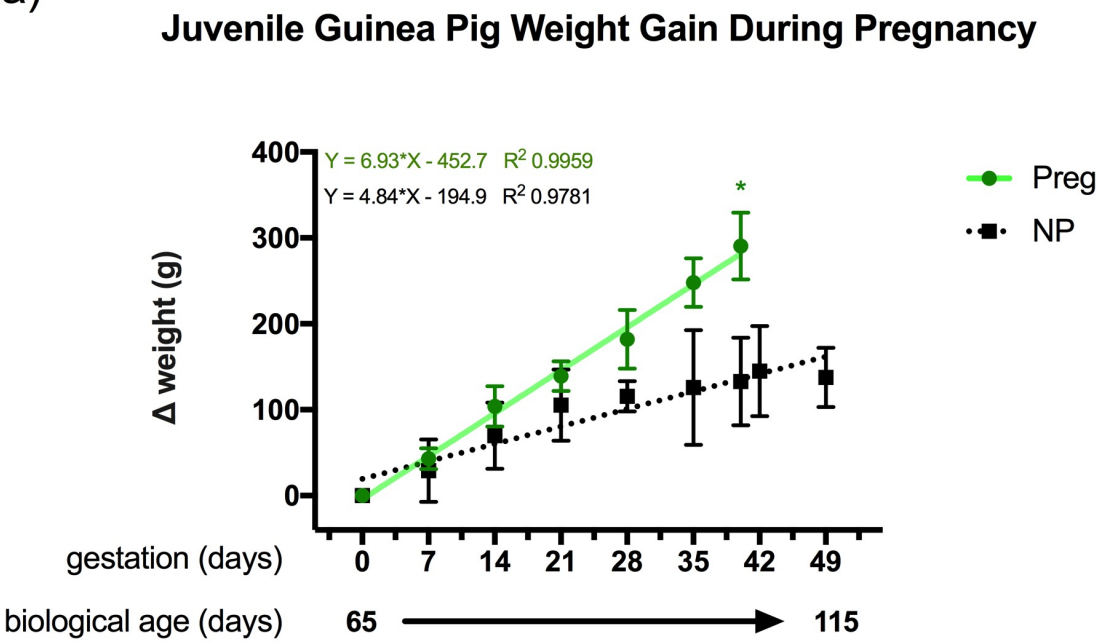

65d pregnant
non-pregnant

Figure 1: *X-ray filmography of pregnant and non-pregnant guinea pig*: X-ray filmography of a non-pregnant adult Dunkin-Hartley guinea pig and a term pregnant guinea pig at 65 days of gestation. Three fetuses (red arrows) are morphologically identifiable.

Figure 2: *Vaginal swab of guinea pig post-mating*: A single sperm, as identified through light microscopy (40x). A vaginal swab was taken following overnight cohabitation and smeared onto a glass slide with 50μl of PBS. No other sperm were identified on the slide.

a)


Juvenile Guinea Pig Weight Gain During Pregnancy
Y = 6.93*X - 452.7 R2 0.9959
Y = 4.84*X - 194.9 R2 0.9781
Preg
NP
Δ weight (g)
400
300
200
100
0
*
gestation (days)
0 7 14 21 28 35 42 49
biological age (days)
65
115

b)

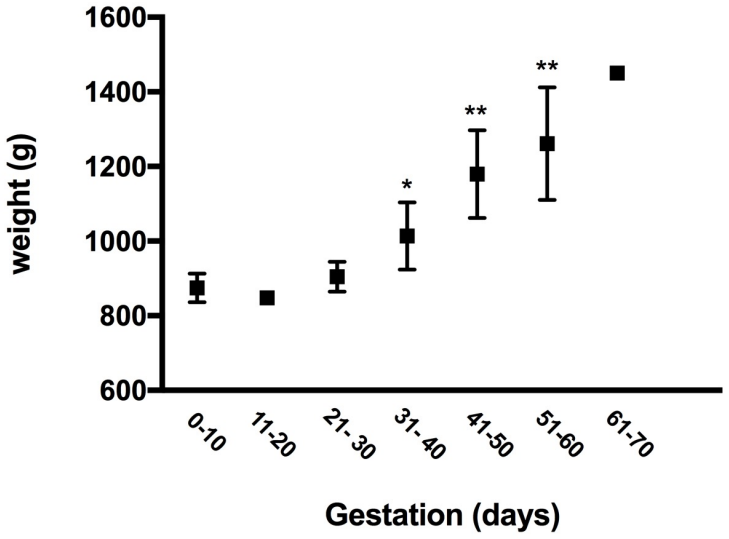
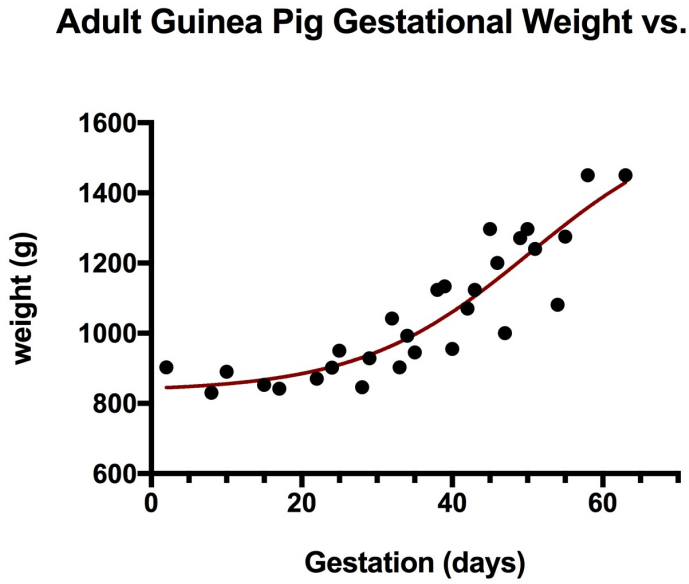

Adult Guinea Pig Weight Gain During Pregnancy
weight (g)
1600
1400
1200
1000
800
600
*
**
**
0-10
11-20
21-30
31-40
41-50
51-60
61-70
Gestation (days)

Figure 3: *Juvenile vs. adult guinea pig weight gain during pregnancy*: (a) Juvenile guinea pigs can be identified as pregnant, using weight gain as the sole metric, at day 42 of gestation, gaining 7.14g ± 0.25 (n=4) of weight per day, as compared to 2.90g ± 0.36 per day in the non-pregnant group (n=8). (b) In mature guinea pigs, in which their individual weight has stabilized when not pregnant, the weight of a pregnant guinea pig can be differentiated from a non-pregnant guinea pig by day 31-40 (P = 0.019), with the average being 34.2d in this cohort.

a)


Adult Guinea Pig Gestational Weight vs. Time
weight (g)
1600
1400
1200
1000
800
600
0
20
40
60
Gestation (days)

b)

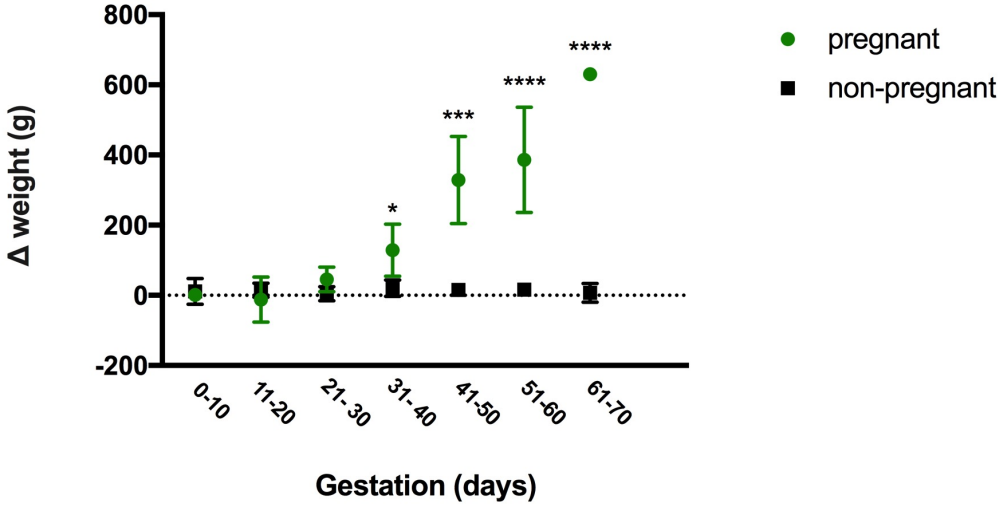

Absolute Weight Gain During Gestation (all ages)
Δ weight (g)
800
600
400
200
0
-200
*
***
****
****
0-10
11-20
21-30
31-40
41-50
51-60
61-70
Gestation (days)
pregnant
non-pregnant

Figure 4: *Aggregate guinea pig weight gain during pregnancy*: (a) A plot of all individual recorded weight of adult guinea pigs during pregnancy reveals a trend that appreciable weight gain does not occur until gestational day ~30+, and continues to term. (b) As expected, the absolute weight gain of all guinea pigs, regardless of age, reveals a significant deviation from non-pregnant guinea pigs between gestational days 31-40, with a more reliable delineation in the 41-50 day period.

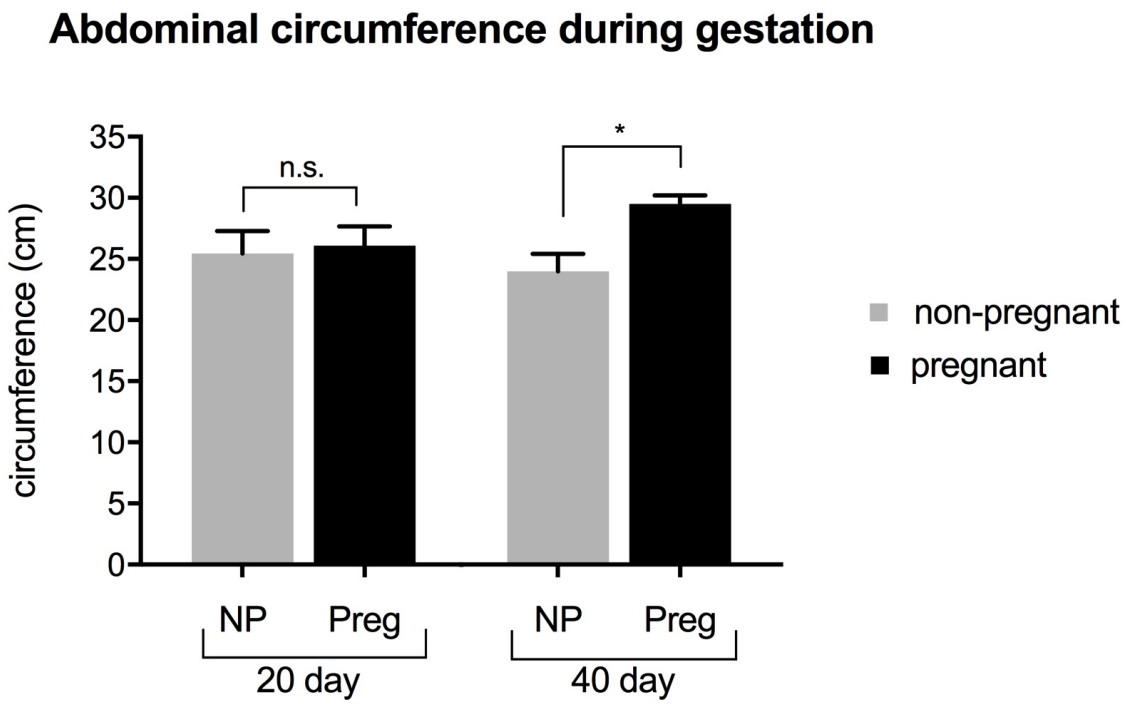
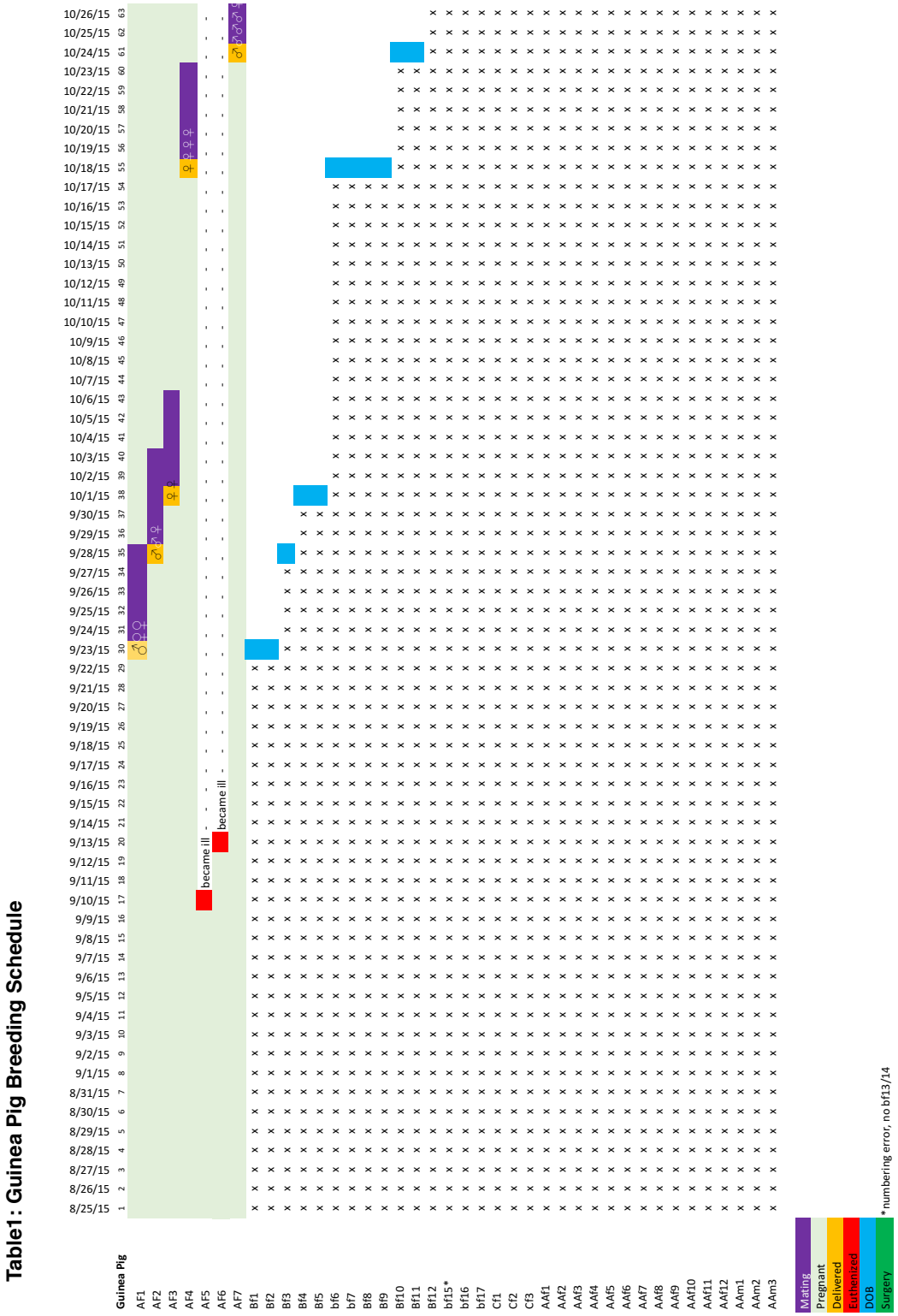
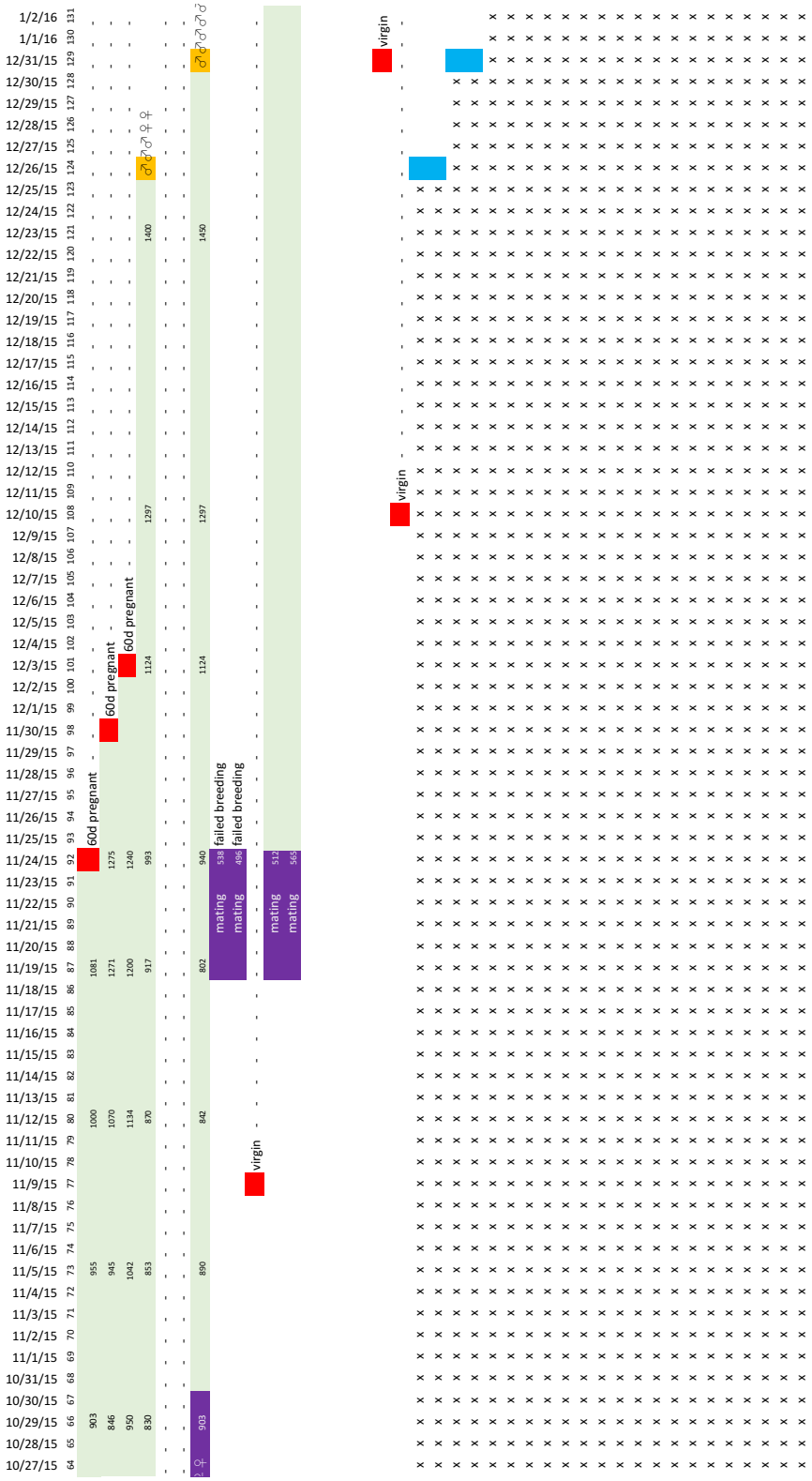
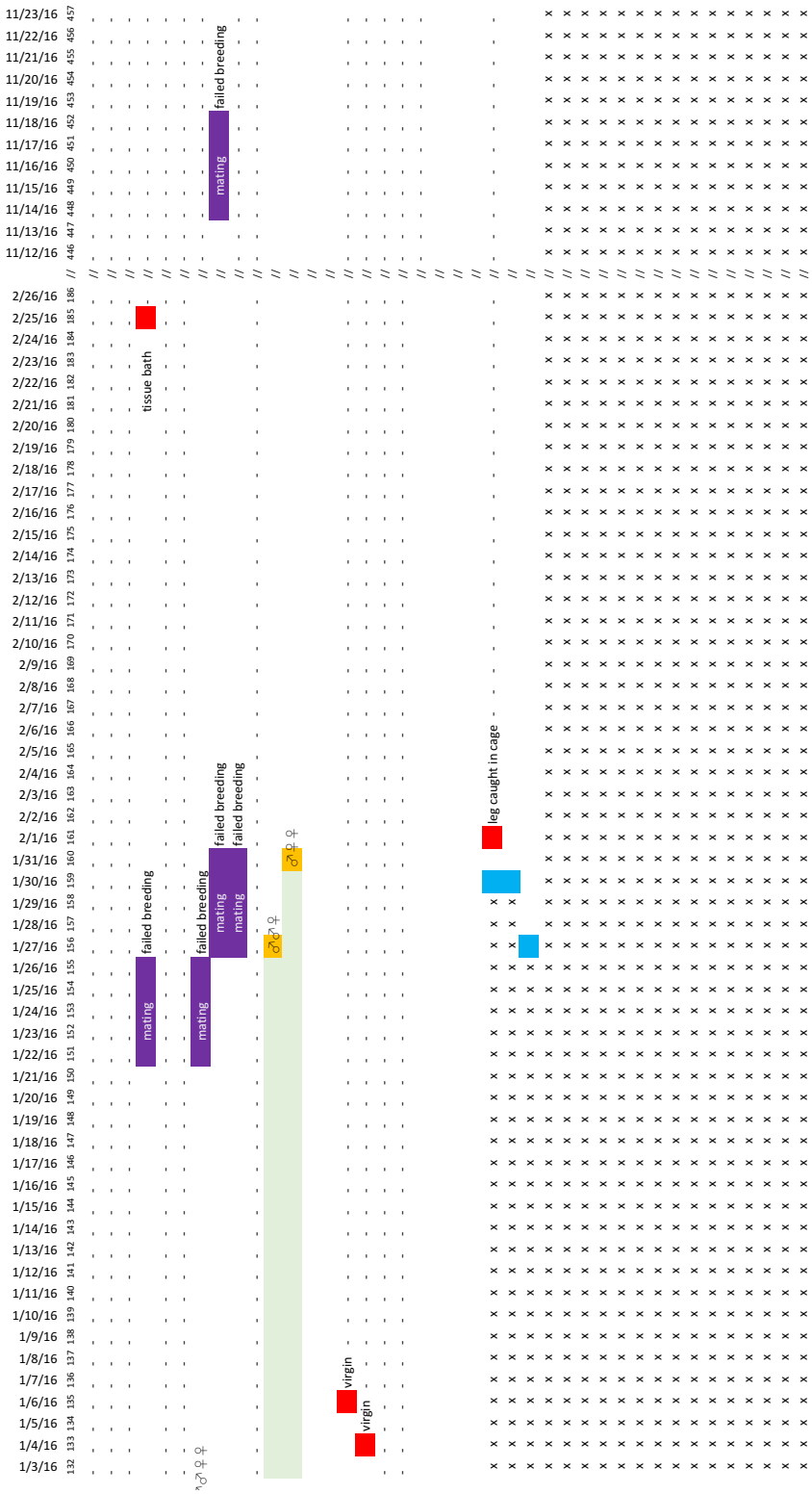

Abdominal circumference during gestation
circumference (cm)
35
30
25
20
15
10
5
0
n.s.
*
NP
Preg
NP
Preg
20 day
40 day
non-pregnant
pregnant

Figure 5: *Abdominal circumference to determine guinea pig pregnancy*: Abdominal circumference was measured weekly in pregnant (n=4) and non-pregnant (N=8) guinea pigs. There was no difference in circumference at gestational day 20 (p=0.5311), but there was by day 40(p= 0.0377), with a 19.6% (± 5.89%) increase in abdominal circumference.

Table1: Guinea Pig Breeding Schedule




virgin
virgin
60d pregnant
60d pregnant
60d pregnant
failed breeding
failed breeding
virgin
mating
mating
mating
mating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2/9/16
2/8/16
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2/6/16
2/5/16
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2/3/16
2/2/16
2/1/16
1/31/16
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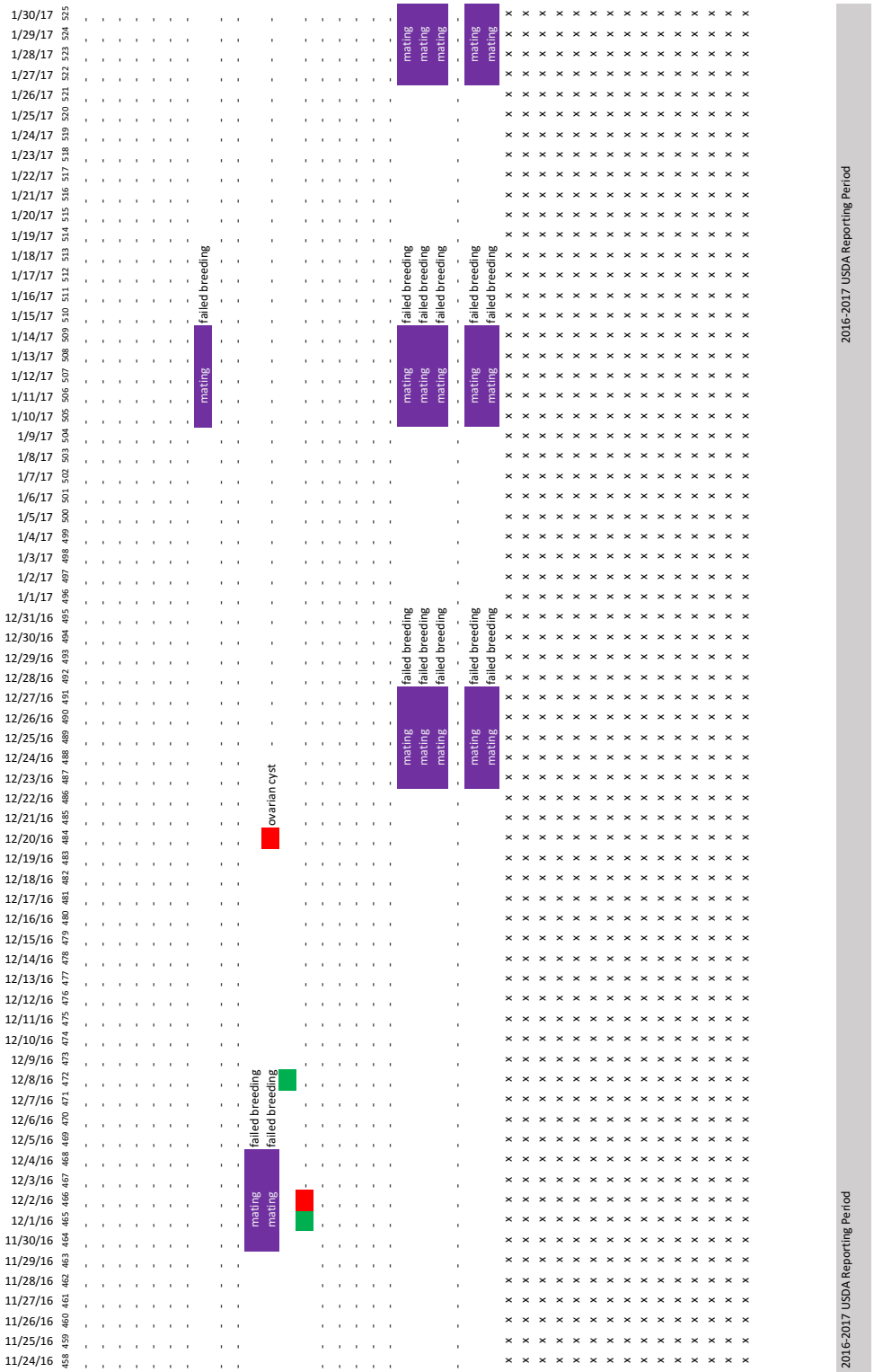
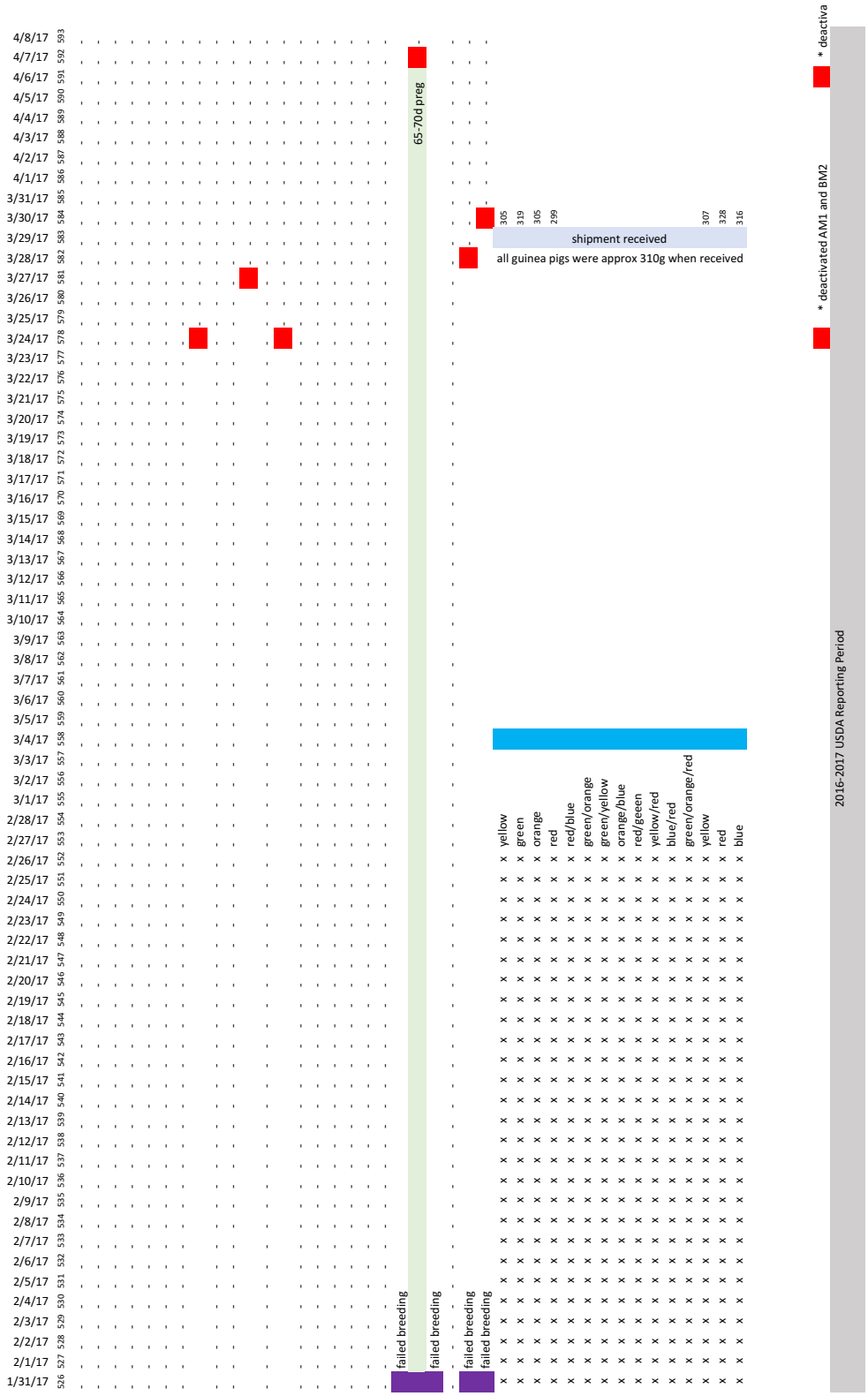
1/13/16
1/12/16
1/11/16
1/10/16
1/9/16
1/8/16
1/7/16
1/6/16
1/5/16
1/4/16
1/3/16
failed breeding
mating
tissue bath
virgin
leg caught in cage
♂♀


mating
failed breeding
ovarian cyst
2016-2017 USDA Reporting Period
2016-2017 USDA Reporting Period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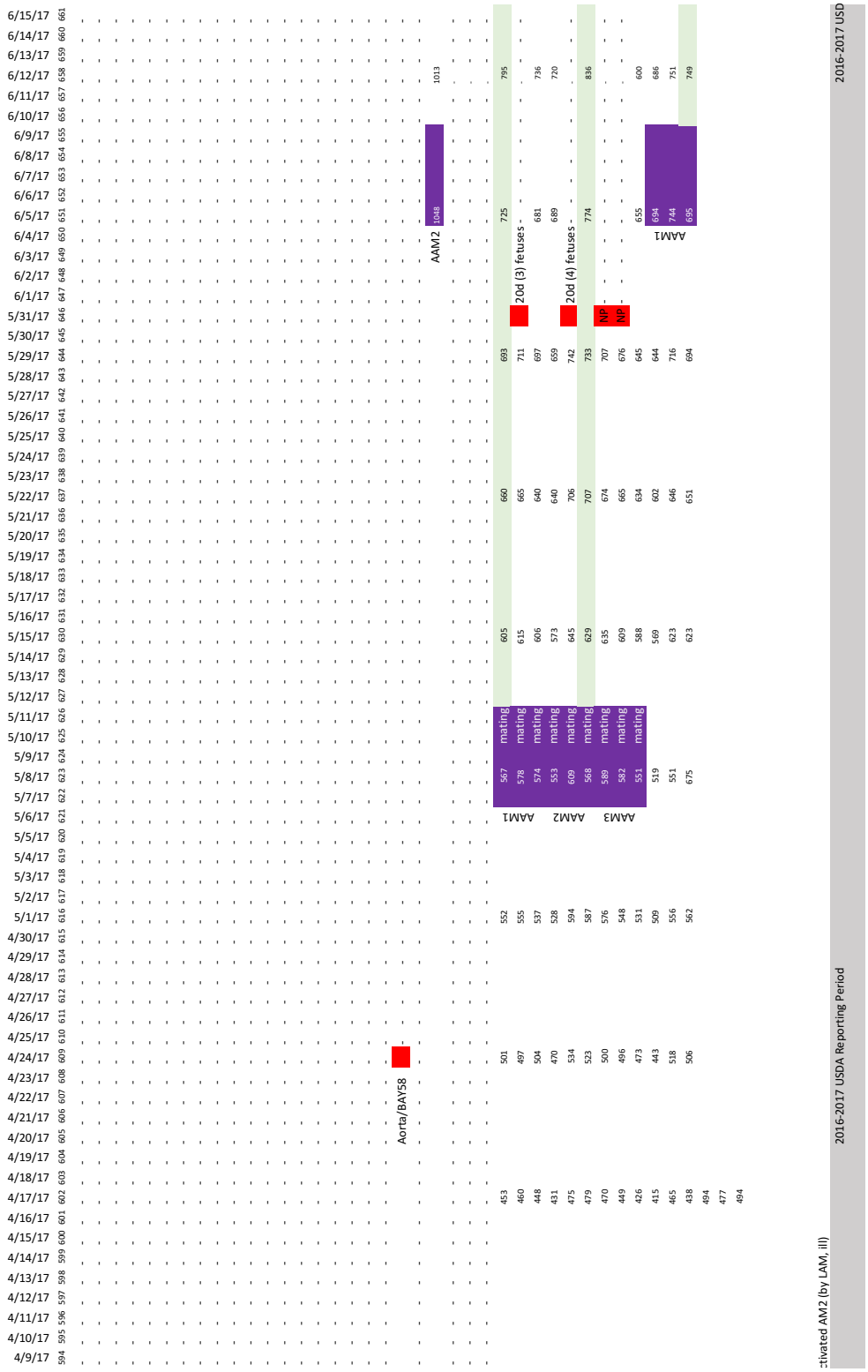
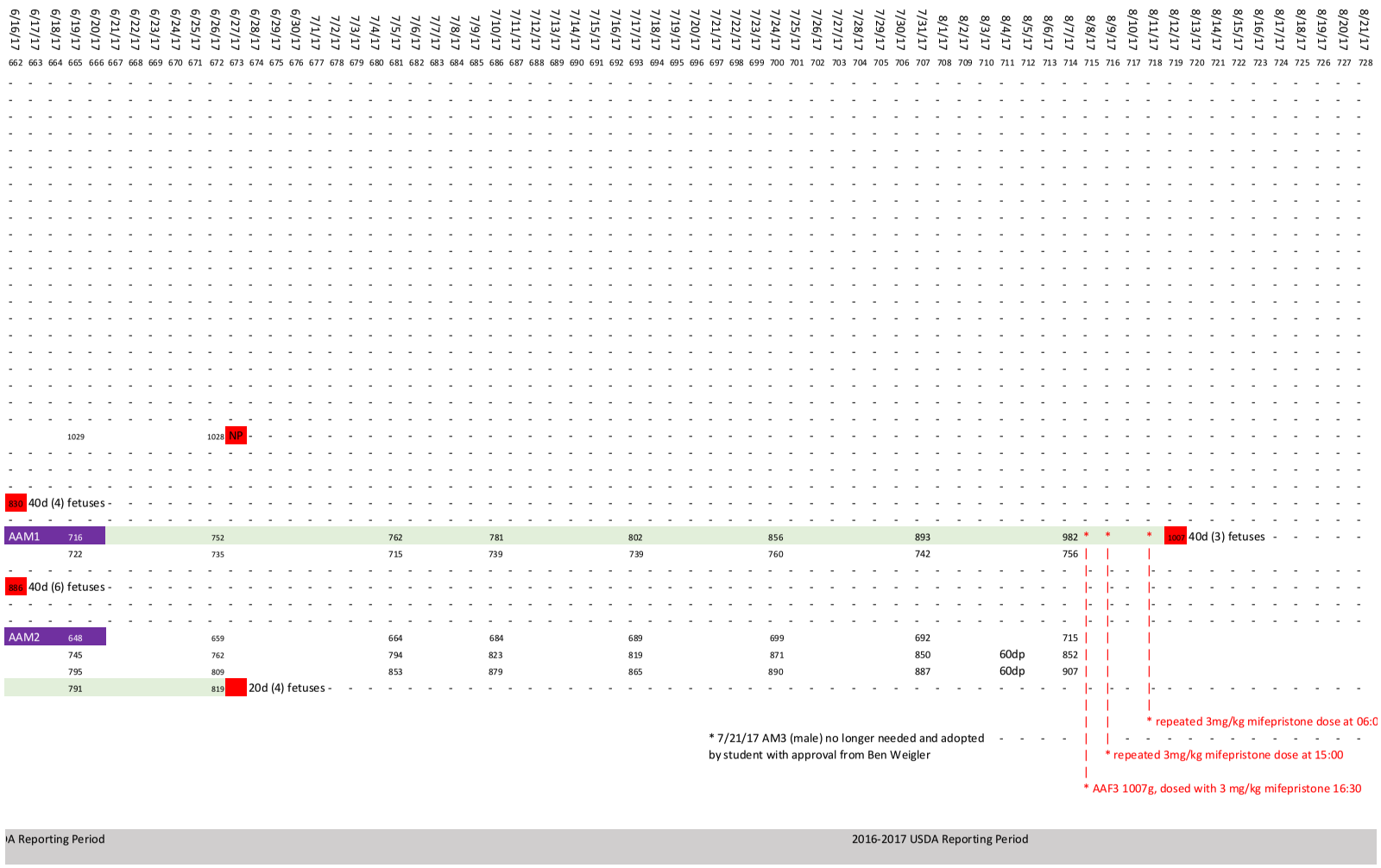
2/8/17
2/7/17
2/6/17
2/5/17
2/4/17
2/3/17
2/2/17
2/1/17
1/31/17
65-70d preg
failed breeding
failed breeding
failed breeding
failed breeding
all guinea pigs were approx 310g when received
shipment received
305
319
305
299
307
328
316
yellow
green
orange
red
red/blue
green/orange
green/yellow
orange/blue
red/geeen
yellow/red
blue/red
green/orange/red
yellow
red
blue
* deactivated AM1 and BM2
* deactiva
2016-2017 USDA Reporting Period


Aorta/BAY58
AAM2
AAM1
20d (3) fetuses
20d (4) fetuses
AAM3
AAM2
AAM1
mating
NP
2016-2017 USDA Reporting Period
2016-2017 USD
ctivated AM2 (by LAM, III)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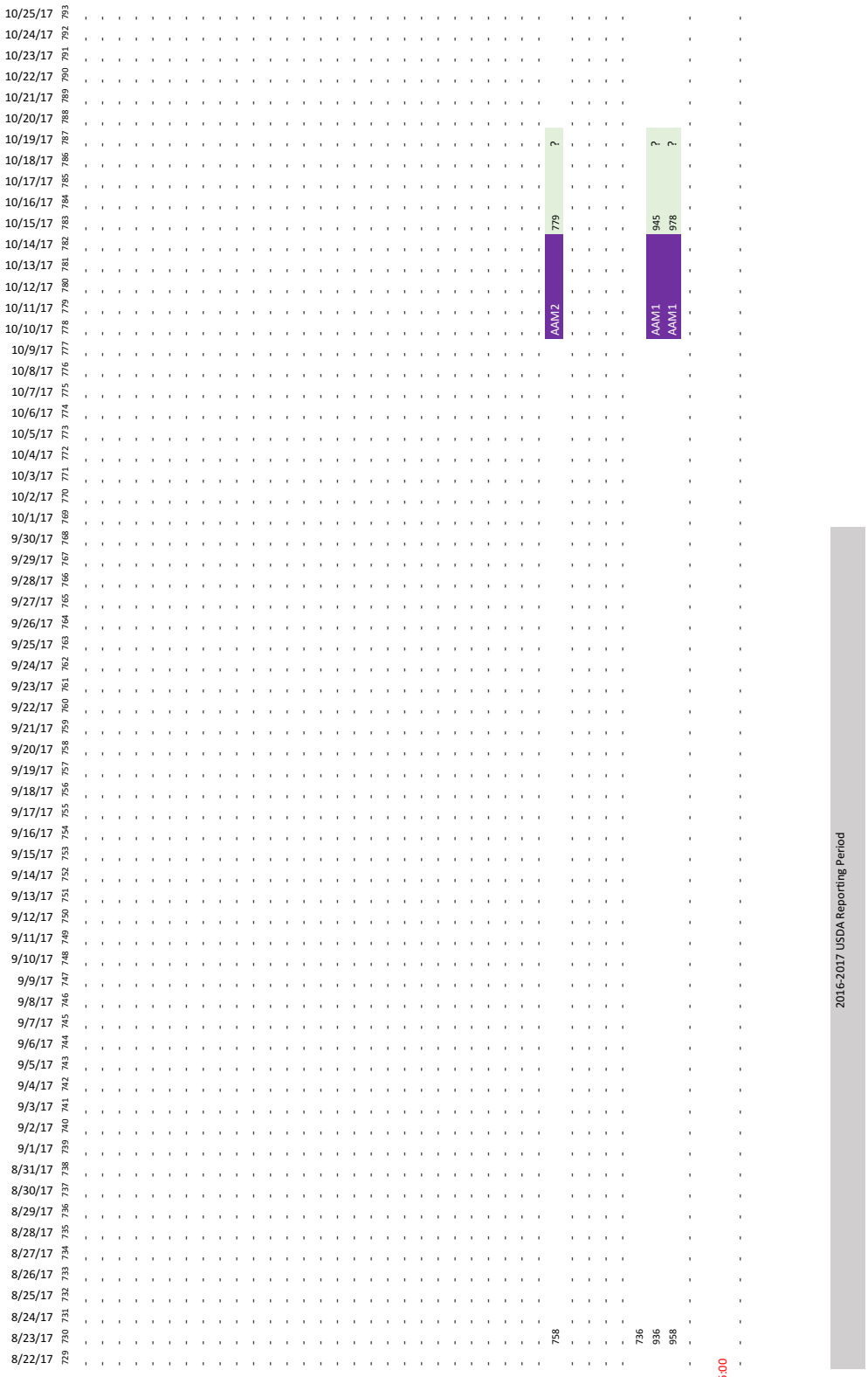
662 663 664 665 666 667 668 669 670 671 672 673 674 675 676 677 678 679 680 681 682 683 684 685 686 687 688 689 690 691 692 693 694 695 696 697 698 699 700 701 702 703 704 705 706 707 708 709 710 711 712 713 714 715 716 717 718 719 720 721 722 723 724 725 726 727 728
1029
1028
NP
830
40d (4) fetuses
AAM1
716
722
752
735
762
715
781
739
802
739
856
760
893
742
982
756
1007
40d (3) fetuses
886
40d (6) fetuses
AAM2
648
745
795
791
659
762
809
819
664
794
853
684
823
879
689
819
865
699
871
890
692
850
887
60dp
60dp
715
852
907
20d (4) fetuses
* repeated 3mg/kg mifepristone dose at 06:0
* 7/21/17 AM3 (male) no longer needed and adopted by student with approval from Ben Weigler
* repeated 3mg/kg mifepristone dose at 15:00
* AAF3 1007g, dosed with 3 mg/kg mifepristone 16:30
A Reporting Period
2016-2017 USDA Reporting Period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8/29/17 736
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8/27/17 734
8/26/17 733
8/25/17 732
8/24/17 731
8/23/17 730
8/22/17 729
AAM2
779
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AAM1
AAM1
945
978
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758
736
936
958
6:00
2016-2017 USDA Reporting Period

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Appendix B

Provisional Patent: Compositions and Methods for Regulating Preterm Labor (UNR16-008Z)

COMPOSITIONS AND METHODS FOR REGULATING PRETERM LABOR

FIELD

This disclosure relates to the field of preterm labor and in particular, to compositions and methods of treating preterm labor and spontaneous preterm labor.

ACKNOWLEDGMENT OF GOVERNMENT SUPPORT

This invention was made with government support under NIH U54 GM 104944-01 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

Approximately 15 million preterm births occur annually worldwide. In 2012, more than 11% of US births were premature. No drugs prevent labor in patients who enter labor preterm to allow pregnancies to go to term. Therapeutic approaches to manage spontaneous preterm labor (sPTL) are off-label and are employed without evidence of benefit for acute or maintenance tocolysis. Because human myometrium does not relax in response to cyclic nucleotide elevation as expected, tocolytics such as Terbutaline are ineffective. Thus, a need exists for new compositions and methods for preventing, inhibiting and/or reducing preterm labor.

SUMMARY

Disclosed herein is the surprising discovery that s-nitrosogluthathione reductase (GSNOR) expression and activity is increased in preterm laboring myometrium as compared to full term myometrial samples. Further, N6022, an inhibitor of GSNOR, is identified as a novel tocolytic by decreasing the peak force of contraction in laboring myometrium. Based upon these findings, methods of diagnosing, treating and/or evaluating the efficacy of a treatment of preterm labor are disclosed. In some examples, a method of treating preterm labor comprises administering an effective amount of a

GSNOR modulatory agent to a subject at risk of developing or having one or more signs or symptoms of preterm labor, to include infection, wherein the GSNOR modulatory agent comprises N6022, modified N6022 or a combination thereof, wherein the GSNOR modulatory agent decreases GSNOR activity as compared to GSNOR expression and/or activity prior to treatment, thereby preventing, inhibiting or reducing one or more signs or symptoms of preterm labor. In some examples, the method further includes diagnosing a subject at risk of developing of having spontaneous preterm labor, such as by detecting expression and/or activity of GSNOR, wherein detection of increased expression and/or activity of GSNOR as compared as a control (e.g., level of GSNOR expression and/or activity in a subject who has reached full term of gestation or not experiencing preterm contractions), indicates the subject has or is at risk of developing spontaneous preterm labor. In some further embodiments, method of evaluating the efficacy of the treatment of preterm labor are disclosed which can comprise monitoring GSNOR expression and/or activity levels prior to, during and following treatment with one or more modulators of uterine contractility, such as N6022, modified N6022 or a combination thereof. Methods of identifying compounds useful for preventing or treating preterm labor are also disclosed, comprising measuring GSNOR activity and/or expression following treatment with a test compound, wherein detection of a compound which decreases and/or inhibits GSNOR activity and/or expression indicates such compound could be an effective modulator of preterm labor.

The foregoing and other features and advantages of the disclosure will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A and 1B illustrate GSNOR protein expression. FIG. 1A is a bar graph illustrating s-nitrosoglutathione Reductase (GSNOR) expression in spontaneous preterm laboring (SPTL) human myometrium as compared to full term laboring (TL) myometrium; 20µg of protein lysate were run at 200 V for 45 min on a 4-20% PAGE

gel and transferred to nitrocellulose, blocked in Licor® blocking buffer and labeled with GSNOR polyclonal primary ab (ab91385) normalized to GAPDH expression. N = 4-5, mean ± SEM. FIG. 1B is a bar graph illustrating guinea pig GSNOR expression near term (term=68 days) is consistent with increased S-nitrosation to promote quiescence. Mean ± range of three replicates.

FIG. 2 is a confocal microscopy image illustrating GSNOR expression in preterm laboring myometrium. Image illustrates a PFA-fixed 10µm slice of preterm laboring myometrial tissue, labeled with anti-ADH5 antibody (ab59134) FITC labeled showing diffuse GSNOR expression.

FIG. 3 is a bar graph illustrating GSNOR Enzyme Activity. The GSNOR enzymatic activity assay was performed using total protein lysate from human uterine smooth muscle tissue taken from the superior (or upper) portion of the incision. Whole tissue was snap frozen in LN2 and stored at -150°C until ready for use. Once frozen tissue showed no depreciable GSNOR enzymatic activity as compared to fresh tissue. Total protein lysate was prepared as follows: (1) crush tissue in mortar/pestle cooled in LN2; (2) suspend crushed tissue (1mg/ml) in buffer containing 20 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 0.1% NP-40 and 1 mM phenylmethylsulphonyl Fluoride (PMSF); (3) centrifuge at 4,000 rcf for 5 minutes, transfer lysate to new tube, then centrifuge at 18,000 rcf for 15 minutes; (4) incubate in 200µM NADH for 5 minutes at room temperature.; (5) apply 100µM GSNO to NADH/lysate mixture and measure absorbance at 340nm once per minute for 15 minutes. GSNOR activity was measured by the decrease in absorbance at 340nm via conversion of NADH (strong absorbance at 340nm) to NAD+ by GSNOR in the presence of GSNO. 8nM N6022, a potent and specific inhibitor of GSNOR, is used to verify GSNOR specific activity.

FIG. 4 illustrates the effect of GSNOR Inhibition on peak force of contraction in guinea pig myometrium. Strips of myometrium from virgin dunkin-hartley guinea pigs were mounted in an organ bath, attached to force transducers by silk thread, and isometrically stretched (1.2x * length). Transducer voltages were amplified and converted to digital signals. Strips were maintained at 37°C while being continuously bubbled with 100% O_2. Following a 3-minute incubation and washout with 60mM KCL

to stimulate contractions, the tissue strips were equilibrated for one-hour period until regular contractions were achieved. All tissues employed in experiments were spontaneously active. After a baseline peak force of contraction was recorded, an increasing dose of N6022 was applied to each tissue strip. Each dose was applied for 15 minutes and the last five contractions prior to the next dose were averaged (+/- SEM). An equivalent volume of DMSO was applied to each control bath. A significant decrease in peak force of contraction was observed with 30μM and 100μM doses of N6022.

DETAILED DESCRIPTION OF SEVERAL EMBODIMENTS

I. Introduction

The posttranslational modification of proteins regulate cellular function. It is known that the S-nitrosation of cysteines via endogenous nitric oxide (NO) acts as a mediator of disease. Smooth muscle contractile proteins are differentially S-nitrosated based upon the state of labor in women. A regulator of protein S-nitrosation is the availability of S-nitrosoglutathione (GSNO), an endogenously expressed NO donor. The enzyme GSNO Reductase (GSNOR) regulates GSNO levels.

Disclosed herein is the surprising discovery that GSNOR is overexpressed in preterm laboring myometrial samples and is a therapeutic target for preventing and/or inhibiting preterm labor. Without being bound to a specific theory, the overexpression of GSNOR in preterm laboring myometrium is believed to decrease the availability of nitric oxide (NO) by eliminating GSNO from the cytosol and contribute to the dysfunctional relaxation seen to exogenous NO. N6022 (a potent and selective inhibitor of GSNOR with an IC50 of 8 nM and is in clinical trial as an airway smooth muscle relaxant) is believed to increase available GSNO by selectivity inhibiting GSNOR and promote relaxation in preterm laboring myometrial tissue that overexpresses GSNOR.

Based upon these findings, disclosed herein are methods of regulating myometrial contractility and in particular, methods of preventing, inhibiting or treating preterm labor. Also disclosed herein are methods of identifying

II. Terms

The following explanations of terms and methods are provided to better describe the present disclosure and to guide those of ordinary skill in the art in the practice of the present disclosure. The singular terms “a,” “an,” and “the” include plural referents unless context clearly indicates otherwise. Similarly, the word “or” is intended to include “and” unless the context clearly indicates otherwise. The term “comprises” means “includes.” Thus, “comprising A or B,” means “including A, B, or A and B,” without excluding additional elements.

It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

Unless indicated otherwise, the nomenclature of substituents that are not explicitly defined herein are arrived at by naming the terminal portion of the functionality followed by the adjacent functionality toward the point of attachment.

All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

In order to facilitate review of the various embodiments of this disclosure, the following explanations of specific terms are provided:

Administration: To provide or give a subject by any effective route one or more agents, such as an agent that prevents, reduces and/or inhibits GSNOR expression, activation and/or biological activity thereby reducing myometrium contraction, such as in a women who is at risk and/or experiencing spontaneous preterm labor. Exemplary routes of administration include, but are not limited to, injection (such as subcutaneous, intramuscular, intradermal, intraperitoneal, and intravenous), oral, sublingual, rectal, transdermal, intranasal, vaginal and inhalation routes.

Agent: Any protein, nucleic acid molecule (including chemically modified nucleic acids), compound, antibody, small molecule, organic compound, inorganic compound, or other molecule of interest. Agent can include a therapeutic agent, a diagnostic agent or a pharmaceutical agent. A therapeutic or pharmaceutical agent is one that alone or together with an additional compound induces the desired response (such as inducing a therapeutic or prophylactic effect when administered to a subject, including treating a subject with preterm labor).

In some examples, an agent can act directly or indirectly to alter the expression and/or activity of GSNOR. In a particular example, a therapeutic agent significantly decreases the activity of GSNOR (which is an enzyme associated with preterm labor) thereby treating one or more signs or symptoms associated with preterm labor. An example of a therapeutic agent is one that can decrease the expression and/or activity of the GSNOR gene or gene product, for example as measured by a clinical response (such as a decrease in one or more signs or symptoms associated with the spontaneous preterm labor, such as a decrease in uterine contractility).

A "**pharmaceutical agent**" is a chemical compound or composition capable of inducing a desired therapeutic or prophylactic effect when administered to a subject, alone or in combination with another therapeutic agent(s) or pharmaceutically acceptable carriers. In a particular example, a pharmaceutical agent significantly decreases the expression and/or activity of GSNOR thereby treating a condition or disease associated with decreased GSNOR, such as spontaneous preterm labor.

Aliphatic: A hydrocarbon, or a radical thereof, having at least one carbon atom to 50 carbon atoms, such as one to 25 carbon atoms, or one to ten carbon atoms, and which includes alkanes (or alkyl), alkenes (or alkenyl), alkynes (or alkynyl), including cyclic versions thereof, and further including straight- and branched-chain arrangements, and all stereo and position isomers as well.

Alkyl: A saturated monovalent hydrocarbon having at least one carbon atom to 50 carbon atoms, such as one to 25 carbon atoms, or one to ten carbon atoms, wherein the saturated monovalent hydrocarbon can be derived from removing one hydrogen atom from one carbon atom of a parent compound (*e.g.*, alkane). An alkyl group can

be branched, straight-chain, or cyclic (*e.g.*, cycloalkyl).

Alkenyl: An unsaturated monovalent hydrocarbon having at least two carbon atoms 50 carbon atoms, such as two to 25 carbon atoms, or two to ten carbon atoms, and at least one carbon-carbon double bond, wherein the unsaturated monovalent hydrocarbon can be derived from removing one hydrogen atom from one carbon atom of a parent alkene. An alkenyl group can be branched, straight-chain, cyclic (*e.g.*, cylcoalkenyl), *cis*, or *trans* (*e.g.*, *E* or *Z*).

Alkynyl: An unsaturated monovalent hydrocarbon having at least two carbon atoms 50 carbon atoms, such as two to 25 carbon atoms, or two to ten carbon atoms and at least one carbon-carbon triple bond, wherein the unsaturated monovalent hydrocarbon can be derived from removing one hydrogen atom from one carbon atom of a parent alkyne. An alkynyl group can be branched, straight-chain, or cyclic (*e.g.*, cycloalkynyl).

Aliphatic-aryl: An aryl group that is or can be coupled to a compound or compound precursor disclosed herein, wherein the aryl group is or becomes coupled through an aliphatic group.

Alkylaryl/Alkenylaryl/Alkynylaryl: An aryl group that is or can be coupled to a compound or compound precursor disclosed herein, wherein the aryl group is or becomes coupled through an alkyl, alkenyl, or alkynyl group, respectively.

Aliphatic-heteroaryl: A heteroaryl group that is or can be coupled to a compound or compound precursor disclosed herein, wherein the heteroaryl group is or becomes coupled through an aliphatic group.

Alkylheteroaryl/Alkenylheteroaryl/Alkynylheteroaryl: A heteroaryl group that is or can be coupled to a compound or compound precursor disclosed herein, wherein the heteroaryl group is or becomes coupled through an alkyl, alkenyl, or alkynyl group, respectively.

Aryl: An aromatic carbocyclic group comprising at least five carbon atoms to 15 carbon atoms, such as five to ten carbon atoms, having a single ring or multiple condensed rings, which condensed rings can or may not be aromatic provided that the point of attachment is through an atom of the aromatic carbocyclic group. Aryl groups

may be substituted with one or more groups other than hydrogen, such as aliphatic, heteroaliphatic, aryl, heteroaryl, other functional groups, or any combination thereof.

Analog or **Derivative**: A compound which is sufficiently homologous to a compound such that it has a similar functional activity for a desired purpose as the original compound. Analogs or derivatives refers to a form of a substance which has at least one functional group altered, added, or removed, compared with the parent compound.

Biological activity: The beneficial or adverse effects of an agent on living matter. When the agent is a complex chemical mixture, this activity is exerted by the substance's active ingredient or pharmacophore, but can be modified by the other constituents. Activity is generally dosage-dependent and it is not uncommon to have effects ranging from beneficial to adverse for one substance when going from low to high doses. In one example, the agent significantly decreases the biological activity of S-nitrosoglutathione (GSNO) which reduces one or more signs or symptoms associated with spontaneous preterm labor.

Contacting: Placement in direct physical association, including both a solid and liquid form. Contacting an agent with a cell can occur *in vitro* by adding the agent to isolated cells or *in vivo* by administering the agent to a subject.

Control: A sample or standard used for comparison with a test sample, such as a biological sample obtained from a patient (or plurality of patients) without a particular disease or condition, such as spontaneous preterm labor. In some embodiments, the control is a sample obtained from a healthy patient (or plurality of patients) (also referred to herein as a "normal" control), such as a normal biological sample, such as a full term myometrial sample. In some embodiments, the control is a historical control or standard value (*e.g.*, a previously tested control sample or group of samples that represent baseline or normal values (*e.g.*, expression values), such as baseline or normal values of a particular gene such as a GSNOR gene, gene product in a subject not experiencing labor, including spontaneous preterm labor, such as a myometrial sample obtained from a subject at full term). In some examples, the control is a standard value representing the average value (or average range of values) obtained from a plurality of

patient samples (such as an average value or range of values of the gene or gene products, such as the GSNOR gene or gene products, in the subjects not experiencing labor, including spontaneous preterm labor, or is at full term).

Decrease: To reduce the quality, amount, or strength of something. In one example, a therapy decreases one or more symptoms associated with spontaneous preterm labor, such as decreasing myometrial contractions, for example, as compared to the response in the absence of the therapy.

Diagnosis: The process of identifying a condition, such as spontaneous preterm labor, by its signs, symptoms and results of various tests. The conclusion reached through that process is also called "a diagnosis." Forms of testing commonly performed include blood tests, medical imaging, urinalysis, and biopsy.

Effective amount: An amount of agent that is sufficient to generate a desired response, such as reducing or inhibiting one or more signs or symptoms associated with a condition or disease. When administered to a subject, a dosage will generally be used that will achieve target tissue/cell concentrations. In some examples, an "effective amount" is one that treats one or more symptoms and/or underlying causes of any of a disorder or disease. In some examples, an "effective amount" is a therapeutically effective amount in which the agent alone with an additional therapeutic agent(s) (for example additional agents for treating spontaneous preterm labor and/or pain), induces the desired response such as treatment of spontaneous preterm labor.

In particular examples, it is an amount of an agent capable of decreasing gene expression or activity of GSNOR by least 20%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, or even at least 100%.

In some examples, an effective amount is an amount of a pharmaceutical preparation that alone, or together with a pharmaceutically acceptable carrier or one or more additional therapeutic agents, induces the desired response.

In another or additional example, it is an amount sufficient to partially or completely alleviate symptoms of spontaneous preterm labor within the subject. Treatment can involve only slowing the progression of the condition temporarily, but can also include halting or reversing the progression of the condition permanently.

Effective amounts of the agents described herein can be determined in many different ways, such as assaying for a reduction in of one or more signs or symptoms associated with spontaneous preterm labor in the subject or measuring the expression level of one or more molecules known to be associated with spontaneous preterm labor. Effective amounts also can be determined through various *in vitro*, *in vivo* or *in situ* assays, including the assays described herein.

The disclosed therapeutic agents can be administered in a single dose, or in several doses, for example daily, during a course of treatment. However, the effective amount can be dependent on the source applied (for example a nucleic acid molecule isolated from a cellular extract versus a chemically synthesized and purified nucleic acid), the subject being treated, the severity and type of the condition being treated, and the manner of administration.

Expression: The process by which the coded information of a gene is converted into an operational, non-operational, or structural part of a cell, such as the synthesis of a protein. Gene expression can be influenced by external signals. Different types of cells can respond differently to an identical signal. Expression of a gene also can be regulated anywhere in the pathway from DNA to RNA to protein. Regulation can include controls on transcription, translation, RNA transport and processing, degradation of intermediary molecules such as mRNA, or through activation, inactivation, compartmentalization or degradation of specific protein molecules after they are produced. In an example, expression, such as expression of GSNOR, can be regulated to treat one or more signs or symptoms associated with spontaneous preterm labor, including uterine contractility.

The expression of a nucleic acid molecule can be altered relative to a normal (wild type) nucleic acid molecule. Alterations in gene expression, such as differential expression, include but are not limited to: (1) overexpression; (2) underexpression; or (3) suppression of expression. Alternations in the expression of a nucleic acid molecule can be associated with, and in fact cause, a change in expression of the corresponding protein.

Protein expression can also be altered in some manner to be different from the expression of the protein in a normal (wild type) situation. This includes but is not necessarily limited to: (1) a mutation in the protein such that one or more of the amino acid residues is different; (2) a short deletion or addition of one or a few (such as no more than 10-20) amino acid residues to the sequence of the protein; (3) a longer deletion or addition of amino acid residues (such as at least 20 residues), such that an entire protein domain or sub-domain is removed or added; (4) expression of an increased amount of the protein compared to a control or standard amount; (5) expression of a decreased amount of the protein compared to a control or standard amount; (6) alteration of the subcellular localization or targeting of the protein; (7) alteration of the temporally regulated expression of the protein (such that the protein is expressed when it normally would not be, or alternatively is not expressed when it normally would be); (8) alteration in stability of a protein through increased longevity in the time that the protein remains localized in a cell; and (9) alteration of the localized (such as organ or tissue specific or subcellular localization) expression of the protein (such that the protein is not expressed where it would normally be expressed or is expressed where it normally would not be expressed), each compared to a control or standard. Controls or standards for comparison to a sample, for the determination of differential expression, include samples believed to be normal (in that they are not altered for the desired characteristic, for example a sample from a subject who is not experiencing spontaneous preterm labor) as well as laboratory values (*e.g.*, range of values), even though possibly arbitrarily set, keeping in mind that such values can vary from laboratory to laboratory.

Laboratory standards and values can be set based on a known or determined population value and can be supplied in the format of a graph or table that permits comparison of measured, experimentally determined values.

For example, "**detecting or measuring expression of GSNOR**" includes quantifying the amount of the gene, gene product or modulator thereof present in a sample. Quantification can be either numerical or relative. Detecting expression of the gene, gene product or modulators thereof can be achieved using any method known in

the art or described herein, such as by measuring nucleic acids by PCR (such as RT-PCR) and proteins by ELISA. In primary embodiments, the change detected is an increase or decrease in expression as compared to a control, such as a reference value or a healthy control subject. In some examples, the detected increase or decrease is an increase or decrease of at least two-fold compared with the control or standard. Controls or standards for comparison to a sample, for the determination of differential expression, include samples believed to be normal (in that they are not altered for the desired characteristic, for example a sample from a subject who is not experiencing spontaneous preterm labor, such as a non-pregnant subject or a pregnant subject not in active labor) as well as laboratory values (*e.g.*, range of values), even though possibly arbitrarily set, keeping in mind that such values can vary from laboratory to laboratory.

Laboratory standards and values can be set based on a known or determined population value and can be supplied in the format of a graph or table that permits comparison of measured, experimentally determined values.

In other embodiments of the methods, the increase or decrease is of a diagnostically significant amount, which refers to a change of a sufficient magnitude to provide a statistical probability of the diagnosis.

The level of expression in either a qualitative or quantitative manner can detect nucleic acid or protein. Exemplary methods include microarray analysis, RT-PCR, Northern blot, Western blot, and mass spectrometry.

Haloaliphatic: An aliphatic group wherein one or more hydrogen atoms, such as one to 10 hydrogen atoms, independently is replaced with a halogen atom, such as fluoro, bromo, chloro, or iodo.

Heteroaliphatic: An aliphatic group comprising at least one heteroatom to 20 heteroatoms, such as one to 15 heteroatoms, or one to 5 heteroatoms, which can be selected from, but not limited to oxygen, nitrogen, sulfur, selenium, phosphorous, and oxidized forms thereof within the group.

Heteroalkyl/Heteroalkenyl/Heteroalkynyl: An alkyl, alkenyl, or alkynyl group (which can be branched, straight-chain, or cyclic) comprising at least one heteroatom to 20 heteroatoms, such as one to 15 heteroatoms, or one to 5 heteroatoms,

which can be selected from, but not limited to oxygen, nitrogen, sulfur, selenium, phosphorous, and oxidized forms thereof within the group.

Heteroaliphatic-aryl: An aryl group that is or can be coupled to a compound or compound precursor disclosed herein, wherein the aryl group is or becomes coupled through a heteroaliphatic group.

Heteroalkylaryl/Heteroalkenylaryl/Heteroalkynylaryl: An aryl group that is or can be coupled to a compound or compound precursor disclosed herein, wherein the aryl group is or becomes coupled through a heteroalkyl, heteroalkenyl, or heteroalkynyl group, respectively.

Heteroalkylheteroaryl/Heteroalkenylheteroaryl/Heteroalkynylheteroaryl: A heteroaryl group that is or can be coupled to a compound or compound precursor disclosed herein, wherein the aryl group is or becomes coupled through a heteroalkyl, heteroalkenyl, or heteroalkynyl group, respectively.

Heteroaryl: An aryl group comprising at least one heteroatom to six heteroatoms, such as one to four heteroatoms, which can be selected from, but not limited to oxygen, nitrogen, sulfur, selenium, phosphorous, and oxidized forms thereof within the ring. Such heteroaryl groups can have a single ring or multiple condensed rings, wherein the condensed rings may or may not be aromatic and/or contain a heteroatom, provided that the point of attachment is through an atom of the aromatic heteroaryl group. Heteroaryl groups may be substituted with one or more groups other than hydrogen, such as aliphatic, heteroaliphatic, aryl, heteroaryl, other functional groups, or any combination thereof.

Inhibiting a disease or condition: A phrase referring to reducing the development of a disease or condition, for example, in a subject who is at risk for a disease or who has a particular disease. Particular methods of the present disclosure provide methods for inhibiting preterm labor or undesired uterine contractions. A **GSNOR-associated condition** is a condition associated with altered GSNOR expression or activity, including preterm labor.

Muscle: Any myoblast, myocyte, myofiber, myotube or other structure composed of muscle cells. Muscles or myocytes can be skeletal, smooth, or cardiac. Myometrium includes uterine smooth muscle or myocytes.

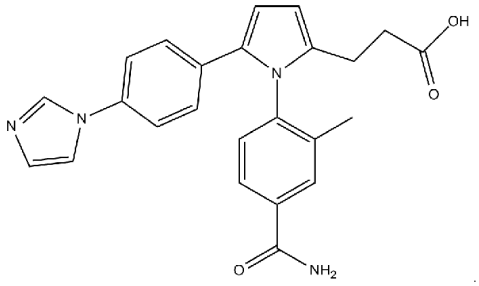
S-nitrosogluthathione reductase (GSNOR): A member of the alcohol dehydrogenase (ADH) family also known as alcohol dehydrogenase 5 (class III) chi polypeptide. GSNOR is encoded by the gene ADH5 and is evolutionarily conserved from bacteria to higher order eukaryotes. GSNOR has many monikers, to include: Formaldehyde dehydrogenase (FDH or FALDH), Glutathione-dependent formaldehyde dehydrogenase (GSH-FDH), and Alcohol Dehydrogenase Class-3. As its name suggests, most alcohol dehydrogenase isozymes have a high specificity for alcohols, however, GSNOR has a low affinity for primary alcohols and its enzymatic activity is almost exclusively limited to S-nitrosoglutathione (GSNO). GSNOR activity is dependent on the presence of nicotinamide adenine dinucleotide (NADH) and GSNO, both of which are oxidized during the reaction to NAD+ and glutathione disulfide (GSSG), respectively.

The emerging field of S-nitrosation and its affect on protein modulation and disease state represents an exciting new branch of research. Nitric oxide (NO) is produced enzymatically in most or all cell types and tissues. S-nitrosation occurs when an NO group is covalently added to the thiol side chain of cysteine residues within proteins and peptides. As with phosphorylation, S-nitrosation regulates cellular mechanisms and affects protein-protein interactions. NO leads to smooth muscle relaxation in a cGMP independent manner. Elevations in cyclic guanosine 5'-monophosphate (cGMP) that accompany relaxation can be prevented by pretreatment of the tissue with the soluble guanylyl cyclase (sGC) inhibitor 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1 (ODQ).

The intracellular availability of nitric oxide (NO) and GSNO affects protein S-nitrosation. GSNOR is a potent negative regulator of GSNO in smooth muscle. In fact, the deletion of the ADH5 gene increases both the levels of GSNO and total protein S-nitrosation *in vivo*. The aberrant expression of many ADH subclasses are associated with disease (Jelski & Szmitkowski, 2008; Jelski et al., 2009; Laniewska-Dunaj et al.,

2013). Of particular interest is the observation that airway hyperesponsivity in wild-type mice correlates to increased expression of GSNOR and decreased S-nitrosothiols, while GSNOR$^{-/-}$ mice are protected from airway hyperesponsivity and maintain higher total SNO levels. In humans GSNOR upregulation can lead to changes in airway smooth muscle tone in asthmatics(Henderson & Gaston, 2005; Moore et al., 2009; Wu et al., 2007). Single nucleotide polymorphisms (SNPs) found in the promoter and 3' UTR of the ADH5 gene in some populations results in an increase of GSNOR expression.

N6022: A specific inhibitor of GSNOR. N6022 has a chemical name of 3-[1-(4-carbamoyl-2-methylphenyl)-5-(4-imidazol-1-ylphenyl)pyrrol-2-yl]propanoic acid and a structural formula of



. N6022 is a first-in-class inhibitor of GSNOR. Inhibition of GSNOR causes the accumulation of GSNO which acts as a vasodilator and anti-inflammatory factor. N6022 presents an IC50 value of 8nM in the GSNO reduction assay and 32nM in the HMGSH oxidation assay. The Ki values are 2.5nM and 3.1nM, respectively. N6022 is selective against GSNOR over other human ADH enzymes. The IC50 values are 21μM, 67μM and 0.5μM for ADH IB, ADH II and ADH IV, respectively. N6022 also shows no effect on the NADPH-dependent enzyme, human carbonyl reductase, with IC50 value of 221μM. Currently, N6022 is under clinical studies for the treatment of inflammatory lung diseases. N6022 and compositions comprising N6022 are disclosed herein for the use of preventing, inhibiting, and/or treating spontaneous preterm labor contractions.

Pharmaceutically acceptable carriers: The pharmaceutically acceptable carriers (vehicles) useful in this disclosure are conventional. Remington's Pharmaceutical Sciences, by E. W. Martin, Mack Publishing Co., Easton, PA, 19th Edition (1995), describes compositions and formulations suitable for pharmaceutical delivery of one or more agents, such as one or more GSNOR modulatory agents.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations can include injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. In addition to biologically-neutral carriers, pharmaceutical agents to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate, sodium lactate, potassium chloride, calcium chloride, and triethanolamine oleate.

Preterm birth: Preterm birth is the birth of a baby at less than 37 weeks gestational age. Some causes of preterm birth are preterm labor induction and spontaneous preterm labor. Although the etiology of spontaneous preterm birth is complex, disparate medical, environmental, and genetic risk factors are thought to converge on effector pathways in the uterine myometrium such as protein S-nitrosation to influence contractility and birth timing in women.

Preterm labor: A woman who experiences forceful contractions of the uterus at 37 weeks of gestation or earlier, she is in labor preterm. Women who enter labor spontaneously preterm (in the absence of any known cause) are at risk of preterm delivery of an underdeveloped fetus. Signs and symptoms of preterm labor include uterine contractions which occur more often than every ten minutes or the leaking of fluid from the vagina. In contrast to false labor, true labor is accompanied by cervical dilatation and effacement. Also, vaginal bleeding in the third trimester, heavy pressure in the pelvis, or abdominal or back pain could be indicators that a preterm birth is about to occur. A watery discharge from the vagina may indicate premature rupture of the membranes that surround the baby. While the rupture of the

membranes may not be followed by labor, usually delivery is indicated as infection (chorioamnionitis) is a serious threat to both fetus and mother. In some cases the cervix dilates prematurely without pain or perceived contractions, so that the mother may not have warning signs until very late in the birthing process. Methods of diagnosing a subject in spontaneous preterm labor can include monitoring uterine contractions for their activity or force, observing cervical effacement in the presence of uterine contractions, uterine contractions associated with fetal distress, or measuring GSNOR activity levels in a sample obtained from the subject. For example, alterations in GSNOR or other biomarkers known to be associated with spontaneous preterm labor may be detected by measuring such levels in serum or urine sample. No drugs reliably prevent labor in patients who enter labor preterm thereby allowing their pregnancies to go to term and none carry an FDA indication for this use. Therapeutic approaches to manage spontaneous preterm labor (SPTL) are employed without clear evidence of benefit for acute or maintenance tocolysis.

Prodrug: A compound that is transformed *in vivo* to yield a parent compound, for example, by hydrolysis in the gut or enzymatic conversion in blood.

Sample (or biological sample): A biological specimen containing genomic DNA, RNA (including mRNA), protein, or combinations thereof, obtained from a subject. Examples include, but are not limited to, peripheral blood, urine, saliva, tissue biopsy, surgical specimen, and autopsy material. In one example, a sample includes a myometrial sample, such as from a subject experiencing spontaneous preterm labor.

Signs or symptoms: Any subjective evidence of disease or of a subject's condition, *e.g.*, such evidence as perceived by the subject; a noticeable change in a subject's condition indicative of some bodily or mental state. A "sign" is any abnormality indicative of disease, discoverable on examination or assessment of a subject. A sign is generally an objective indication of condition or disease. Signs include, but are not limited to any measurable parameters such as tests for detecting spontaneous preterm labor, including measuring GSNOR levels and/or uterine contractility. In one example, reducing or inhibiting one or more symptoms or signs associated with spontaneous preterm labor, includes decreasing the activity or

expression of GSNOR by a desired amount, for example by at least 20%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, or even at least 100%, as compared to the activity and/or expression in the absence of the treatment. Symptoms of spontaneous preterm labor include, but are not limited to, undesired myometrial contractions, such as uterine contractions which occur more often than every ten minutes, or the leaking of fluid from the vagina. In contrast to false labor, true labor is accompanied by cervical dilatation and effacement. Also, vaginal bleeding in the third trimester, heavy pressure in the pelvis, or abdominal or back pain could be indicators that a preterm birth is about to occur. Patients with a short cervix less than or equal to 25 mm incur higher rates of preterm labor.

Subject: Living multi-cellular vertebrate organisms, a category that includes human and non-human mammals.

Tissue: An aggregate of cells, usually of a particular kind, together with their intercellular substance that form one of the structural materials of an animal and that in animals include connective tissue, epithelium, muscle tissue, such as myometrium, and nerve tissue.

Treating a disease: A therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition related to preterm labor. Treatment can induce remission or cure of a condition or slow progression, for example, in some instances can include inhibiting the full development of a disease, for example preventing development of preterm labor. Prevention of a disease does not require a total absence of disease. For example, a decrease of at least 20% can be sufficient.

Treating a disease can be a reduction in severity of some or all clinical symptoms of the disease or condition, a reduction in the number of relapses of the disease or condition, an improvement in the overall health or well-being of the subject, by other parameters well known in the art that are specific to the particular disease or condition, and combinations of such factors.

Under conditions sufficient for: A phrase that is used to describe any environment that permits the desired activity. In one example, includes administering a

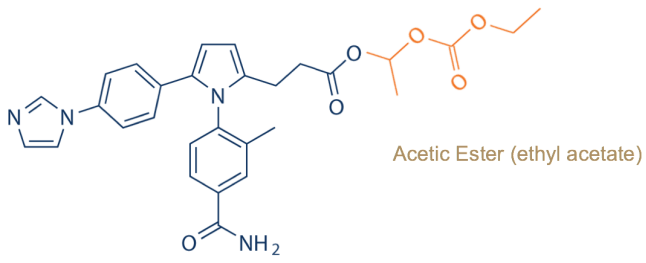
disclosed agent to a subject sufficient to allow the desired activity. In particular examples, the desired activity is decreasing the expression or activity of GSNOR.

III. Compositions for Regulating Myometrium

Disclosed herein are compositions that may be used as GSNOR modulatory agents in methods disclosed herein. In particular disclosed embodiments, the composition is effective in preventing, reducing or inhibiting preterm labor. The composition includes N6022 which has been modified to increase bioavailability. In some examples, a composition includes modified N6022 which increases the permeability of N6022 and thus, decreases the concentration needed to achieve a therapeutic dose.

In some examples, N6022 is modified with various functional groups, such as on the C(O)OH portion of N6022. In some embodiments, N6022 is modified on the C(O)OH portion with esters or carbonate esters such as an ester = $-(R^c)_mC(O)O(R^c)_n$ wherein each R^c can be selected from aliphatic, aryl, or aliphatic-aryl, m can range from 0-30, and n can range from 1 to 30. In one example, N6022 is modified with $-(CH_2)_{0-10}C(O)O(CH_2)_{1-10}$. In some examples, N6022 is modified with a carbonate ester = $-(R^c)_mOC(O)O(R^c)_n$ wherein each R^c can be selected from aliphatic, aryl, or aliphatic-aryl and each of m and n can range from 1 to 30, such as $-(CH_2)_{1-10}C(O)O(CH_2)_{1-10}$, $-C(Me)H\text{-}CH_2O(O)O(CH_2)_{1-10}$. In some examples, N6022 is modified with an alkylene oxide (e.g., , $-[(CH_2)_2O]_m-$, wherein m can range from 1 to 30) or phosphate esters, $-(R^c)_mOP(O)(OR^d)_2$, wherein R^c can be selected from aliphatic, aryl, or aliphatic-aryl and each R^d independently can be selected from hydrogen, aliphatic, aryl, heteroaliphatic, aliphatic-aryl, heteroaryl, aliphatic-heteroaryl, heteroaliphatic-aryl, or heteroaliphatic-heteroaryl, and m can be 1-30. Some specific examples include N6022 modified with $-(CH_2)_{1-10}OP(O)(OH)_2$ or $-(CH_2)_{1-10}OP(O)(OMe)_2$.

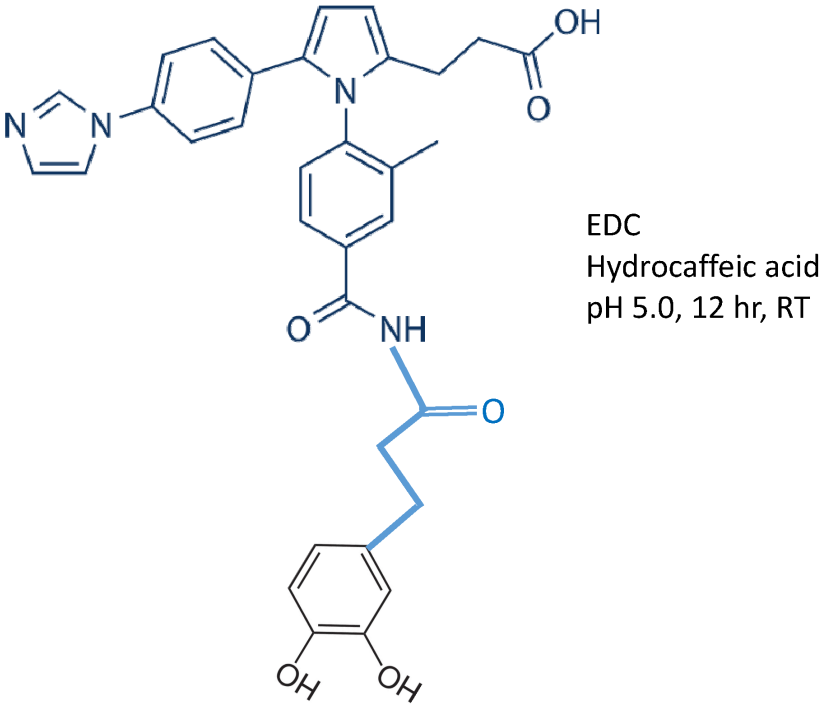
In certain disclosed embodiments, a disclosed composition has a formula illustrated below:



Once in the blood stream resorption esterfication is cleaved by endogenous esterases, returning the chemical to its native state.

In some examples, N6022 is modified with various functional groups, such as on the C(O)NH2 portion of N6022. In some examples, a C(O)NH2 portion of N6022 is modified with an imidoester, such as $-C(NH_2^+)R^c$, wherein R^c can be selected from aliphatic, aryl, heteroaliphatic, aliphatic-aryl, heteroaryl, aliphatic-heteroaryl, heteroaliphatic-aryl, or heteroaliphatic-heteroaryl), including, but not limited to, $-C(NH_2^+)(CH_2)_{1\text{-}30}CH_3$, $-C(NH_2^+)(CH_2)_{1\text{-}30}Ph$, or $-C(NH_2^+)(CH_2)_{1\text{-}30}$(3,4-OH-Ph).

In some examples, a C(O)NH2 portion of N6022 is modified with a carbonyl-containing moiety, carbonyl-containing moiety structure $-C(O)R^c$ wherein R^c can be selected from aliphatic, aryl, heteroaliphatic, aliphatic-aryl, heteroaryl, aliphatic-heteroaryl, heteroaliphatic-aryl, or heteroaliphatic-heteroaryl, such as $-C(O)(CH_2)_{1\text{-}30}CH_3$, $-C(O)(CH_2)_{1\text{-}30}Ph$, $-C(O)(CH_2)_{1\text{-}30}$(3,4-OH-Ph). In one specific embodiment, a disclosed composition has a structural formula as follows:



III. Methods of Use

As disclosed herein, GSNOR expression is increased in preterm labor myometrial samples and inhibition of GSNOR results in inhibition of myometrial contractions. Based on these observations, methods of treatment of preterm labor by regulating GSNOR activity and/or expression are disclosed.

In one example, the method includes administering an effective amount of a GSNOR modulatory agent to a subject with preterm labor or suspected of having or developing spontaneous preterm labor, in which the agent decreases the biological

activity or expression of GSNOR and thereby, decreasing the peak force of contraction in laboring myometrium and thus treating preterm labor in the subject.

In some example, the method of treatment inhibits or reduces one or more signs or symptoms associated with spontaneous preterm labor in the subject.

In some examples, the GSNOR modulatory agent includes an inhibitor of GSNOR, such as N6022, an N6022 analog or like molecule which has been modified to increase bioavailability as described herein. In some examples, the GSNOR modulatory agent is a modified N6022 compound designed and synthesized according to the chemical principles known to one of ordinary skill in the art and identified as a GSNOR modulatory agent by methods known to those of ordinary skill in the art, including those disclosed in the Examples.

The disclosed GSNOR modulatory agents can alter the expression of nucleic acid sequences (such as DNA, cDNA, or mRNAs) and proteins of GSNOR. A decrease in the expression or activity does not need to be 100% for the agent to be effective. For example, an agent can decrease the expression or biological activity by a desired amount, for example by at least 10%, for example at least 20%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, or even at least 100%, including about 15% to about 98%, about 30% to about 95%, about 40% to about 80%, about 50% to about 70%, including about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 98% or about 100%, as compared to activity or expression in a control. Methods of assessing GSNOR expression and activity are described in the Examples below.

In a particular example, the subject is a pregnant woman.

In additional aspects, the method involves selecting a subject at risk of developing or having spontaneous preterm labor. In some example, a subject is selected for treatment following diagnosing the subject with spontaneous preterm labor. For example, the method can include diagnosing the subject with spontaneous preterm labor.

Methods of diagnosing a subject with spontaneous preterm labor or at risk of developing spontaneous preterm labor can include monitoring uterine contractions or

measuring GSNOR expression or activity levels in a sample obtained from the subject. For example, alterations in GSNOR or other biomarkers known to be associated with spontaneous preterm labor may be detected by measuring such levels in serum or urine sample. In some examples, a method of diagnosing includes identifying a patient with a short cervix less than or equal to 25 mm before 25 weeks of gestation. In some examples, a patient with a short cervix less than or equal to 25 mm before 25 weeks of gestation is identified and then is administered a GSNOR inhibitor alone or in combination with progesterone to reduce the incidence of preterm birth.

Alterations in the expression can be measured at the nucleic acid level (such as by real time quantitative polymerase chain reaction or microarray analysis) or at the protein level (such as by Western blot analysis or ELISA). These methods are known to those of skill in the art.

In some examples, following the measurement of the expression levels of GSNOR or GSNOR biological activity, the assay results, findings, diagnoses, predictions and/or treatment recommendations are recorded and communicated to technicians, physicians and/or patients, for example. In certain embodiments, computers are used to communicate such information to interested parties, such as, patients and/or the attending physicians. The therapy selected for administered is then based upon these results.

In one embodiment, the results and/or related information is communicated to the subject by the subject's treating physician. Alternatively, the results may be communicated directly to a test subject by any means of communication, including writing, such as by providing a written report, electronic forms of communication, such as email, or telephone. Communication may be facilitated by use of a computer, such as in case of email communications. In certain embodiments, the communication containing results of a diagnostic test and/or conclusions drawn from and/or treatment recommendations based on the test, may be generated and delivered automatically to the subject using a combination of computer hardware and software which will be familiar to artisans skilled in telecommunications. One example of a healthcare-oriented communications system is described in U.S. Pat. No. 6,283,761; however, the present

disclosure is not limited to methods which utilize this particular communications system. In certain embodiments of the methods of the disclosure, all or some of the method steps, including the assaying of samples, diagnosing of diseases, and communicating of assay results or diagnoses, may be carried out in diverse (*e.g.*, foreign) jurisdictions.

In several embodiments, identification of a subject as having spontaneous preterm labor results in the physician treating the subject, such as prescribing one or more disclosed GSNOR agents for inhibiting or delaying one or more signs and symptoms associated with spontaneous preterm labor. In additional embodiments, the dose or dosing regimen is modified based on the information obtained using the methods disclosed herein.

i. Administration of an Effective amount of a GSNOR modulatory agent

For any of the disclosed methods, an effective amount of a GSNOR modulatory agent is one when administered by a particular route and concentration induces the desired response (*e.g.*, preventing, reducing or treating spontaneous preterm labor).

i. Administration routes, formulations and concentrations

Methods of administration of the disclosed GSNOR modulatory agents are disclosed herein. The disclosed GSNOR modulatory agents or other therapeutic substance are in general administered topically, nasally, intravenously, orally, intramuscularly, parenterally or as implants, but even rectal or vaginal use is possible in principle. The disclosed GSNOR modulatory agents also may be administered to a subject using a combination of these techniques.

Suitable solid or liquid pharmaceutical preparation forms are, for example, aerosols, (micro)capsules, creams, drops, drops or injectable solution in ampoule form, emulsions, granules, powders, suppositories, suspensions, syrups, tablets, coated tablets, and also preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as binders, coating agents, disintegrants, flavorings, lubricants, solubilizers, sweeteners, or swelling agents are customarily used as described above. The pharmaceutical agents are suitable for use in a variety of drug delivery systems. For a brief review of various methods for drug delivery, see Langer,

"New Methods of Drug Delivery," *Science* 249:1527-1533 (1990), incorporated by reference herein to the extent not inconsistent with the present disclosure.

The disclosed GSNOR modulatory agents or other therapeutic agents of the present disclosure can be formulated into therapeutically-active pharmaceutical agents that can be administered to a subject parenterally or orally. Parenteral administration routes include, but are not limited to epidermal, intraarterial, intramuscular (IM and depot IM), intraperitoneal (IP), intravenous (IV), intrasternal injection or infusion techniques, intranasal (inhalation), intrathecal, injection into the stomach, subcutaneous injections (subcutaneous (SQ and depot SQ), transdermal, topical, and ophthalmic.

The disclosed GSNOR modulatory agents or other therapeutic agents can be mixed or combined with a suitable pharmaceutically acceptable excipients to prepare pharmaceutical agents. Pharmaceutically acceptable excipients include, but are not limited to, alumina, aluminum stearate, buffers (such as phosphates), glycine, ion exchangers (such as to help control release of charged substances), lecithin, partial glyceride mixtures of saturated vegetable fatty acids, potassium sorbate, serum proteins (such as human serum albumin), sorbic acid, water, salts or electrolytes such as cellulose-based substances, colloidal silica, disodium hydrogen phosphate, magnesium trisilicate, polyacrylates, polyalkylene glycols, such as polyethylene glycol, polyethylene-polyoxypropylene-block polymers, polyvinyl pyrrolidone, potassium hydrogen phosphate, protamine sulfate, group 1 halide salts such as sodium chloride, sodium carboxymethylcellulose, waxes, wool fat, and zinc salts, for example. Liposomal suspensions may also be suitable as pharmaceutically acceptable carriers.

Upon mixing or addition of one or more disclosed GSNOR modulatory agents and/or or other therapeutic agents, the resulting mixture may be a solid, solution, suspension, emulsion, or the like. These may be prepared according to methods known to those of ordinary skill in the art. The form of the resulting mixture depends upon a number of factors, including the intended mode of administration and the solubility of the agent in the selected carrier. Pharmaceutical carriers suitable for administration of the disclosed GSNOR modulatory agents or other therapeutic agents include any such carriers known to be suitable for the particular mode of administration. In addition, the

disclosed GSNOR modulatory agents or other therapeutic substance can also be mixed with other inactive or active materials that do not impair the desired action, or with materials that supplement the desired action, or have another action.

Methods for solubilizing may be used where the agents exhibit insufficient solubility in a carrier. Such methods can include, but are not limited to, dissolution in aqueous sodium bicarbonate, using cosolvents such as dimethylsulfoxide (DMSO), and using surfactants such as TWEEN® (ICI Americas, Inc., Wilmington, DE).

The disclosed GSNOR modulatory agents or other therapeutic agents can be prepared with carriers that protect them against rapid elimination from the body, such as coatings or time-release formulations. Such carriers include controlled release formulations, such as, but not limited to, microencapsulated delivery systems. A disclosed GSNOR modulatory agents or other therapeutic agent is included in the pharmaceutically acceptable carrier in an amount sufficient to exert a therapeutically useful effect, typically in an amount to avoid undesired side effects, on the treated subject. The therapeutically effective concentration may be determined empirically by testing the compounds in known *in vitro* and *in vivo* model systems for the treated condition.

Injectable solutions or suspensions can be formulated, using suitable non-toxic, parenterally-acceptable diluents or solvents, such as 1,3-butanediol, isotonic sodium chloride solution, mannitol, Ringer's solution, saline solution, or water; or suitable dispersing or wetting and suspending agents, such as sterile, bland, fixed oils, including synthetic mono- or diglycerides, and fatty acids, including oleic acid; a naturally occurring vegetable oil such as coconut oil, cottonseed oil, peanut oil, sesame oil, and the like; glycerine; polyethylene glycol; propylene glycol; or other synthetic solvent; antimicrobial agents such as benzyl alcohol and methyl parabens; antioxidants such as ascorbic acid and sodium bisulfite; buffers such as acetates, citrates, and phosphates; chelating agents such as ethylenediaminetetraacetic acid (EDTA); agents for the adjustment of tonicity such as sodium chloride and dextrose; and combinations thereof. Parenteral preparations can be enclosed in ampoules, disposable syringes, or multiple dose vials made of glass, plastic, or other suitable material. Buffers, preservatives,

antioxidants, and the like can be incorporated as required. Where administered intravenously, suitable carriers include physiological saline, phosphate-buffered saline (PBS), and solutions containing thickening and solubilizing agents such as glucose, polyethylene glycol, polypropyleneglycol, and mixtures thereof. Liposomal suspensions, including tissue-targeted liposomes, may also be suitable as pharmaceutically acceptable carriers.

For topical application, one or more disclosed GSNOR modulatory agents, or other therapeutic agent may be made up into a cream, lotion, ointment, solution, or suspension in a suitable aqueous or non-aqueous carrier. Topical application can also be accomplished by transdermal patches or bandages which include the therapeutic substance. Additives can also be included, e.g., buffers such as sodium metabisulphite or disodium edetate; preservatives such as bactericidal and fungicidal agents, including phenyl mercuric acetate or nitrate, benzalkonium chloride, or chlorhexidine; and thickening agents, such as hypromellose.

If the disclosed GSNOR modulatory agent, or other therapeutic agent is administered orally as a suspension, the pharmaceutical agents can be prepared according to techniques in the art of pharmaceutical formulation and may contain a suspending agent, such as alginic acid or sodium alginate, bulking agent, such as microcrystalline cellulose, a viscosity enhancer, such as methylcellulose, and sweeteners/flavoring agents. Oral liquid preparations can contain conventional additives such as suspending agents, e.g., gelatin, glucose syrup, hydrogenated edible fats, methyl cellulose, sorbitol, and syrup; emulsifying agents, e.g., acacia, lecithin, or sorbitan monooleate; non-aqueous carriers (including edible oils), e.g., almond oil, fractionated coconut oil, oily esters such as glycerine, propylene glycol, or ethyl alcohol; preservatives such as methyl or propyl p-hydroxybenzoate or sorbic acid; and, if desired, conventional flavoring or coloring agents. When formulated as immediate release tablets, these agents can contain dicalcium phosphate, lactose, magnesium stearate, microcrystalline cellulose, and starch and/or other binders, diluents, disintegrants, excipients, extenders, and lubricants.

If oral administration is desired, one or more disclosed GSNOR modulatory agents, or other therapeutic substances can be provided in a composition that protects it from the acidic environment of the stomach. For example, the disclosed GSNOR modulatory agents or other therapeutic agents can be formulated with an enteric coating that maintains its integrity in the stomach and releases the active compound in the intestine. The disclosed GSNOR modulatory agents, or other therapeutic agent can also be formulated in combination with an antacid or other such ingredient.

Oral compositions generally include an inert diluent or an edible carrier and can be compressed into tablets or enclosed in gelatin capsules. For the purpose of oral therapeutic administration, one or more of the disclosed GSNOR modulatory agents, or other therapeutic substances can be incorporated with excipients and used in the form of capsules, tablets, or troches. Pharmaceutically compatible adjuvant materials or binding agents can be included as part of the composition.

The capsules, pills, tablets, troches, and the like can contain any of the following ingredients or compounds of a similar nature: a binder such as, but not limited to, acacia, corn starch, gelatin, gum tragacanth, polyvinylpyrrolidone, or sorbitol; a filler such as calcium phosphate, glycine, lactose, microcrystalline cellulose, or starch; a disintegrating agent such as, but not limited to, alginic acid and corn starch; a lubricant such as, but not limited to, magnesium stearate, polyethylene glycol, silica, or talc; a gildant, such as, but not limited to, colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; disintegrants such as potato starch; dispersing or wetting agents such as sodium lauryl sulfate; and a flavoring agent such as peppermint, methyl salicylate, or fruit flavoring.

When the dosage unit form is a capsule, it can contain, in addition to material of the above type, a liquid carrier, such as a fatty oil. In addition, dosage unit forms can contain various other materials that modify the physical form of the dosage unit, for example, coatings of sugar and other enteric agents. One or more of the disclosed GSNOR modulatory agents, or other therapeutic agent can also be administered as a component of an elixir, suspension, syrup, wafer, tea, chewing gum, or the like. A syrup

may contain, in addition to the active compounds, sucrose or glycerin as a sweetening agent and certain preservatives, dyes and colorings, and flavors.

When administered orally, the compounds can be administered in usual dosage forms for oral administration. These dosage forms include the usual solid unit dosage forms of tablets and capsules as well as liquid dosage forms such as solutions, suspensions, and elixirs. When the solid dosage forms are used, they can be of the sustained release type so that the compounds need to be administered less frequently.

In some examples, one or more of the disclosed GSNOR modulatory agents and/or a therapeutic agent is injected into the stomach of a subject is incorporated systemically in the subject, such as in diverse muscle groups. Examples of methods and compositions for administering therapeutic substances which include proteins include those discussed in Banga, *Therapeutic Peptides and Proteins: Formulation, Processing, and Delivery Systems* 2ed. (2005); Mahato, *Biomaterials for Delivery and Targeting of Proteins and Nucleic Acids* (2004); McNally, *Protein Formulation and Delivery*, 2ed. (2007); and Kumar *et al.*, "Novel Delivery Technologies for Protein and Peptide Therapeutics," *Current Pharm. Biotech.*, **7**:261-276 (2006); each of which is incorporated by reference herein to the extent not inconsistent with the present disclosure.

In some implementations, the effective amount of one or more of the disclosed GSNOR modulatory agents is administered as previously described in order to directly target the muscle of the uterus. In some examples, the effective amount of one or more of the disclosed GSNOR modulatory agents is administered as a single dose per time period, such as every hour, day, biweekly, weekly or it can be divided into at least two unit dosages for administration over a period. Treatment may be continued as long as necessary to achieve the desired results. For instance, treatment may continue for about 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks or longer. The compound can also be administered in several doses intermittently, such as every hours (for example, at least once, twice a day every few days (for example, at least about every two, three, four, five, or ten days) or every few weeks (for example at least about every two, three, four, five, or ten weeks).

Particular dosage regimens can be tailored to a particular subject and severity of symptoms, such as severity of myometrial contractions. For example, when the methods of the present disclosure are used to treat preterm labor, an initial treatment regimen can be applied to arrest the condition. Such initial treatment regimen may include administering a higher dosage of one or more of the disclosed GSNOR modulatory agents, or administering such material more frequently, such as daily. After a desired therapeutic result has been obtained, such as reduced uterine contractions, a second treatment regimen may be applied, such as administering a lower dosage of one or more of the disclosed GSNOR modulatory agents or administering such material less frequently, such as every couple of days, weekly, monthly, or bi-monthly. In such cases, the second regimen may serve as a "booster" to restore or maintain a desired level of uterine relaxation.

Amounts effective for various therapeutic treatments of the present disclosure may, of course, depend on the severity of the condition, (e.g., preterm contractions) and the weight and general state of the subject, as well as the absorption, inactivation, and excretion rates of the therapeutically-active compound or component, the dosage schedule, and amount administered, as well as other factors known to those of ordinary skill in the art. The exact dosage and frequency of administration will depend on the particular GSNOR modulatory agent, or other therapeutic substance being administered, the particular condition being treated, the severity of the condition being treated, the age, weight, general physical condition of the particular subject, and other medication the subject may be taking. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for *in vivo* administration of the pharmaceutical composition, and animal models may be used to determine effective dosages for treatment of particular disorders. For example, guinea pig models of spontaneous preterm labor may be used to determine effective dosages (mg/kg) that can then be translated to dosage amount for other subjects, such as humans, as known in the art.

In specific examples, the one or more disclosed GSNOR modulatory agents is administered to a subject in an amount sufficient to provide a dose of the agent of via a single or multiple administrations between 0.1-100 mg of agent per patient (70kg mean

weight) as indicated. 3.5 nmol/g and about 3.5 µmol/g, such as between about 1.4 µg/kg and about 1.4 mg/kg or between about 45 nM and 45µM, such as between about 0.019 µg/ml 0.019 mg/ml.

ii. Desired Response

One or more disclosed GSNOR modulatory agents and/or additional therapeutic agents are administered by a specific route and/or concentration to generate a desired response. In some examples, a desired response refers to an amount effective for lessening, ameliorating, eliminating, preventing, or inhibiting at least one symptom of a disease, disorder, or condition treated and may be empirically determined. In various embodiments of the present disclosure, a desired response is reduced frequency and/or intensity of myometrial contractions, reductions or prevention of myometrial contractions, reduction or prevention in one more signs or symptoms associated with spontaneous preterm labor.

In particular, indicators of spontaneous preterm labor, include monitoring uterine contractions as well expression and/or activity of spontaneous preterm labor associated molecules, such as GSNOR. For example, increased expression of such markers can indicate that spontaneous preterm labor is occurring or a subject is at risk of developing spontaneous preterm labor.

iii. Additional treatments or therapeutic agents

In particular examples, prior to, during, or following administration of an effective amount of an agent that reduces or inhibits one or more signs or symptoms associated with spontaneous preterm labor, the subject can receive one or more other therapies. In one example, the subject receives one or more treatments prior to administration of a disclosed GSNOR modulatory agent. Examples of such additional therapies include, but are not limited to, administration of tocolytics, antibiotics, progesterone and/or antenatal corticosteroids. Additional therapies may also include cervical cerclage or bed rest.

The following examples are provided to illustrate certain particular features and/or embodiments. These examples should not be construed to limit the invention to the particular features or embodiments described.

EXAMPLES

Example 1

GSNOR Protein Expression is Significantly Greater in Spontaneous preterm laboring Myometrium

This example illustrates GSNOR protein expression in Term laboring and Spontaneous preterm laboring myometrium.

Samples of term laboring and preterm laboring myometrium were obtained and S-nitrosoglutathione Reductase (GSNOR) expression in preterm laboring human myometrium as compared to full term laboring myometrium was determined. Twenty µg of protein lysate were run at 200 V for 45 minutes on a 4-20% PAGE gel and transferred to nitrocellulose, blocked in Licor® blocking buffer. The Western blot was labeled with GSNOR polyclonal primary ab (ab91385, Abcam, Cambridge, MA) and normalized to GAPDH expression. As shown in FIG. 1A, significantly greater amounts of GSNOR expression was detected in preterm laboring myometrial samples as compared to full term laboring myometrial samples. FIG. 1B illustrates that guinea pig GSNOR expression near term (term=68 d.) is consistent with increased S-nitrosation to promote quiescence. Mean ± range of three replicates.

To further validate expression of GSNOR in pregnancy, confocal microscopy was used to view the pattern of myocyte expression in human preterm laboring myometrial tissue (FIG. 2). GSNOR was widely distributed in the cytoplasm. (A confocal image of PFA-fixed pHUSMC cells labeled with anti-ADH5 antibody (ab59134) FITC labeled showing diffuse GSNOR expression.

Example 2

GSNOR Enzyme Activity Assay

This example illustrates GSNOR enzyme activity in Term laboring (TL) and preterm laboring (PTL) myometrium.

The GSNOR enzymatic activity assay was performed using total protein lysate from human uterine smooth muscle tissue taken from the superior (or upper) portion of the incision. Whole tissue was snap frozen in liquid nitrogen (LN2) and stored at -150°C until ready for use. Once frozen tissue showed no depreciable GSNOR enzymatic activity as compared to fresh tissue. Total protein lysate was prepared as follows: (1) crushing tissue in mortar/pestle cooled in LN2; (2) suspending crushed tissue (1mg/ml) in buffer containing 20 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 0.1% NP-40 and 1 mM phenylmethylsulphonyl Fluoride (PMSF); (3) centrifuging at 4,000 rcf for 5 minutes, transferring lysate to new tube, then centrifuging at 18,000 rcf for 15 minutes; (4) incubating in 200µM NADH for 5 minutes at room temperature (r.t.); and (5) applying 100µM GSNO to NADH/lysate mixture and measure absorbance at 340nm once per minute for 15 minutes. GSNOR activity was measured by the decrease in absorbance at 340nm via conversion of NADH (strong absorbance at 340nm) to NAD+ by GSNOR in the presence of GSNO. 8nM N6022, a potent and specific inhibitor of GSNOR, was used to verify GSNOR specific activity. As shown in FIG. 3, GSNOR activity was dramatically increased in TL and PTL samples. Further, the noted increase was inhibited by 8nM N6022.

Example 3

N6022 Inhibits GSNOR Activity in Myometrium

This example illustrates the effect of GSNOR inhibition on peak force of contraction in guinea pig myometrium.

Strips of myometrium from virgin Dunkin-Hartley guinea pigs were mounted in an organ bath, attached to force transducers by silk thread, and isometrically stretched (1.2x * length). Transducer voltages were amplified and converted to digital signals. Strips were maintained at 37°C while being continuously bubbles with 100% O2. Following a 3-minute incubation and washout with 60mM KCL stimulate contractions, the tissues were equilibrated for one-hour period until regular contractions were achieved. All tissues employed in studies were spontaneously active. After a baseline peak force of contraction was recorded, an increasing dose of N6022 was applied to each tissue strip. Each dose was applied for 15 minutes and the final five contractions prior to the next dose were averaged (+/- SEM). An equivalent volume of DMSO was applied to each control bath. A significant decrease in peak force of contraction was observed with 30μM and 100μM doses of N6022.

In view of the many possible embodiments to which the principles of the disclosed invention may be applied, it should be recognized that the illustrated embodiments are only preferred examples of the invention and should not be taken as limiting the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

We claim:

1. A method, comprising:

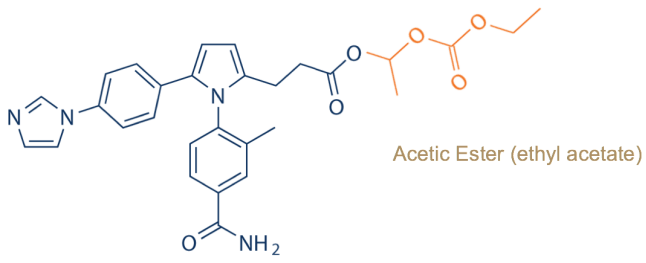
administering an effective amount of a GSNOR modulatory agent to a subject at risk of developing or having one or more signs or symptoms of spontaneous preterm labor, wherein the GSNOR modulatory agent comprises N6022, modified N6022 or a combination thereof, wherein the GSNOR modulatory agent decreases GSNOR expression and/or activity as compared to GSNOR expression and/or activity prior to treatment, thereby preventing, inhibiting or reducing one or more signs or symptoms of spontaneous preterm labor.

2. The method of claim 1, wherein the composition comprises N6022.

3. The method of claim 1, wherein the composition comprises modified N6022, wherein C(O)OH portion of N6022 is modified with an ester or carbonate ester, such as an ester, $-(R^c)_mC(O)O(R^c)_n$ wherein each R^c can be selected from aliphatic, aryl, or aliphatic-aryl, m can range from 0-30, and n can range from 1 to 30, such as $-(CH_2)_{1-10}C(O)O(CH_2)_{1-10,}$ $-C(Me)H\text{-}CH_2O(O)O(CH_2)_{1-10}$.

4. The method of claims 3, wherein C(O)OH portion of N6022 is modified with $-(CH_2)_{0-10}C(O)O(CH_2)_{1-10.}$

5. The method of claim 3, wherein the composition comprises modified N6022 with a structural formula of



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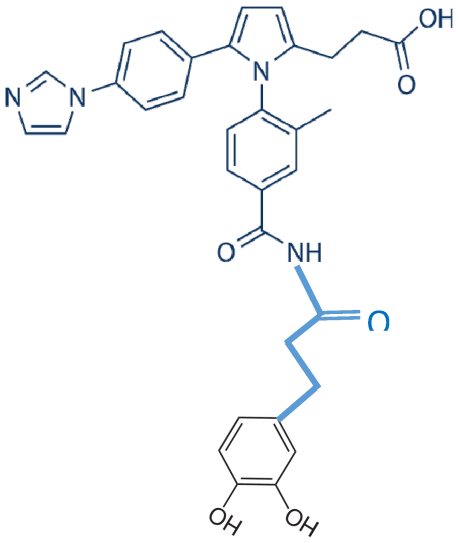
6. The method of claim 1, wherein C(O)OH portion of N6022 is modified with an alkylene oxide (e.g., , $-[(CH_2)_2O]_m-$, wherein m can range from 1 to 30) or phosphate esters, $-(R^c)_mOP(O)(OR^d)_2$, wherein R^c can be selected from aliphatic, aryl, or aliphatic-aryl and each R^d independently can be selected from hydrogen, aliphatic, aryl, heteroaliphatic, aliphatic-aryl, heteroaryl, aliphatic-heteroaryl, heteroaliphatic-aryl, or heteroaliphatic-heteroaryl, and m can be 1-30, such as $-(CH_2)_{1-10}OP(O)(OH)_2$ or $-(CH_2)_{1-10}OP(O)(OMe)_2$.

7. The method of claim 1, wherein the composition comprises modified N6022, wherein C(O)NH2 portion of N6022 is modified with one or more functional groups, including an imidoester, such as $-C(NH_2^+)R^c$, wherein R^c can be selected from aliphatic, aryl, heteroaliphatic, aliphatic-aryl, heteroaryl, aliphatic-heteroaryl, heteroaliphatic-aryl, or heteroaliphatic-heteroaryl), including $-C(NH_2^+)(CH_2)_{1-30}CH_3$, $-C(NH_2^+)(CH_2)_{1-30}Ph$, or $-C(NH_2^+)(CH_2)_{1-30}$(3,4-OH-Ph).

8. The method of claim 1, wherein the composition comprises modified N6022, wherein C(O)NH2 portion of N6022 is modified with one or more functional

groups, including a carbonyl-containing moiety, carbonyl-containing moiety structure $-C(O)R^c$ wherein R^c can be selected from aliphatic, aryl, heteroaliphatic, aliphatic-aryl, heteroaryl, aliphatic-heteroaryl, heteroaliphatic-aryl, or heteroaliphatic-heteroaryl, such as $-C(O)(CH_2)_{1-30}CH_3$, $-C(O)(CH_2)_{1-30}Ph$, $-C(O)(CH_2)_{1-30}$(3,4-OH-Ph).

9. The method of claim 1, wherein the composition comprises modified N6022 with a structural formula of



10. The method of any one of claims 1-9, further comprising selecting a subject at risk of developing or having one or more signs or symptoms of spontaneous preterm labor.

11. The method of claim 10, wherein selecting a subject at risk of developing or having one or more signs or symptoms of spontaneous preterm labor

comprises diagnosing the subject prior to administering an effective amount of the GSNOR modulatory agent to the subject by detecting the presence of GSNOR expression and/or activity in the subject as compared to GSNOR expression and/or activity in a control.

12. The method of claim 11, wherein the control is GSNOR expression and/or activity in a term myometrial sample.

13. The method of any one of claims 1-12, wherein administration comprises systemic administration using IV administration of the solubilized GSNOR modulatory agent or administration by transdermal patch placed on the abdomen or other effective location on the subject.

14. The method of claim 13, wherein an effective amount is between 0.1-100 mg of agent per subject (70kg mean weight), 3.5 nmol/g and about 3.5 µmol/g, such as between about 1.4 µg/kg and about 1.4 mg/kg nmol/g or between about 45 nM and 45µM, such as between about 0.019 µg/ml 0.019 mg/ml of N6022, modified N6022 or a combination thereof.

15. The method of any one of claims 1-14, wherein the GSN0R modulatory agent is administered with an additional therapeutic agent.

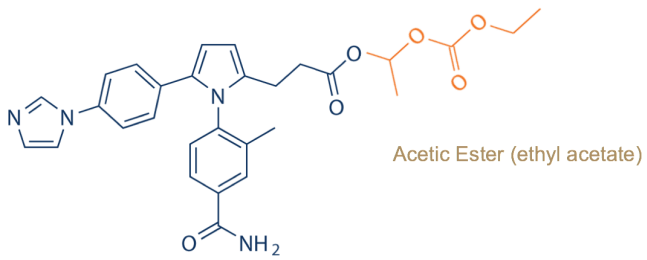
16. A composition for modulating GSNOR, comprising modified N6022 wherein C(O)OH portion of N6022 and/or C(O)NH2 portion of N6022 are modified with one or more functional groups.

17. The composition of claim 16, wherein C(O)OH portion of N6022 is modified with an ester or carbonate ester, such as an ester, $-(R^c)_mC(O)O(R^c)_n$ wherein each R^c can be selected from aliphatic, aryl, or aliphatic-aryl, m can range from 0-30,

and n can range from 1 to 30, such as $-(CH_2)_{1\text{-}10}C(O)O(CH_2)_{1\text{-}10,}$ $-C(Me)H-CH_2O(O)O(CH_2)_{1\text{-}10}$.

18. The composition of claims 16, wherein C(O)OH portion of N6022 is modified with $-(CH_2)_{0\text{-}10}C(O)O(CH_2)_{1\text{-}10.}$

19. The composition of claim 16, wherein the composition comprises modified N6022 with a structural formula of



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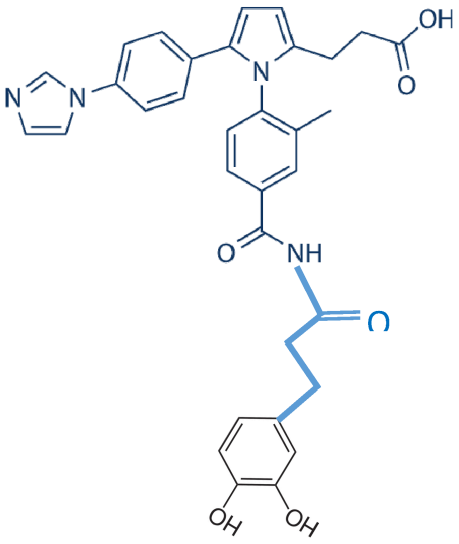
20. The composition of claim 16, wherein C(O)OH portion of N6022 is modified with an alkylene oxide (e.g., , $-[(CH_2)_2O]_m-$, wherein m can range from 1 to 30) or phosphate esters, $-(R^c)_mOP(O)(OR^d)_2$, wherein R^c can be selected from aliphatic, aryl, or aliphatic-aryl and each R^d independently can be selected from hydrogen, aliphatic, aryl, heteroaliphatic, aliphatic-aryl, heteroaryl, aliphatic-heteroaryl, heteroaliphatic-aryl, or heteroaliphatic-heteroaryl, and m can be 1-30, such as $-(CH_2)_{1\text{-}10}OP(O)(OH)_2$ or $-(CH_2)_{1\text{-}10}OP(O)(OMe)_2$.

21. The composition of claim 16, wherein C(O)NH2 portion of N6022 is modified with one or more functional groups, including an imidoester, such as $-C(NH_2^+)R^c$, wherein R^c can be selected from aliphatic, aryl, heteroaliphatic, aliphatic-aryl, heteroaryl, aliphatic-heteroaryl, heteroaliphatic-aryl, or heteroaliphatic-heteroaryl), including $-C(NH_2^+)(CH_2)_{1\text{-}30}CH_3$, $-C(NH_2^+)(CH_2)_{1\text{-}30}Ph$, or $-C(NH_2^+)(CH_2)_{1\text{-}30}$(3,4-OH-Ph).

21. The method of claim 16, wherein C(O)NH2 portion of N6022 is modified with one or more functional groups, including a carbonyl-containing moiety, carbonyl-containing moiety structure $-C(O)R^c$ wherein R^c can be selected from aliphatic, aryl, heteroaliphatic, aliphatic-aryl, heteroaryl, aliphatic-heteroaryl, heteroaliphatic-aryl, or heteroaliphatic-heteroaryl, such as $-C(O)(CH_2)_{1\text{-}30}CH_3$, $-C(O)(CH_2)_{1\text{-}30}Ph$, $-C(O)(CH_2)_{1\text{-}30}$(3,4-OH-Ph).

22. The method of claim 16, wherein the composition comprises modified N6022 with a structural formula of


OH
O
N
N
N
O
NH
O
OH
OH

COMPOSITIONS AND METHODS FOR REGULATING SPONTANEOUS PRETERM LABOR

ABSTRACT OF THE DISCLOSURE

Compositions and methods for diagnosing, treating and/or evaluating the efficacy of a treatment of spontaneous preterm labor are disclosed. In some examples, a method of treating spontaneous preterm labor comprises administering an effective amount of a GSNOR modulatory agent to a subject at risk of developing or having one or more signs or symptoms of spontaneous preterm labor, wherein the GSNOR modulatory agent comprises N6022, modified N6022 or a combination thereof, wherein the GSNOR modulatory agent decreases GSNOR expression and/or activity as compared to GSNOR expression and/or activity prior to treatment, thereby preventing, inhibiting or reducing one or more signs or symptoms of spontaneous preterm labor.

FIG. 1A

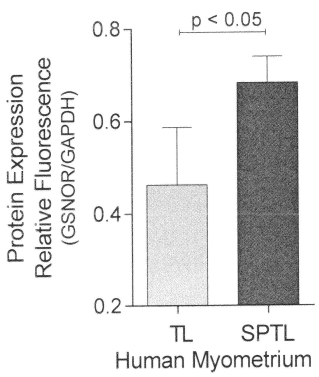

p < 0.05
0.8
0.6
0.4
0.2
Protein Expression
Relative Fluorescence
(GSNOR/GAPDH)
TL
SPTL
Human Myometrium

FIG. 1B

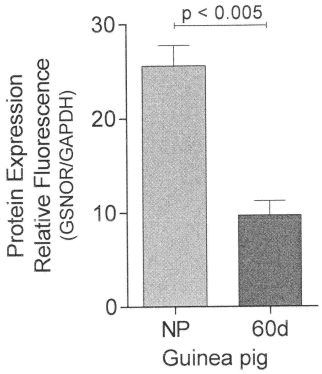

p < 0.005
30
20
10
0
Protein Expression
Relative Fluorescence
(GSNOR/GAPDH)
NP
60d
Guinea pig

FIG. 2

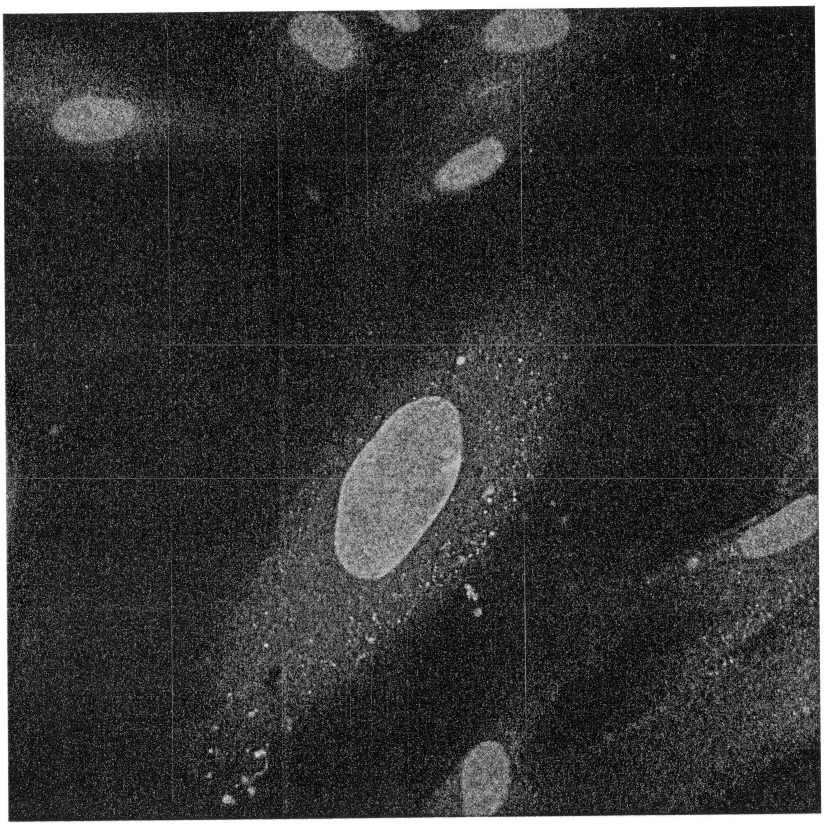


FIG. 3

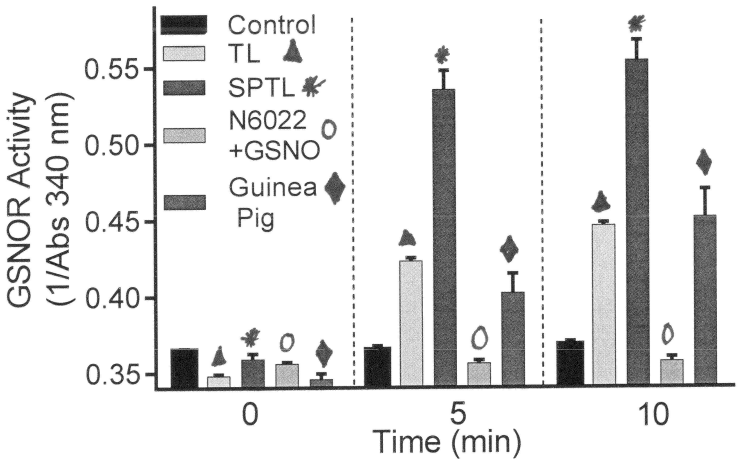
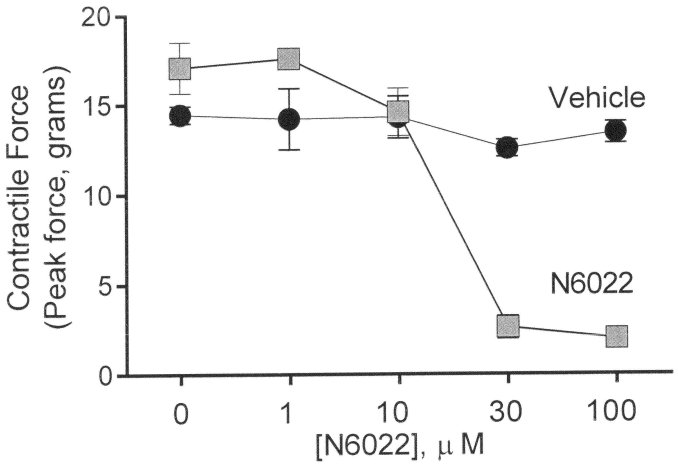

Control
TL
SPTL
N6022 +GSNO
Guinea Pig
GSNOR Activity (1/Abs 340 nm)
0.55
0.50
0.45
0.40
0.35
0
5
10
Time (min)

FIG. 4


20
15
10
5
0
Contractile Force
(Peak force, grams)
Vehicle
N6022
0
1
10
30
100
[N6022], μ M

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Appendix C

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November 6, 2017

Scott Barnett
Pharmacology Department
University of Nevada, Reno School of Medicine
1664 N Virginia St, CMM308 MS0573
Reno, NV

Email: sdbarnett@med.unr.edu

Dear Scott Barnett:

This is to grant you permission to include the following article in your dissertation entitled "Intimations on the Pathophysiology of Human Preterm Labor: The Unique Actions of Nitric Oxide in the Myometrium and the Consequences of its Dysregulation" for the University of Nevada, Reno:

> ILO Buxton, D Milton, SD Barnett, and SD Tichenor (2010) Agonist-Specific Compartmentation of cGMP Action in Myometrium, *J Pharmacol Exp Ther*, 335(1):256-263; DOI: https://doi.org/10.1124/jpet.110.171934

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Alternatively Spliced Human TREK-1 Variants Alter TREK-1 Channel Function and Localization

TREK-1 currents in smooth muscle cells from pregnant human myometrium

Nathanael S. Heyman,* Chad L. Cowles,* Scott D. Barnett, Yi-Ying Wu, Charles Cullison, Cherie A. Singer, Normand Leblanc, and Iain L. O. Buxton

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