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APPROPRIATE PRECONDITIONING OF THE UTERINE ENDOPLASMIC RETICULUM STRESS RESPONSE INHIBITS PRETERM LABOR

by

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DISSERATION

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Advisor Date

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DEDICATION

I dedicate this work to my Mom and Dad.

for loving me more than the moon at night and chocolate milkshakes

for making me feel safe and important in this world

for teaching me to live one day at a time.

I dedicate this work to my Sisters and soon to be Husband..

for loving me through my very worst and my very best

for making me laugh when I wanted to cry

for teaching me the true meaning of friendship.

I dedicate this work to my Grandparents.

for loving me from my first breath to my last

for making me believe I could change the world

for teaching me that family is the most important gift of all.

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PREFACE

The objective of this dissertation is to determine the role of uterine unfolded protein response (UPR) preconditioning in the maintenance of myometrial quiescence. The overarching hypotheses is that preconditioning the myometrial UPR would allow for the maintenance of non-apoptotic caspase 3 (CASP3) activity, and thus sustain uterine quiescence. In chapter one, we test the specific hypothesis that preconditioning the UPR *in vitro* in the immortalized human myocyte would be effective in activating and maintaining CASP3 in a non-apoptotic state. Following Tunicamycin (TM) or Thapsigargin (Thaps) preconditioning, ERSR activation and apoptosis will be examined along with inflammatory responses. In the second chapter, we expand our hypothesis and test the role of endogenous pregnancy-generated stress stimuli in preconditioning the myometrium for the maintenance of uterine quiescence. Using a pregnant mouse model with phenyl butyric acid (PBA)-dependent sub-preconditioned mice, we will analyze the effects of inappropriate UPR preconditioning on gestational length, the regulation of uterine CASP3, inflammation and the process of luteolysis. In the final chapter we will 1) characterize the UPR-generated secretome in stressed uterine myocytes, 2) test the functional capacity of the UPR-secretome to transmit the stress response in a paracrine and endocrine manner and 3) evaluate changes in the UPR secretome with pregnancy associated pathologies. We hypothesize endoplasmic reticulum stress (ERS) in the uterine myocyte produces a unique secretome that has the capability of propagating the ERS response in a paracrine and endocrine manner and may be used in modulating systemic adaptations to pregnancy. The following work will review our results and discuss the main implications of this collection of studies.

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General Introduction

Preterm Birth

Preterm birth (PTB), which is classically described as the delivery of a baby prior to 37 weeks of gestation, is the number one cause of mortality in children under 5 years of age.[1] Each year approximately 15 million babies are born premature worldwide, and this number continues to rise.[2,3] Due to the incomplete development of vital organs at birth, premature infants that are fortunate enough to survive often suffer major health complications. Consequently, the risk of health problems and mortality associated with preterm delivery is inversely correlated to gestational age and organ development at the time of birth.[4] As a result of immature organ development, babies born preterm have an increased risk of neurologic and developmental disabilities, such as cerebral palsy, hearing and vision impairments, as well as respiratory complications.[5,6] Recent studies have also demonstrated direct correlations between PTB and latent diseases such as asthma, insulin resistance and hypertension.[7-9] Unfortunately, the treatment of such conditions, in addition to direct complications of preterm birth, poses as a major financial hardship for affected individuals. In the United States alone, the treatment of PTB and resulting acute and chronic disorders costs nearly 26.2 billion dollars annually.[10]

To reduce the burden of premature delivery and improve subsequent maternal and neonatal treatment a large portion of clinical research has begun to examine maternal risk factors associated with preterm delivery. As such, a few of the major discernable risk factors for preterm labor include low socioeconomic status, advanced age, tobacco use, high stress, inflammation, infection, short cervical length and race.[11-17] In 2014, the final U.S birth reports revealed a continuation of extreme preterm birth rates in the Black population; compared to Caucasians or Hispanics, Black women had a 50% greater chance of delivering prematurely.[14] While the exact genetic component responsible for the disproportionate rate of preterm birth remains elusive, one study examining genetic predispositions for PTB identified multiple single nucleotide polymorphisms in black women that augment infection and inflammatory responses, which could increase the individual's risk of preterm delivery.[18] Independent of race, women who have reduced cervical length at term, multiple fetuses, or previously undergone preterm birth are also at an increased risk of early delivery.[19]

Currently there is no cure for preterm birth, but three preventative treatments are available 1) mid-trimester progesterone, 2) cervical cerclage and 3) cervical pessary. To date, four key randomized, double-blinded, placebo-controlled clinical trials have been performed examining the effectiveness of mid-trimester vaginal progesterone treatments in delaying the onset of labor in women at high risk for delivering preterm.[20-23] In each of these studies all women included were found to be at risk of undergoing preterm birth due to 1) having a cervical length between 10-20mm, 2) previously having a spontaneous preterm birth, 3) having a uterine malformation or 4) having a twin pregnancy. Overall, vaginal progesterone significantly reduces the risk of preterm birth at less than <32 weeks of gestation (RR, 0.47; 95% CI, 0.24-0.91) and decrease composite perinatal morbidity and mortality (RR, 0.43; 95% CI, 0.20-0.94).[24] Unfortunately, mid-trimester vaginal progesterone treatment did not reduce the frequency of preterm birth in a multicenter randomized control trial in which women with a cervix of less than <30mm were included. Besides mid-trimester vaginal progesterone, cervical cerclage has also been examined as a potential treatment for reducing the risk of preterm birth.[25-29] In a recent retrograde meta-analysis reviewing five control trials, cervical cerclage significantly decreased the risk of preterm birth less than <32 weeks of gestation (RR, 0.66; 95% CI, 0.48-0.91) and lessened composite perinatal morbidity and mortality (RR, 0.64; 95% CI, 0.45-0.91) in women previously identified as being at risk of preterm birth primarily due to having a cervical length of <25mm.[24] When comparing the effectiveness between mid-trimester vaginal progesterone or cervical cerclage in women with a cervical length less than <25mm or who have previously had a spontaneous preterm birth, no differences were found.[24] The last treatment method currently being used/studied as a preventative option for preterm birth is cervical pessary. Studies examining the use of cervical pessary have given more convoluted results than either progesterone or cervical cerclage. One multicenter randomized control trial performed in Spain, cervical pessary in women between the ages 18-43, with a cervical length of 25mm or less found spontaneous delivery less than 34 weeks was significantly reduced (12 [6%] *vs* 51 [27%], odds ratio 0.18, 95% CI 0.08–0.37; p<0.0001) with cervical pessary compared to the expected management group.[30] Further, Goya and colleague found no significant differences between the effectiveness of 1) vaginal progesterone, 2) cervical cerclage or 3) cervical pessary as a management strategy for preterm birth in women with singleton pregnancies, a history of preterm birth and a sonographic short cervix.[31] On the contrary, a more recent large multicenter randomized control-trial with 932 participant only including women with a cervix length of 25mm or less did not find cervical pessary reduced the risk of spontaneous preterm delivery before 34 weeks of gestation.[32] Similarly, in a randomized control trial of 1180 women, the use of cervical pessary did not reduce the risk of preterm birth in women undergoing a twin pregnancy when compared to routine treatment.[33] Subsequently, more research is necessary accurately elucidate the effectiveness of cervical pessary.

With the number of preterm births occurring annually, it is clear there is still much work needed to increase the effectiveness of preventative treatments and additionally, the tocolytic drugs given to women who are actively undergoing premature contractions. Currently, the best tocolytic agents available are only effective in impeding the immediate processes of active labor by 24-48hrs.[34,35] The most common of these agents used include Nifedipine, a calcium channel blocker, Indomethacin, a cyclooxygenase 2 inhibitor (COX-2).[36,37] While 24-48hrs is not a long period of time it does allow for the administration of antenatal corticosteroids and magnesium sulfate to improve respiratory and neurological fetal development, thereby reducing fetal morbidities.[38,39] Unfortunately, once a woman presents with premature contractions, there is nothing that can be done to stop at that point to stop the labor from occurring. Consequently, for the continuation of therapeutic development and successful prevention of preterm birth it is imperative that we first understand the complex regulatory networks responsible for the maintenance of uterine quiescence and subsequent transition into parturition during both term and preterm birth.

The Female Reproductive System

For a complete understanding of gestational regulation, it is necessary to start by reviewing the female reproductive system including the primary female sex organ (ovary), secondary female sex organs (oviducts, uterus and vagina) and the menstrual cycle (ovarian and uterine).

Ovary

In a developed female there are two ovaries located slightly superior and bilateral to the uterus within the pelvic girdle, which are held in place via the broad ligament.[40] Each ovary can be separated into two distinct regions, the medulla and the cortex. The innermost region of the ovary is referred to as the medulla, and is comprised of loose connective tissue, spiral arteries, autonomic nerves and hilus cells. The cortex surrounding the medulla, which is made up primarily of dense connective tissue, functions to maintain primordial follicles. Primordial follicles are the most basic form of a female gamete. Following puberty, a small proportion of stored primordial follicles will undergo folliculogenesis, which will lead to the development of a mature oocyte. At the time of ovulation, an oocyte is released into the oviducts for further fertilization. Subsequent to ovulation, the corpus luteum will remain within the cortex until fertilization and/or luteolysis (degradation of the corpus luteum) occurs.[41]

Oviducts

Directly adjacent to the ovaries are bilateral tube-like structures known as the oviducts. These structures function to transport the ovum from the ovary to the uterus, as well as provide an appropriate environment for fertilization of the ovum by sperm. The luminal membrane of the oviducts consists primarily of cuboidal and columnar epithelial cilia and secretory cells, which are surrounded by multiple layers of smooth muscle.[42,43] Each oviduct can be divided into three sections: the infundibulum, the ampulla, and the isthmus. The infundibulum is the distal most part of the oviduct and is primarily comprised of finger-like structures projecting towards the ovary, known as fimbriae. During ovulation when the cumulus-oocyte complex is released from the ruptured follicle in the cortex of the ovary it is collected by the fimbriae of one of the oviducts.[44] Upon retrieval, the ovum then passes through the ampulla, located proximal to the infundibulum, and is fertilized within the ampulla/isthmus junction.[45] While smooth muscle contraction and mucosal secretions play a role in ovum transport, it is generally accepted that ciliary action is largely responsible for propelling the ovum through the oviduct.[46] Approximately 80 hours after being taken in by the oviducts, the fertilized embryo exits through the isthmus into the superior region of the uterus.[47]

Uterus

The uterus is a hollow organ made up of multiple components. Most notably, the uterine body can be separated into two functional domains: the fundus and corpus. For this review, I will not discuss tissue specific differences between the fundus and corpus regions but will instead describe the body of the uterus as a whole. The uterine body can be delineated into three specific layers of tissue: the endometrium, the myometrium, and the perimetrium. The endometrium lines the lumen of the uterine cavity, and functions to support the implantation and growth of a fetus. The endometrium, exposed to the uterine cavity, is a single layer of columnar epithelial cells. These cells are bordered by the functionalis, which is primarily composed of epithelial and stromal cells. The endometrium, along with the majority of the functionalis is shed from the uterus following the secretory phase of the menstrual cycle in a non-pregnant uterus.[48] In contrast, the basement layer of the endometrium, termed the basalis, remains largely intact throughout menstruation. The basalis is rich in stromal cells, as well as secretory glands that have been demonstrated to extend proximally into the functionalis and deep into the circular layer of the neighboring myometrium.[49,50] The myometrium is the smooth muscle layer of the uterus located between the endometrium and the perimetrium. Structurally, it is made up of two distinct muscle layers arranged in a circular and then longitudinal pattern, respective to the endometrium, to enhance the contractile potential of the tissue.[51,52] However, the myometrium remains in a quiescent state through the active suppression of contraction throughout the majority of gestation to allow for proper fetal development. It is not until the onset of labor at approximately 40 weeks gestation that the contracting myometrium must expel the fetus from the uterus.[53,54] The perimetrium, a serosal membrane collectively surrounding the myometrium, functions to support the uterus and acts as a protective barrier.[55]

Cervix

The cervix is a tightly regulated canal structure which connects the uterine cavity to the vagina.[56] In the average woman, the cervical canal is between 3-4 centimeters in length and is comprised of: the endocervix located superior to the vagina, the external and internal os junctions located where the cervix meets the vagina and uterine cavity, respectively and the exocervix located inferior to the vagina.[57] The interior portion of the cervix is comprised of the extracellular matrix and to a lesser extent a cellular component.[58] The extracellular matrix is predominantly comprised of collagens type 1 (70% and type 3 (30%), but also contains elastin fibers intertwined between the collagen.[58] The inner cellular component is made up of smooth muscle, fibroblasts, blood vessels and the epithelium, which lines the cervical canal.[58,59] Specifically, simple columnar epithelia within the endocervix and both columnar and squamous epithelia within the exocervix.[57] Similar to the endometrium, the cervical epithelia contains glands, which secrete a hormone regulated-sugar rich mucosal substance, which primarily contains water, electrolytes and mucins.[60,61] Together, the three main functions of the cervix/cervical mucosa are 1) act as a protective barrier against external microorganisms, 2) maintain intrauterine integrity during fetal development and 3) undergo cervical remodeling late in gestation to facilitate parturition.[61-63] The detailed process of cervical remodeling is discussed in the following parturition section.

Vagina

The most distal organ in the reproductive tract is the vagina, which functions as an intermediate organ connecting the uterus to the external genitalia.[64] Unlike the cervix, the epithelial lining of the vagina is a thick layer of stratified squamous cells approximately 0.15-0.2 centimeters in length. This epithelial lining can be categorized into three sections: the basalis comprised of round-shaped cells with prominent nuclei, an intermediate zone with flatter cells, and the hormone responsive superficial layer of cornified cells.[59] Encasing the vaginal epithelium is a smooth muscle layer, continuous with the uterine muscle previously discussed.

Parturition

Much of how the dynamic regulatory network functions throughout the course of gestation remains largely unknown. Subsequently, the advancements in tocolytic therapies over the past decade have been minimal. To improve upon the development of future preventative treatments for preterm delivery, it is necessary to further our understanding of the basic mechanisms that maintain uterine quiescence and those that coordinate the progression of the myometrium from a quiescence state to a fully functioning contractile unit with the onset of parturition.

Initiation of Uterine Activation

While the exact mechanisms responsible for the commencement of parturition remains undefined, it is well described that induction of parturition is demarcated by heightened uterine inflammation in both term and preterm labor.[65,66] Uterine inflammation is observed as the infiltration of both activated innate (macrophages, neutrophils, mast cells) and adaptive (B-lymphocytes and T-lymphocytes) immune cells, and increased pro-inflammatory cytokines in the amniotic fluid, myometrium, cervix, decidua and fetal membranes.[67-69] As term approaches integrated endocrine signaling and mechanical stretch of uterine myocytes induce uterine chemoattractant proteins, i.e. SP-A, CXCL8-

CXCL11, CCL2 and CCL5.[70] Chemoattractant proteins then act to propagate macrophage and neutrophil migration, as well as alter the ratio of adaptive pro-inflammatory CD4$^+$ effector T-cells to immunosuppressive naïve regulatory T cells in favor of increased inflammation.[70-75] Subsequently, both innate and adaptive inflammatory cells secrete major labor modulating cytokines such as IL-1β, interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor α (TNFα) into the surrounding uterine tissue.[76-78] Within uterine tissues, increased pro-inflammatory cytokines leads to the activation of the multifactorial transcription factor nuclear factor kappa B (NFκB) and the activating protein 1 (AP-1) family of transcription factors (*jun*, *fos* or *ATFs*).[75] Prior to stimulation, NFκB is held inactive within the cytoplasm bound to the inhibitor protein termed inhibitor kappa B alpha (IκBα).[79] With the binding of the pro-inflammatory cytokines IL-1β, IL-6 and TNFα to their appropriate receptors proteasome-mediated degradation of IκBα occurs and NFκB is able to translocate to the nucleus.[80-84] Upon nuclear translocation, NFκB increases the expression of inflammatory and contractile associated proteins (CAPs), as described in the following section Contractile Associated Proteins.[80,82,85-88] Consequently, the AP-1 family of transcription factors, which are primarily regulated at the level of transcription, are also induced upon cytokine stimulation.[89] Together, AP-1 and NFκB mediated increases in CAP expression transform the uterus into a uterotonic sensitive, synchronous contractile organ which allows for the successful expulsion of the fetus at term.[90,91]

Contractile Associated Proteins

Cyclooxygenase 2- Prostaglandin endoperoxide synthase 2 (cyclooxygenase 2, or COX-2) is an inducible contractile associated enzyme upregulated in human gestational tissues with the onset of spontaneous labor.[92,93] The biochemical role of COX-2 *in vivo* is to mediate prostaglandin synthesis.[94] Accordingly, COX-2 assists in the conversion of arachidonic acid, which has been liberated from phospholipids in the membrane, into the unstable intermediate endoperoxide.[94] Thereafter, prostaglandin specific synthases further transform endoperoxide into their respective prostaglandin products. Analysis of NFκB targets in human myometrial cells revealed IL-1-mediated binding of the NFκB subunit RelA to the promoter region of the *COX-2* gene increases mRNA levels of COX-2.[82,95] Furthermore, increasing mechanical stretch and estrogen signaling within the myometrium at term directly increases the expression of COX-2 via AP-1 mediated induction of COX-2 transcription.[91,96] Consequently, the upregulation of COX-2 leads to the increase in prostaglandin synthesis, specifically prostaglandins E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$), which act as uterotonic agents within the pregnant uterus.

Prostaglandins- PGE_2 and $PGF_{2\alpha}$ have classically been recognized as major contributors to the onset of parturition, and as previously mentioned, are both highly upregulated during the process of labor.[97-99] Through activation of G-coupled protein receptors EP_{1-4} and FP respectively, PGE_2 and $PGF_{2\alpha}$ primarily function to 1) stimulate smooth muscle contraction of the myometrium, 2) prime the cervix for delivery of the fetus and 3) initiate rupture of the fetal membranes. Prostaglandins are potent CAPs, as the application of exogenous $PGF2_{2\alpha}$ alone is enough to stimulate myometrial contractions.[100] The mechanism in which $PGF_{2\alpha}$ is capable of stimulating myometrial contractility is by directly and indirectly altering intracellular calcium concentrations.[101] With the binding of $PGF_{2\alpha}$ to its $G_{\alpha q}$-coupled protein receptor, phospholipase C (PLC) is activated. Subsequently, PLC second messenger signaling transduction through inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) stimulate ligand-regulated calcium channels within the cell membrane and calcium channels within the sarcoplasmic reticulum to increase mobilization of intracellular calcium.[102] Increased intracellular calcium concentrations decrease the electrochemical gradient across the plasma membrane which in turn activates membrane bound voltage-gated calcium channel causing further mobilization of calcium into the cell.[103] Consequently, increased calcium concentrations trigger the cycling of actin and myosin to generate smooth muscle contractions; further outlined in the upcoming section Smooth Muscle Contraction. PGE_2 binding to the EP_1 receptor also triggers intracellular calcium mobilization. However, this is through a loosely defined PLC-independent process which involves activation of G inhibitory protein.[104] Secondary to the regulation of myometrial contractility, prostaglandins also partake in cervical remodeling. As the major role of the cervix prior to parturition is to act as a mechanically competent protective barrier for the uterine cavity, extensive remodeling of the cervical tissue must occur before the delivery of the fetus through the cervical-vaginal canal. The process of cervical ripening, in which the cervix becomes soft, thin and easily stretched, is typically characterized by the reorganization and altered biochemical properties of cervical collagen.[105] One mechanism in which prostaglandins in the endocervical canal contribute to cervical ripening is by stimulating the upregulation of matrix metalloproteases (MMPs) secretion and collagenase activity.[106] Increased MMP-1 and collagenase activity leads to decreased collagen concentrations which increases the compliance of the cervical canal.[107] Similarly, prostaglandin stimulation has also been shown to increase the synthesis of hydrophilic glycosaminoglycans, which act to increase collagen solubility and thereby increase cervical pliability.[108] However, as cervical mucosal concentrations of prostaglandins do not increase during late gestation and inhibitors of prostaglandin synthesis inhibit cervical ripening, further studies are necessary to elucidate the dynamic prostaglandin-mediated regulation of cervical ripening.[109,110] In addition to cervical ripening, prostaglandins also participate in the remodeling (i.e. rupture) of fetal membranes during parturition. In both term and preterm labor, the rupture of fetal membranes *in utero* is an integral step in the expulsion of the fetus from the uterine cavity.[111,112] As in the cervix, PGE_2 and $PGF_{2\alpha}$ in fetal membranes have been shown to stimulate the production of MMPs, specifically MMP-2 and MMP-9.[113,114] As a result, increases in MMPs synthesis, concomitant to a labor associated decline in the expression of tissue inhibitors of MMPs, leads to the decreased structural integrity of fetal membranes and membrane rupture via extracellular matrix degradation.[115]

Prostaglandin Receptor Regulation- In addition to increased concentrations of COX-2 and prostaglandin E_2 and $F_{2\alpha}$, recent studies have also demonstrated augmented prostaglandin receptor expression in the uterus at the time of parturition.[80] When comparing expression of both EP and FP receptors in non-pregnant versus pregnant myometrium, there is a significant reduction in pregnant uterine tissues to suppress contractility while the fetus develops.[116] Subsequently, in the laboring versus non-laboring pregnant myometrium prostaglandin FP receptors are highly upregulated.[116,117] Further studies have since shown, increased FP receptor expression late in gestation is induced by IL-1β/NFκB-mediated transcriptional regulation.[80,118] Unlike the FP receptor, which only participates in myometrial contractility, it has been demonstrated that various EP receptors isoforms can propagate both myometrial relaxation (EP_2 and EP_4), as well as myometrial contractility (EP_1 and EP_3). Multiple studies examining gestational regulation of the EP receptor isoforms have found that the relaxation associated EP_2 receptor is high in the myometrium throughout early and mid-gestation, but significantly declines as term approaches, which is in agreement with receptor function.[119,120] Taken together, differential regulation of COX-2, prostaglandins and prostaglandin receptors during late gestation contributes to the multifactorial process of parturition indirectly by priming the uterus for optimal prostaglandin activity and directly through the initiation and propagation of myometrial contractility, cervical ripening and fetal membrane rupture.

Oxytocin- Unlike prostaglandins E_2 and $F_{2\alpha}$, the uterotonic nonapeptide hormone oxytocin is highly upregulated in the myometrium only after parturition has been initiated.[121] Subsequently, oxytocin is not responsible for stimulation of uterine contractions, but instead increases contractile force after the commencement of labor. Typically, oxytocin is synthesized in the magnocellular neurons in the supraoptic and periventricular nuclei of the hypothalamus in the form of a pro-peptide.[122] The pro-peptide is processed into the mature nonapeptide via neurophysin, while being transported via secretory vesicles down the neuronal axons, which terminate in the posterior lobe of the pituitary. Upon depolarization, oxytocin is released into circulation through exocytosis.[123] However, during pregnancy oxytocin is also locally produced in the myometrium, endometrial epithelium, corpus luteum and placenta.[124-126] At the time of parturition in particular, oxytocin mRNA is approximately 70-fold greater in the uterus than in the hypothalamus.

Oxytocin Receptor- Within target tissues, such as the myometrium, oxytocin exerts its physiological effect via ligand binding to the G-coupled rhodopsin-type class 1 oxytocin receptor (OTR).[127] Activation of the OTR at the end of gestation is thought to increase myometrial contractility through multiple mechanisms, one being the alteration of myometrial calcium dynamics. Like prostaglandins, oxytocin acts to increase intracellular Ca^{2+} concentrations through a multifactorial process, which involves the mobilization of Ca^{2+} entry, as well as inhibition of Ca^{2+} efflux from the cytosol. Currently, there is evidence to support two modalities of OTR-mediated Ca^{2+} entry: 1) through the activation of PKC and downstream signaling events which stimulate store-operated calcium entry (SOCE) channels within the plasma membrane and 2) via the release intracellular calcium stores from the sarcoplasmic reticulum to directly increase the intracellular concentrations of calcium.[128-130] Secondary to both SR Ca^{2+} release and SOCE channel dependent increases in the membrane potential, voltage operated L-type calcium channels open to further increase the influx of extracellular Ca^{2+}.[131] In addition, oxytocin/OTR activity inhibits the SR Ca^{2+} ATPase (SERCA) responsible for sequestering cytoplasmic Ca^{2+} and relocating it back into the lumen of the SR; this process again, indirectly raises cytoplasmic Ca^{2+} to concentrations which are necessary for calmodulin activation and subsequent myosin/actin cross-bridge cycling.[132] More recently, there has been evidence to suggest oxytocin receptor activity may also indirectly increases contractility through the inhibition of myosin light chain phosphatase, which is an enzyme that de-phosphorylates myosin light chain kinase (MLCK). As phosphorylation is necessary for MLCK to take part in Ca^{2+}/calmodulin mediated myosin/actin cross-bridge cyclin, an increase in its phosphorylation state leads to heightened uterine contractility.[133,134] Conclusively, oxytocin has also been demonstrated to upregulate the expression of the CAPs $PGF_{2\alpha}$, in which we have previously discussed, and connexin 43.[135-137]

Oxytocin Receptor Regulation- An additional level of oxytocin signaling regulation occurs through the augmentation of oxytocin receptor expression. Unlike increases in uterine oxytocin expression, which occurs subsequent to the initiation of parturition, it is well established that the concentration of uterine OTR mRNA is significantly increased prior to the onset of labor.[138-140] Similar to FP receptor expression, IL-1β mediated NFκB activity plays a synergistic role with CCAAT/enhancer-binding protein-β to increase OTR promoter activity and concomitantly OTR mRNA late in gestation.[86,141] Additional studies have demonstrated that estrogen also significantly stimulates OTR gene expression.[142,143] Furthermore, independent of inflammatory or estrogenic effects, increasing mechanical stretch, as experienced by the myometrium near term, was shown to increase OTR mRNA in uterine myocytes.[144] Not surprisingly, following the multifactorial increase in OTR mRNA, OTR protein expression is significantly increased within the myometrium at term compared to early gestation and post parturition.[145] The physiological consequence of augmented OTR expression at term is heightened myometrial sensitivity to oxytocin at the time of parturition and thus increased myometrial contractility.[146]

<u>Connexin 43-</u> The ultimate responsibility of the myometrium at the time of parturition is to produce contractions forceful enough to expel a fetus from the uterine cavity, through the cervical canal and out of the vagina. As the contraction of a single uterine myocyte does not produce the amount of force required for successful parturition of the fetus, the myometrium must contract as a synchronous unit. To do this, regional intercellular communication must be established which allows for the propagation of periodic contractile signaling. Similar to the majority of other cell types in the body, myocytes achieve cellular connectivity through the formation of permeable gap junction channels within the plasma membrane.[147,148] Generally, gap junction channels are formed between adjacent cells through the attachment of porous hexameric structures termed connexins, located within the plasma membrane. The formation of permeable channels between series of cells allows for chemical and electrical signals to pass freely through intracellular compartments, thereby speeding up the processes of intracellular communication.[149] In the uterus, the establishment of electrical conductance between myocytes is particularly important for the propagation of calcium signaling at the time of

parturition.[150,151] With intercellular coupling, calcium signals that originate in the fundal region are transduced throughout the body of the myometrium creating a powerful synchronous contraction that is capable of generating sufficient force to expel the fetus.[152,153] While there are numerous types of connexins present throughout the human body, connexin 43 (Cx43) is the primary gap junction protein expressed within the myometrium. During a normal pregnancy multiple factors e.g. the biological withdrawal of progesterone activity further discussed later in this chapter, myometrial stretch, estrogen, prostaglandins and AP-1/*c-jun*, stimulate the induction of Cx43 expression in the myometrium as late as 24hrs prior to start of labor.[154-158] It is known that increased Cx43 late in gestation is necessary for successful parturition, because multiple studies in mice have demonstrated that the functional loss of Cx43 prior to labor causes impaired uterine contractility and delays the onset of birth.[159,160]

Smooth Muscle Contraction

Thus far, each of the CAPs discussed contributes to myometrial contractility by altering intracellular calcium ($[Ca^{2+}]_i$) signaling and/or sensitivity in uterine myocytes. Appropriate regulation of $[Ca^{2+}]_i$ across gestation is extremely important, as the level of $[Ca^{2+}]_i$ is the primary determinant of myometrial contractile potential.[161] In the myometrium, $[Ca^{2+}]_i$ is derived from two separate compartments, the plasmalemma and the sarcoplasmic reticulum. The primary method of extracellular Ca^{2+} mobilization is through voltage-gated Ca^{2+} channels e.g. transient receptor potential channel (TrpC) 1 and TrpC6.[162] However, as previously mention, receptor-operated and store-operated Ca^{2+} channels also facilitate the internalization Ca^{2+}.[102] Within the cell, Ca^{2+} release from the sarcoplasmic reticulum is mediated through the ryanodine receptor.[163] While type 1, 2 and 3 of the ryanodine receptor exist within the myometrium, type 2 is the only receptor differentially upregulated during pregnancy.[164] Once in the intracellular space, four Ca^{2+} molecules will cooperatively bind calmodulin, which in turn activates MLCK through n-terminal binding and conformational changes in the enzyme.[165,166] Active MLCK then initiates contraction through the modulation of smooth muscle cell (SMC) contractile architecture. The canonical SMC contractile architecture consists of two primary components, the thick filament (myosin) and the thin filament (actin). The myosin filament can further be broken into three units, two heavy chains and a pair of regulatory and essential light chains (MLC_{20} and MLC_{17}, respectively).[167] The heavy chains, are comprised of a globular head domain attached to the end of long rod-like base.[168] The thin filament is comprised mainly of actin polymers that form an alpha helical coil. Upon activation, MLCK phosphorylates Ser-19 on the MLC_{20}. In the phosphorylated state, MCL_{20} significantly increases actin-dependent myosin ATPase activity which produces the energy necessary for cross-bridge formation.[169] In a study utilizing Wortmannin and ML-9, inhibitors of MLCK, diminished MLCK activity in both human and rat myometrium completely abolished uterine force, illustrating its importance during labor.[170] During cross-bridge cycling, myosin ATPase activity perpetuates continual binding and release of the myosin head to the actin filament in a specific motion that collectively shortens the longitudinal axis of a SMC to generate contractile force.[168] It is then, only through coordinated regional contractions of uterine SMCs, that sufficient force is produced and parturition commences.

Transition of the Uterus from Quiescence to Contractility

Progesterone

Thus far, we have discussed the role in which inflammation and CAPs participate in promoting myometrial contractility and the cellular mechanisms required to produce a contraction. However, as premature labor is deleterious to fetal development the myometrium must be maintained in a quiescent state for 40 weeks until the fetus has reached full term. Current evidence suggests that the steroid hormone progesterone (P_4), acting through the P_4 receptor (PR), may be the master regulator of uterine quiescence.[171] For most mammals, such as mice, rats, horses, cows and rabbits, circulating P_4 is maintained by the corpus luteum throughout the length of gestation.[172] Whereas, in humans and non-human primates, luteal P_4 synthesis by the ovary declines between 6-8 weeks due to reduced human chorionic growth factor (hCGF) stimulation, and placental trophoblast instead become the major source of P_4 production for the remainder of gestation.[173] Compared to pre-ovulatory concentrations of less than 1ng/ml, circulating P_4 concentrations are significantly higher, ranging from 11-90ng/ml during the first and second triamesters.[174-178] P_4 activity is mediated through ligand binding to the PR. The PR exists as two isoforms transcribed from the same gene, termed PR_A and PR_B.[179] *In vivo*, PR_B is the predominant transcription factor, which acts to regulate the expression of progesterone-responsive genes.[180] In contrast, PR_A is a ligand-dependent dominant negative transcription factor that functions to inhibit the genomic action of PR_B.[181] Differential expression of PR_A/PR_B in the myometrium throughout gestation is thought to play a role in the maintenance of quiescence, as well as the transition to uterine contractility.[182] Two major pieces of evidence support the role of P_4/PR as the main regulator of myometrial quiescence 1) the inhibition of P_4/PR activity in mammals at any point throughout gestation, via progesterone receptor antagonist e.g. RU486 or misoprostol, results in spontaneous abortion of the fetus and 2) the treatment of exogenous P_4 to mice late in gestation blocks the onset of labor indefinitely.[70,183] Encouragingly, as previously mentioned, the administration of vaginal P_4 to women with high risk of preterm delivery reduces the rate of spontaneous preterm birth by 45%.[23] Examination of the mechanisms in which P_4, through the PR, inhibits uterine contractility, a select set of anti-contractile processes have continued to present as important downstream consequences of P_4 activity. These processes primarily include, but may not be limited to, the inhibition of inflammation, estrogen signaling and expression of CAPs.

Inhibition of Inflammation, Estrogen Signaling and CAPs by Progesterone

Inflammation- As previously described, the induction of labor is demarcated by an upregulation of uterine inflammation largely characterized by infiltration of leukocytes and a subsequent induction of cytokines and chemokines within the myometrium.[69] Prior to labor however, the myometrium is subjected to relatively low levels of inflammation due to the inhibitory actions of P_4 and the PR. The effect of hormones, including P_4, on uterine inflammation was first characterized by examining leukocyte infiltration during a normal menstrual cycle.[184,185] These studies found that macrophage and neutrophil invasion is highest during the estrous cycle when concentrations of estrogen are elevated and the concentration of P_4 remain low. In contrast, when levels of P_4 increase throughout diestrus, and estrogen availability declines, the uterine macrophage and neutrophil population is substantially reduced. Further examination of the antagonistic properties of P_4/PR using normal and PR knockout mice validated P_4-dependent inhibition of estrogen mediated macrophage and neutrophil infiltration.[186,187] In both studies, PRKO mice did not respond to exogenous P_4 treatment, reiterating the importance of PR activity in the prevention leukocyte invasion. One mechanism by which P_4/PR action can reduce leukocyte infiltration is through direct inhibition of the expression of certain chemoattractants. During a normal pregnancy the concentration of uterine chemoattractants, such as monocyte chemoattractant protein-1 (MCP-1), do not increase until late in gestation prior to the onset of labor. In a rat, MCP-1 expression is not seen to increase until gestation day 21-22.[70] The delivery of RU486 to pregnant rats on gestation day 19 however, causes significant premature increases in both mRNA and protein levels of MCP-1, as well as substantial macrophage infiltration demonstrating that P_4 action prevents myometrial inflammation.[70] Whereas, rats given exogenous P_4 during late gestation (E21-24) maintain low concentrations of MCP-1, comparable to levels seen during early gestation where there is minimal leukocyte infiltration.[70] Secondary to P_4/PR-mediated inhibition leukocyte invasion in the myometrium, concentrations of canonical inflammatory cytokines (IL-β, TNFα and IL-6) are significantly reduced during early gestation compared to late in gestation.[188,189] Consequently, downstream inflammatory signaling events, e.g. NFκB and AP-1 activation, are depressed throughout early and mid-gestation in a P_4/PR-dependent manner.[190,191]

Estrogen- Similar to P_4, estrogens (estrone [E_1], estradiol [E_2], estriol [E_3]) are a major reproductive hormone derived from cholesterol.[192] During human pregnancy, a large concentration of circulating estrogen is synthesized in the placenta where maternal cholesterol is aromatized via fetal aromatases.[193] In all mammalian species, the level of circulating estrogens steadily increases across gestation, peaking prior to onset in parturition.[176,194,195] In humans, estrogen concentrations peak at approximately 38 weeks' gestation.[196,197] Of the three ER agonists, E_2 is the most abundant in circulation during pregnancy.[196] The genomic effects of estrogen are mediated through ligand binding to the nuclear receptors ERα and ERβ.[192] However, characterization studies of ER isoform expression within myometrium have demonstrated that ERα is the dominant receptor.[182] While the expression of myometrial ERβ remains relatively low and unchanged across gestation, there is a sharp increase in ERα expression late in gestation.[182] Consequently, increased levels of ERα leads to increased estrogen responsiveness within uterine tissues. Functionally increased estrogen responsiveness near term is important because, as previously mentioned; estrogen activity contributes to uterine activation through the up-regulation of AP-1 transcriptional activity, as well as COX-2, OTR and Cx43 expression.[96,158,198-200] P_4 inhibits estrogen activity in the uterus, with the purpose of preventing E_2-mediated premature myometrial contractility.[201] Initial studies identified differential ER expression as a mechanism for decreased estrogen responsiveness; the dynamic regulation of P_4 and estrogen signaling within the uterus was not clearly defined until recently. Mesiano and colleagues demonstrated for the first time in 2002, that the ratio of PR_B/PR_A expression was important for the regulation of ERα expression in-utero.[182] Consequently, increased expression of PR_B/PR_A is inversely correlated to the expression of ERα. It is thus extrapolated that similarly to inflammation induced proteins, P_4 through PR_B inhibits ERα expression and subsequently minimizes myometrial estrogen responsiveness and prevents estrogen mediated uterine activation.

Nuclear Factor Kappa B- In addition to the downregulation of upstream inflammatory and estrogen signaling, P_4 acting through the PR is able to directly disrupt myometrial NFκB activity.[191] While multiple mechanisms contribute to P_4/PR mediated NFκB inhibition the most conventional method is binding of the PR to the RelA subunit (p65).[202] In doing so, PR physically inhibits the DNA binding domain, located on p65, from attaching to the promoter region of target genes and altering transcription. Interestingly, *in vitro* evidence suggests PR binds p65 in a ligand-independent, as well as ligand-dependent manner. Both mechanisms however, require an intact DNA binding domain on the PR for successful antagonism.[202] Secondary to direct inhibition, P_4/PR also regulates inflammatory signaling by reducing NFκB nuclear translocation.[190] In order for nuclear translocation to occur the inhibitor protein, IκBα, must be phosphorylated and degraded by the proteasome to expose the nuclear localization sequence (NLS) on the p65 subunit.[203] To prevent exposure of the NLS, PR-mediated transcriptional regulation of the IκBα gene leads to increased intracellular concentrations of both IκBα mRNA and protein.[190] P_4/PR activity has also been shown to inhibit IL-β dependent decreases in IκBα protein concentrations, suggesting PR action may prevent IκBα degradation.[190] Subsequently, increased transcription and decreased degradation leads to an upregulation of active IκBα and the reduction of NFκB nuclear translocation. A third, less defined, method of NFκB inhibition has been proposed in which downstream events of P_4/PR-mediated activation of MAPK-phosphatase-1 (MKP-1) lead to inhibition of NFκB nuclear translocation.[171] Using T47D human breast cancer cells, Chen and colleagues demonstrated PR binding to P_4 response elements downstream of the MKP-1 transcription start site induces MKP-1 expression.[204] An additional study, examining the effects of MKP-1 on NFκB nuclear translocation in prostate tissue, discovered MKP-1 expression inversely correlates with nuclear translocation of NFκB.[205] These findings suggest MKP-1-mediated inhibition of NFκB is due to decreased p38 MAPK expression, which is known to activate NFκB.[206] Together, these P_4/PR regulated mechanisms work to depress nuclear NFκB activity and therefore reduce overall inflammatory signaling.

Activating Protein 1- Like NFκB, the AP-1 family of transcription factors must be gestationally regulated to prevent premature induction of labor. However, the regulatory actions of P_4/PR on AP-1 transcription factors (*fos*, *jun* and *ATFs*) within the uterus remains largely undefined compared to the regulation of NFκB. Never the less, it has been demonstrated within the myometrium specifically, the pretreatment of P_4 attenuates stretch induced activation of *c-fos* and *fosB*.[207] Beyond inhibiting AP-1 protein expression, PR activity also decreases AP-1 DNA binding and therefore transcriptional activity. In the endothelial cancer cell line Hec50, chromatin immunoprecipitation findings demonstrate reduced AP-1 binding at the promoter of its target gene cyclin D1 in the presence of P_4.[208] Based on these studies, it is reasonable to propose P_4/PR activity further attenuates uterine inflammation by augmenting AP-1 signaling.

<u>Contractile Associated Proteins-</u> AP-1 and NFκB signaling increase expression of CAPs e.g. COX-2, prostaglandins, OTR and Cxn43, which function to prime the myometrium for labor and initiate uterine contractions. However, the expression of CAPs is also tightly controlled via P_4/PR action during early and throughout mid-gestation to prevent the uterus from establishing premature contractility. The main mechanism in which P_4 represses the expression of CAPs, such as COX-2, OTR and Cxn43, is by inhibiting AP-1 and NFκB transcriptional activity.[190,209,210] Subsequently, the direct inhibition of inflammatory signaling pathways indirectly prevents uterine activation and therefore facilitates the maintenance of quiescence. Interestingly, certain P_4 responsive microRNAs (miRNAs) and downstream targets have also recently been shown to regulate the expression of certain CAPs.[171] More specifically, Renthal and colleagues demonstrated the zinc finger E-box binding homeobox transcriptional repressor proteins (ZEB)-1 and ZEB-2 directly inhibit transcription of the contractile associated genes encoded for OTR and Cx43.[211] Moreover, ZEB-1 and ZEB-2 were later found to upregulate the microRNA cluster miR-199a and miR-214, during early and mid-gestation in a P_4 dependent manner.[212] Functionally, active miR-199a and miR-214 target and inhibit transcription of the COX-2 gene. In the absence of uterine COX-2, OTR and Cx43, prostaglandin synthesis is limited, calcium signaling is disrupted and the contractile

potential of the myometrium is insufficient to induce labor.[213]

Luteolysis and Progesterone Decline in Lower Mammalian Species

Just as it is important that the uterus remains quiescent during pregnancy, it is also imperative the uterus undergoes appropriate transformation prior to onset of labor to allow for successful deliveries of the term neonate. In most lower mammal species, such as rabbits, rats and mice, prostaglandin-mediated regression of the corpus luteum near term induces a sharp decline in circulating P_4.[214] As term approaches heightened estrogen action in the uterus, increases the concentration of oxytocin receptors within the endometrium.[210] Together, endometrial estrogen and oxytocin activity stimulate the production of prostaglandins (e.g. PGE2 and PGF2α) by regulating the enzymes necessary for prostaglandin synthesis, particularly phospholipase A2 (PLA2) and prostaglandin synthase.[215-217] PLA2 exists in three forms: calcium sensitive cytosolic (cPLA2), secretory (sPLA2) and calcium independent PLA2, which all function to enzymatically convert membrane bound arachidonic acid into free arachidonic acid.[218] In the context of prostaglandin synthesis, free arachidonic acid is important as it is the initial substrate for prostaglandin endoperoxide synthase-dependent synthesis of PGG2, which is then converted into PGH2 in a COX1/2-dependent reaction.[219] Subsequently, as COX expression is regulated by NFκB activation as previously mentioned, the classical increase in inflammation in the uterus as term approaches further augments prostaglandin synthesis.[82] PGH2 is then converted by tissue specific prostaglandin synthase into multiple prostaglandins, including PGF2α and PGE2.[219] Following synthesis within the endometrium, PGF2α leaves through the uterine vein and enters the ovarian artery to act through its receptor at the level of the corpus luteum.[220] Within the corpus luteum, PGF2α acts to reduce the enzymes necessary for the P4, cytochrome

P450 side-chain cleavage enzyme and 3-beta-hydroxysteroid-dehydrogenase (HSD3B2).[221] As circulating P4 acts in an autocrine manner to preserve the corpus luteum, PGF2α-dependent decreases in P_4 cause the subsequent regression of the corpus luteum and further decreases in circulating P_4 necessary for the induction of labor in lower mammalians.[222]

Functional Progesterone Withdrawal

In higher mammal species e.g. chicken, sheep and baboons, a developmental-dependent increase in fetal corticotrophin-releasing hormone results in elevated levels of adrenocorticotropic hormone and thus increased circulating cortisol.[223,224] Heightened cortisol, augments steroidogenesis in the placenta promoting the synthesis of estrogen from C21 steroids e.g. P_4.[225] For many years it was accepted that the withdrawal of circulating P_4 increased inflammatory signaling pathways, E_2/ERα activity and expression of CAPs to allow for the cessation of labor.[182,195,226] However, it has since been established that although the levels of P_4 in the circulation are significantly reduced, they still remain above the K_d for binding to the PR, meaning the levels of P_4 are sufficient to bind PR and induce P_4/PR downstream signaling.[227] Furthermore, in humans and primate species, circulating levels of P_4 continue to rise until parturition has commenced and the placenta is expelled.[177] Subsequently, despite having circulating concentrations of approximately ≤300ng/ml P_4 during the third trimester, the majority of women mount an appropriate contractile response within the myometrium and undergo active labor around 40 weeks' gestation.[178] Taken together, these observations suggested the existence of a secondary mechanism for diminished P_4 activity at term. Thus, in 1965 Csapo theorized a mechanism of functional withdrawal of P_4 within the uterine tissues, which then would allow for the progression of labor.[228] In decades since, multiple regulatory mechanisms have been shown to contribute to the functional withdrawal of P_4 as seen in the uterus prior to the onset of labor independent of circulating levels of P_4.

Progesterone Availability- While in circulation, P_4 can be found in two major forms 1) unbound or free and 2) bound to the corticosteroid-binding globulin transcortin.[229] Characterization of plasma samples taken from pregnant women across gestation revealed the binding capacity of transcortin for P_4 increases linearly between 10 and 20 weeks' gestation.[230] Furthermore, increased transcortin binding capacity was found to be estrogen dependent. These results suggest estrogen-mediated increases in the sequestration of active P_4 by transcortin over time may contribute to a reduction in the bioavailability of P_4. Additional studies have also established a secondary mechanism in which is P_4 availability is diminished through the regional upregulation of P_4 metabolism. Initially, increases in the P_4 metabolite 20α-dihydroxyprogesterone were observed in human myometrial tissue late in gestation.[231] These results can be explained by additional studies examining local P_4 metabolism, which established an upregulation of myometrial P_4 metabolizing enzyme 20α-hydroxysteroid-dehydrogenase (20 α-HSD).[232-234] Importantly, mice deficient in 20 α-HSD experience delayed parturition, delivering the fetuses several days late. Recently, novel clusters of miRNAs have been identified that further regulate P_4 metabolism. In particular the miR-200 family of miRNAs, under the control of E_2/ERα mediated signaling processes, act to oppose P_4/PR_B function.[211,233] Microarray analysis of miRNA and gene expression of uterine tissues revealed concentrations of miR-200 family members to be abundant at term.[233] Analysis in mice models of term and preterm labor validated these findings and further demonstrated an increase in the expression of miR-200s late in gestation, concomitant to a downregulation in the P_4 responsive anti-contractile transcriptional repressors ZEB1, ZEB2, miR199a and miR214. Importantly, the upregulation of miR-200s facilitated the inhibition of signal transducer and activator of transcription (STAT5b), which had previously been shown to repress the expression of 20α-HSD in reproductive tissues.[235] Taken together, the increased metabolism of myometrial P_4 and decreased availability of circulating P_4 limits the bioactivity of P_4 and thus its anti-contractile properties.

Regulation of Progesterone Receptors- In addition to the direct inhibition of P_4 activity through decreased regional P_4 concentrations, indirect inhibition occurs via modulation of downstream P_4 signaling pathways. One mechanism in which myometrial P_4 signaling is regulated is by differential PR isoform expression.[236] As previously mentioned, the P_4 receptor exists as two functionally distinct isoforms PR_A, the dominant negative receptor, and PR_B, the transcriptionally active form.[179] In term laboring tissues, the ratio of PR_A/ PR_B mRNA and protein levels are 2-fold greater than in non-laboring term tissues.[182,236] Furthermore, across gestation the ratio of PR_A/PR_B mRNA positively correlates with the expression of ERα suggesting that differential PR isoform expression both diminishes P_4 responsiveness and heightens estrogen responsiveness, leading to increased myometrial contractility at term. Another mechanism in which P_4/PR signaling modulated at term is through differential expression of PR co-activators. Specifically, mRNA and protein expression of cAMP-response element-binding protein and steroid receptor co-activators 2 in the uterus are reduced during labor.[237] As nuclear receptor co-activators typically increase PR transcriptional activity through the stabilization of the pre-initiation complex, a decrease in PR co-activators inherently reduces the transcription of P_4-responsive genes.[238] Thus, the differential expression of P_4 co-activators at term further hampers the transcriptional activity of P_4/PR_B indirectly increasing myometrial contractility.

Endoplasmic Reticulum

The endoplasmic reticulum (ER) is an intracellular reticular membrane structure that is contiguous with the nuclear envelope. The ER can be defined by two functionally distinct sub-domains, the rough ER and the smooth ER.[56] Structurally, there is no definitive separation between the two compartments.[239] However, the rough ER is demarcated by an increased number of ribosomes embedded within the cytosolic portion of the membrane, which is lacking in the smooth ER. Functionally, the smooth ER is primarily responsible for lipid synthesis and drug metabolism, while the rough ER is associated with managing intracellular calcium stores, regulating the synthesis and folding of secretory and membrane bound proteins, and coordinating protein trafficking to the adjacent Golgi apparatus.[240-244]

Endoplasmic Reticular Milieu

To maximize the process of protein folding, the ER compartment maintains a distinct luminal milieu.[245] For example, the ER lumen maintains a greater oxidative state than the cytosol.[246] While the major intracellular redox buffer for both compartments is glutathione, the ratio of reduced to oxidized glutathione is between 1:1 and 3:1 in the ER lumen, whereas the ratio is greater than 50:1 in the cytosol.[247] This unique oxidative environment is optimal for protein disulfide isomerase (PDI) mediated disulfide bond formation in the ER, which is necessary for proper protein folding.[247] In addition to altered redox state, Ca^{2+} concentrations are augmented in the ER lumen. As the major site of intracellular storage, ER Ca^{2+} concentrations are 50 times that of the cytosol, i.e. 5mM versus 0.1mM respectively.[248] Increased Ca^{2+} availability in the ER is advantageous as Ca^{2+} participates in electrostatic interactions with newly synthesized proteins in a manner that further propagates appropriate hydrophobic interactions essential for protein maturation.[249] Additionally, Ca^{2+} binding is required for chaperone protein function.[250,251] Chaperone proteins by definition aid in proper protein folding during the process of protein maturation.[252] Compared to all other compartments, the ER lumen is equipped with a heightened quantity of chaperone folding proteins that refine protein-folding processes. These chaperones include glucose regulated protein 78 (GRP78), calreticulin, calnexin and PDI.[253-256] Another important factor involved in chaperone protein function is adenosine triphosphate (ATP).[257] To maintain energy demands the ER actively translocates ATP through multiple antiporters located in the ER membrane.[258] In addition to participation in chaperone function, ATP further assists in disulfide bond formation and protein glycosylation.[257,259] Taken together, the distinct chemical and protein composition of the ER lumen enhances the processes of protein folding to allow for more dynamic and complex protein structures to be synthesized.

Co-Translational Translocation, Folding and Protein Trafficking

It is important that secretory and membrane bound proteins are synthesized within the tightly controlled environment of the ER lumen, rather than the cytoplasm. In general, these proteins consist of both hydrophobic/transmembrane and hydrophilic/cytoplasmic domains, which require precisely, coordinated post-translational modification, e.g. disulfide bond formation, hydrophobic interactions and glycosylation, for successful maturation.[260,261] In addition, the ER secretory pathway, which is responsible for trafficking proteins to the cytoplasmic membrane, is necessary to ensure secretory and membrane bound proteins reach their appropriate destination following completion of translation.[262] However, as messenger RNA is secreted into the cytosol following transcription, it must first be targeted to the ER membrane via a signal recognition sequence located within the nascent peptide.[263] When the signal recognition sequence is initially translated by the ribosomal complex, it is immediately identified by free cytosolic signal recognition particles (SRPs).[264] Binding of the SRP to the nascent peptide-ribosomal complex within the cytoplasm has two effects 1) it temporarily inhibits protein translation and 2) it shuttles and attaches the nascent peptide-ribosomal complex to a protein-conducting channel within the ER membrane.[265] These protein-conducting channels, termed translocons, are aqueous pores that span the entire length of the ER membrane.[266] After binding to the translocon, elongation of the nascent peptide is re-initiated and co-translational translocation of the protein into the ER lumen occurs.[267,268] With the help of chaperone proteins the N-terminus of the nascent peptide begins to undergo post-translational modifications, such as protein folding, glycosylation, disulfide bond formation, etc., immediately after entering into the ER lumen.[267,269] Upon completing translation, proteins 1) further undergo oligomer formation, if necessary 2) are recognized by cargo receptors and 3) are sorted via surveillance protein complexes composed of a small ras-related GTPase (Sar1p) and two Sec proteins (Sec23p-Sec24p) or membrane adaptor protein complexes located within in the ER transition zone.[270,271] Clathrin or coat protein complex II vesicles packaging cargo proteins are then trafficked out of the ER lumen to the cis-Golgi network.[272] In the event of inappropriate maturation however, proteins are inhibited from being trafficked to the cis-Golgi network, and instead are retained within the ER lumen by a series of quality control processes.

Protein Quality Control

To prevent cellular dysfunction, abnormal protein products e.g. proteins with point mutations, deletions, insertions or intermediate glycosylation states, are withheld from cis-Golgi apparatus trafficking.[273,274] While some proteins are recycled through the protein folding process and eventually become folded properly, others become are unable to reach their appropriate conformational state. In the case of soluble proteins, terminally misfolded proteins collect as misfolded aggregates within the ER.[275,276] These protein aggregates are cross-linked by inter-chain disulfide bonds, and are irreversibly bound by the chaperone folding protein BiP.[276-278] Evidence suggests, binding of aggregates to GRP78 is necessary for maintaining proteins in a retrotranslocation competent state and recognition of unfolded proteins by ER associated degradation (ERAD) proteins.[279,280] In the instance of misfolded glycoproteins, targeting and recognition of ERAD substrates is instead mediated by cleavage of α1,2-linked mannose via α1,2 exomannosidase.[281,282] Targeted ERAD substrates are then retrotranslocated into the cytosol, via the Sec61 translocon complex.[283,284] Specifically, the ubiquitin-conjugating enzyme Ubc7p ubiquitinates ERAD substrates, which are then targeted for cytosolic proteosomal degradation via the 26S proteasome.[285] Typically, compensatory-targeted protein degradation is sufficient to prevent cytotoxic protein aggregation and cellular distress. Though, in the event of increased protein synthesis, dysregulation of calcium, etc. ERAD may not be adequate to relieve ER stress and the ER stress response (ERSR), also known as the unfolded protein response (UPR) is activated to restore ER homeostasis and avoid cell death.

Activation of the Unfolded Protein Response

The ERSR is comprised of three distinct molecular networks that function harmoniously to deplete unfolded proteins from the ER lumen to regain homeostasis. With the accumulation of unfolded proteins, the chaperone protein GRP78 is released from three transmembrane receptors, inositol-requiring kinase 1 alpha (IRE1α), protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6), to aid in proper folding. Upon release of GRP78, IRE1α, PERK, and ATF6 are activated; initiating signal transduction pathways collectively termed the UPR.

Glucose Regulated Protein 78

It has been well established that GRP78 dissociates from IRE1α, PERK and ATF6 in the presence of unfolded proteins.[286] The active form of GRP78, a monomeric structure, contains a C-terminal peptide binding domain and an N-terminal ATPase.[287] The C-terminal domain is capable of binding a variety of synthetic peptide sequences that exhibit vast sequence diversity.[288] However, GRP78 does show preferential interaction for hydrophobic nascent peptide sequences that activate the N-terminal ATPase upon binding.[289,290] As a chaperone folding protein, GRP78 does not contribute to the enzymatic action necessary for the folding of protein substrates; it instead assists by binding to exposed intramolecular hydrophobic regions on unfolded proteins, subsequently maintaining the substrate in a folding-competent state.[252] Upon substrate binding, the bound N-terminal ATP is hydrolyzed to ADP and substrate affinity is significantly increased.[290] In order for GRP78 to then release its substrate, the co-chaperone glucose related protein E must catalyze an ADP/ATP exchange to return GRP78 to its low affinity ATP-bound confirmation.[291] The shuttling of unfolded proteins through the ATP/ADP GRP78 cycle and folding pathways is the major mechanism in which GRP78 assists in the depletion of unfolded proteins within the ER. It is also capable of transporting permanently unfolded proteins to the translocon for retrograde translocation and further ubiquitin/proteasomal mediated degradation.[280] By facilitating activation of ER stress signaling transducers, and assisting in protein folding and protein degradation GRP78 acts as a multifaceted protein to aid in the restoration of ER luminal homeostasis in the event of stress.

Inositol Required Kinase 1α

IRE1α is an ER stress sensitive, kinase/endoribonuclease type I transmembrane glycoprotein receptor, which contains functional luminal and cytoplasmic domains. During times of ER homeostasis, IRE1α is locked in a monomeric inactive state through N-terminal luminal binding of GRP78.[286] Upon the accumulation of unfolded proteins and subsequent dissociation of GRP78, luminal domains of IRE1α form disulfide-linked heterodimers.[292] While this suggests relief of GRP78 mediated inhibition of a luminal dimerization motif may be responsible for IRE1α dimerization, additional studies have demonstrated dimerization of IRE1α through direct binding of unfolded proteins to the luminal domain.[292,293] Therefore, further studies are necessary to elucidate the exact mechanism of IRE1α dimerization. Following heterodimerization, oligomerization-dependent conformational changes in the cytosolic tyrosine kinase domain induce *trans*-autophosphorylation of IRE1α.[294,295] Unlike traditional kinase signaling cascades, phosphorylation of IRE1α leads to the activation of its own endoribonuclease activity.[296,297] Currently, the only known substrate of IRE1α RNase is mRNA encoding the transcription factor X-box binding protein 1 (XBP1) in humans or homologous to ATF/CREB1 (Hac1) in yeast.[298,299] The alternative splicing of XBP1 mRNA results in the excision of an intronic region and leads to a frame shift during translation. In contrast to the naïve splice product, which represses UPR target genes, the alternative splice product is subsequently more stable and a strong activator of UPR gene targets.[300] XBP1 was originally discovered as a bZIP protein that bound the major histocompatibility complex two gene promoter in the cis-acting X-box region.[301] More recently it has been demonstrated XBP1 is also capable of binding the endoplasmic reticulum stress response element (ERSE) in the presence of nuclear factor Y (NF-Y), the unfolded protein response element (UPRE) and the endoplasmic reticulum stress response element II (ERSE II).[298,302] XBP1 binding to ERSE, in conjunction with ATF6 and NF-Y, enhances the transcription of ER-localized molecular chaperone proteins e.g. GRP78 which act to reduce the accumulation of unfolded proteins and restore ER homeostasis.[303] Similarly, XBP1 binding to the UPRE induces the transcription of ER degradation-enhancing alpha-mannosidase-like protein (EDEM), an important mediator of the ERAD processes.[304] Furthermore, XPB1-ERSE II binding was recently shown to increase the expression of the homo-cysteine-induced endoplasmic reticulum protein (Herp), an ubiquitin-domain containing protein that also participates in ERAD.[302,305] Upon diminished ER stress, IRE1α endoribonuclease activity is repressed, there is decline in XBP1 alternative splicing and increased un-spliced XBP1 acts as a negative feedback regulator of sXBP1.[306] Therefore, UPR-dependent transcription levels are reestablished to a basal state upon restoration of ER homeostasis.

Activating Transcription Factor 6

ATF6 is a type II transmembrane protein that acts separately from IRE1α as a signal transducer of the ERSR. Similarly, in a state of ER homeostasis, the luminal domain of ATF6 is bound by GRP78.[307] Unlike IRE1α and PERK, the dissociation of GRP78 does not induce oligomerization, but instead unmasks a Golgi localization sequence in the luminal domain.[307] Exposure of the Golgi localization sequence to the ER lumen subsequently induces translocation of ATF6 to the Golgi complex. Within the Golgi complex, ATF6 is further processed from a 90kD protein to an active 50kD bZIP transcription factor through the cleavage of the N-terminal domain by site-1 and site-2 proteases (S1P and S2P, respectively).[308] Following processing, ATF6 is translocated to the nucleus where it augments the expression of multiple UPR regulated genes. Similar to XBP1, ATF6 is capable of binding three response elements 1) the ATF/cAMP response element (CRE) 2) the ERSE I and 3) the ERSEII.[298,302,309] In concert with NF-Y, the binding of ATF6 to ERSE I and ERSE II acts to upregulate ERAD gene products, as well as increase the expression of chaperone folding proteins, particularly GRP78.[302,310,311] Furthermore, ATF6, in collaboration with XBP1, also binds the XBP1 promoter to increase expression of XBP1.[298] Therefore, through both indirect and direct mechanisms of action, ATF6 reduces the unfolded protein load within the ER lumen in the event of ER stress.

Protein Kinase RNA-like ER Kinase

Protein kinase RNA-like ER kinase is a type I transmembrane receptor that constitutes the last of three unfolded protein response mediated signal transducers. The luminal portion of the receptor contains an ER stress-regulated oligomerization domain.[312] As previously mentioned, the exact mechanism of PERK receptor oligomerization is yet to be determined. However, with the release of GRP78 from PERK an oligomerization domain is exposed and the receptor forms an oligomer structure.[313] Oligomerization of the receptor induces trans-autophophorylation of the cytoplasmic tyrosine kinase domain. Phosphorylation dependent activation of the cytoplasmic kinase then leads to further phosphorylation of the α-subunit of eukaryotic translation initiation factor-2 (eIF2α) and nuclear factor erythroid 2-related factor 2 (Nrf2).[314] Subsequently, the phosphorylation of eIF2α functionally inhibits GTP/GDP cycling, which significantly reduces the rate of global cellular translation. In return, the load of newly synthesized proteins in the ER is concomitantly decreased, helping to relieve the accumulation of unfolded proteins. Not surprisingly, the whole cell knockout of PERK increases cellular sensitivity to ER stress. This phenotype was then partially relieved with the addition of protein synthesis inhibitors such as cycloheximide, reiterating the importance of PERK-mediated translational inhibition in the event of ER stress.[315] The inhibition of eIF2α provides the opportunity for active translation of mRNAs that contain short upstream open reading frames (uORFs).[316] In particular, ER stress mediated translational repression enhances the expression of activating transcription factor 4 (ATF4).[317] Within two hours of being exposed to stress, the upregulation of ATF4 can further induce an increase in the expression of the basic-region leucine zipper (bZIP) protein activating transcription factor 3 (ATF3). When the cell is unable to restore ER homeostasis due to prolonged or severe ER stress, ATF3 can activate the apoptosis-inducing protein GADD153, which has been demonstrated to trigger cell death through caspase 3 (CASP3) mediated apoptosis.[318]

Extracellular Functions of the Unfolded Protein Response

Beyond the ER, components of the UPR, such as GRP78, are expressed on the extracellular surface of many cell types and in the extracellular space in addition to the ER.[319,320] GRP78 has also been found in the serum, synovial fluid, saliva and oviductal fluid.[320-323] The mechanism whereby components of the UPR signaling cascade traffics from the ER to the cell surface and the extracellular space is not fully resolved. It has been confirmed however that extracellular GRP78, does not arise from apoptotic cell death mediated protein leakage during ER stress, as GRP78 release into the extracellular space precedes any evidence of apoptotic cell death.[324] Moreover, in cells exposed to Brefeldin A, which inhibits ER to Golgi protein transport, extracellular levels of GRP78 were significantly decreased while intracellularly they continued to increase, suggesting that extracellular GRP78 is actively trafficked.[324] In addition, it has been observed *in vitro* that the relative amount of GRP78 versus non-secreted proteins, is increased within the media when compared to whole cell lysates.[324,325] More recently the extracellular trafficking of UPR proteins was confirmed by the finding that prostate apoptosis response-

4 (Par4) was identified as a partner for GRP78 that was necessary for its translocation to the cell surface during periods of ER stress in normal and cancer cells, underscoring the regulated nature of GRP78 translocation to the cell surface.[326] In this study TM-treated telomerase immortalized human myometrial cells (hTERT-HM) were all found to be viable at the time of media collection, adding to the observation that GRP78 is actively secreted due to activation of the UPR and not leaked out of the cell in an apoptotic manner (Figure 15). However, as the GRP78 amino acid sequence contains the classical ER retention signal (KDEL) in its C-terminal it is not entirely resolved how GRP78 is actively secreted. The KDEL sequence should dictate that GRP78 is a lumen bound, ER resident protein, which cannot traffic to the surface of cell or be secreted extracellularly. It is thought that an oversaturation of the specific KDEL receptors in the ER during periods of ER stress, may allow for GRP78 to escape the KDEL retention system and accumulate in the plasma membrane and the extracellular space.[325,327-329] The severity of ER stress needed to facilitate GRP78 secretion into the extracellular space however, remains ambiguous and further studies are needed to characterize cell-type and stimulus specific thresholds. Extracellular GRP78 has been demonstrated to play a critical role in the transmission of biochemical stress signals from one cell to another. Specifically, this form of paracrine and potentially endocrine signaling allows for amplification and expansion of a local tissue response to a systemic alarm or danger signal. GRP78 mediated transmission of ER stress has been observed when conditioned media isolated from stressed tumor cells was exposed to naïve macrophages and resulted in UPR induction in the naïve cells.[330] Cell free GRP78 has also been demonstrated to confer an anti-inflammatory pro-survival phenotype by binding to target cell surface receptors such as Cripto-1, which allows for the attenuation of transforming growth factor beta tumor suppressor functions.[331] Further,

extracellular GRP78 has been demonstrated to block p53 action and thereby inhibit its pro-apoptotic targets, BOK and NOXA.[319] Taken together these data suggest that activation of the UPR intracellularly and secretion of the UPR into the extracellular space has the capacity to confer an anti-inflammatory, pro-survival phenotype.

CASP3-Dependent Apoptosis

As previously mentioned, if the UPR is unable to restore cellular homeostasis PERK/ATF4/ATF3-dependent activation of GADD153 induces CASP3-mediated apoptosis. Overall, the activation of CASP3 is the final step in three cellular signaling pathways that result in apoptotic cell death: the extrinsic pathway, the intrinsic pathway or the UPR.[332,333] In the extrinsic pathway, ligand-induced activation of a well characterized subset of death receptors, such as tumor necrosis factor receptor 1, fatty acid synthetase receptor or death receptor 3 leads to the formation of the death-inducing signaling complex, the autocatalytic activation of initiator caspase 8 and subsequent activation of CASP3.[334] In the intrinsic pathway, damaging cellular stimuli cause dysregulation of mitochondrial homeostasis that results in the opening of the mitochondrial permeability transition pore.[335] Increased mitochondrial membrane permeability facilitates the construction a multi-protein complex called the apoptosome, containing the active initiator caspase 9, which proceeds to activate CASP3.[336] As previously mentioned, ERSR-mediated activation of CASP3 occurs through the signaling cascade of ATF4, ATF3 and GADD153 resulting in the activation of initiator caspase 12, which activates the terminal caspase, CASP3.[337] Following activation, CASP3 acts in a multi-factorial manner to induce cellular apoptosis. Specifically, within the nucleus CASP3 will 1) degrade inhibitor of caspase activated DNAse (ICAD) resulting in chromosomal degradation and chromatin condensation via active DNAse, 2) further target and degrade

DNA repair molecules, such as PARP and 3) disrupt cytoskeleton organization and intra-nuclear transport through the degradation of gelsolin, an actin binding protein necessary for actin polymerization.[338-340] We know cellular apoptosis is extremely important process in the maintenance of cellular homeostasis and many normal physiological functions, as the CASP3 knockout in mice reduces the rate of live births and results in premature death of the live pups.[341-343] Subsequently, CASP3 apoptotic action has been found to be critical for the development of the nervous and immune system; wound healing and overall remodeling in adult tissues.[344-346] In contrast, the dysregulation of normal apoptotic processes can lead to various pathophysiological states. In cancer a resistance to apoptosis, decreased cell death, cell cycle dysfunction and abnormal proliferation leads to the formation of a tumor.[347] Additionally, CASP3-mediated apoptosis in tumors undergoing radiation has been demonstrated to increase growth and proliferation in the surviving tumor cell population further promoting tumorigenesis.[348] In other disease like autoimmune deficiency syndrome, increased apoptosis of the T-cell population causes extreme immunodeficiency and in some cases death.[349]

Non-Apoptotic CASP3 Function

While CASP3 activity is typically a hallmark of cellular apoptosis or programmed cell death, non-apoptotic CASP3 function has been described in many physiological processes such as muscle tocolysis, cellular differentiation and synaptic plasticity.[350-352] As a protease with over 34,000 identified protein targets containing a cleavage motif (DXXD) within the human genome, it is not entirely surprising that emerging evidence supports critical non-apoptotic functions of CASP3.[353] Our laboratory, as well as others, have previously demonstrated the capacity of non-apoptotic CASP3 to selectively target and degrade actin isoforms (e.g. α and γ).[354] In the context of myometrial smooth muscle, selective actin degradation inhibits contraction from occurring. Further, in catabolic conditions such as diabetes, skeletal muscle CASP3 activity has been observed to degrade actomycin and actin, resulting in muscle atrophy.[355] Beyond regulating contraction and muscle proteolysis, non-apoptotic CASP3 activity is also involved in skeletal muscle differentiation.[356] In the CASP3 knockout mouse, myoblasts displayed deficiency of myofiber and myotube formation, presumably due to loss of Mammalian Sterile Twenty-like kinase activity.[357] Non-apoptotic and incomplete apoptotic CASP3 action has similarly been shown to facilitate differentiation lens epithelial cells, monocytes, erythrocytes, keratinocyte, megakaryocytes and potentially neurons.[358-363] Interestingly, increased non-apoptotic CASP3 expression in hippocampal CA1 neurons is also thought to facilitate neuroplasticity through propagation of long-term potentiation.[352] Multiple neuronal CASP3 targets are thought to aid in CASP3-dependent neuroplasticity (e.g. GluR1, IP3R and PKC), but the exact mechanism responsible for long-term potentiation is still unresolved.[353] Taken together, these studies and more suggest CASP3, acting in both an apoptotic and non-apoptotic manner, is important for both normal and pathophysiological conditions and warrants further inquiry.

The UPR and Inflammation

Inflammation and the ERSR are two distinct signaling cascades that are highly interconnected during periods of cellular distress through various mechanisms. Briefly, activation and nuclear translocation of the inflammatory transcription factor NFκB has been achieved through both the IRE1α and PERK signaling pathways.[364] With the autophosphorylation of IRE1α, a cytosolic conformational change of the receptor leads to the recruitment of tumor-necrosis factor-α receptor-associated factor 2 and subsequent activation of IκB kinase resulting in degradation of IκB and nuclear translocation of

NFκB.[365] Separately, as the half-life of IκB is significantly shorter than that of NFκB, PERK-dependent phosphorylation of eIF2α and the inhibition of protein translation have been shown to free NFκB from IκB-dependent cytosolic localization and thus allow for nuclear translocation.[366] Lastly, within the mouse liver it has been demonstrated that ER stress initiates the translocation of CREBH from the ER to the Golgi, where it is processed and activated in a similar manner to ATF6.[367] Typically, CREBH is induced by inflammatory cytokines, e.g. TNFα and IL-1β and mediates an acute phase response that activates serum amyloid P component and C-reactive protein.[367] However, CREBH does not increase the expression of UPR regulated genes, and further studies are necessary to delineate the functional role of CREBH in this ER stress-mediated inflammatory response.[367]

Preconditioning

A broad definition of preconditioning is the act of preparing for a subsequent action. The process of preconditioning biological systems against pathophysiological events can be observed in multiple forms, one example is vaccination. In 1796, Dr. Edward Jenner demonstrated that the inoculation of a small titer of smallpox virus into a young boy thereafter provided him with life-time immunity against the deadly disease.[368] It has since been shown, that presentation of a non-lethal dose of bacterial or viral infection into the body acts paradoxically to increase the production of neutralizing antibodies within the immune system.[369,370] Increased antibody production will 1) eliminate the acute infection and 2) boost the immune response in the event of future exposure to that same infection, increasing one's chances of overcoming the disease and its effects.[369] Recent evidence suggests novel-preconditioning paradigms may prepare patients and limit damage from other lethal events such as heart attacks, strokes, or liver failure.[371-373]

Ischemia/reperfusion preconditioning has become an increasingly active area of interest over the last three decades.[374] In the event of acute ischemia, i.e. myocardial infarction (MI), transient brain ischemia or liver transplant, persistent hypoxia induces both cellular necrosis, and apoptosis in the area of infarct, as well as apoptosis in the bordering tissue zone.[371-373] Studies examining the effects of ischemia/reperfusion preconditioning in these tissues have demonstrated that multiple applications of brief ischemic events prior to prolonged ischemia reduces subsequent tissue damage.[373,375,376] Murry and colleagues demonstrated the application of brief ischemic events prior to a prolonged myocardial infarction reduced the infarct size by approximately 25 percent compared to control animals that did not receive ischemic preconditioning.[375] While the methods and modalities conditioning are continuously evolving, many preconditioning-mediated cardio-protective effects have been linked to mitochondria stabilization via G-protein coupled receptor activation and canonical downstream events, release of circulating humoral factors and neurogenic activation of protein kinase C and the ERSR.[374,377-381] As ongoing studies continue to examine the molecular mechanisms responsible for ischemia/reperfusion preconditioning effects in the various tissue paradigms, it has become abundantly clear that low dose stress facilitated pre-induction of cellular readiness has paradoxical effects against further cellular damages.

Preconditioning of the Endoplasmic Reticulum Stress Response

The benefits of preconditioning are not isolated to ischemia/reperfusion injury alone. There is a growing body of evidence demonstrating that preconditioning of the cellular ERSR with minor stresses enhances cell viability upon exposure to a subsequent more damaging stress. Various cellular stressors such as hypoxia, inflammation and glucose deprivation activate the ERSR.[382,383] Importantly, it has been demonstrated that the pre-activation of adaptive ER stress signaling pathways provide cytoprotection and enhance cell viability against exposure to various cellular insults such as oxidative stress, inflammation, and hypoxia.[384-388]

Oxidative Stress- In 2003, Hung and colleagues examined the effect of ER stress preconditioning against oxidative injury in LLC-PK$_1$ renal epithelial cells, utilizing both TM and Thaps. In this study, cells were pretreated with TM and Thaps (1.5µg/ml and 0.3µg/ml respectively) for 12-16 hours prior to the exposure of 0.5-1mM H_2O_2. As determined by LDH release, increased concentrations of intracellular Ca^{2+}, cell injury was significantly reduced in cells preconditioned with either TM and Thaps, compared to non-preconditioned controls. The preconditioning effects against oxidative injury however, were negated in LLC-PK$_1$ cells expressing a GRP78 antisense RNA. Therefore, the authors suggested the expression of GRP78 is necessary for successful ER stress preconditioning against H_2O_2 induced oxidative injury. These results corroborated other preconditioning models of ischemia/reperfusion, which demonstrate that increased expression of ER chaperones is positively correlated with cell survival.[389] In addition, increased phosphorylation of extracellular signaling-kinase regulated protein (ERK) was found to be necessary for ER stress mediated resistance against oxidative stress and was associated to increased GRP78 expression following TM and Thaps preconditioning.[386] A later study, utilizing three additional renal cell lines (NRK-52E, HEK293 and MDCK), similarly examined the effect of 24hr pretreatment with TM, Thaps or oxidized DTT, which alters protein folding by augmenting the ER redox state, against subsequent oxidative insults.[385,390] In agreement with previous studies, ER stress preconditioning significantly increased cell viability. However, they found both the cell type and the method of ER stress induction affected the quality of cytoprotection. Originally, the authors speculated the variation in cytoprotective effects to be linked to the cell type specific dose-dependent induction of GRP78 protein levels, but no relationship was found between the extent of GRP78 induction and the afforded cytoprotection. Consequently, it is important to note that these results suggest the cytoprotective effects mediated by ER stress preconditioning are cell type specific and are influenced by the mechanism of ER stress induction. Moreover, because GRP78 expression levels in this study did not directly correlate to the amount of cytoprotection provided, it is likely supplementary factors are responsible for modifying ER stress preconditioning effects.

Inflammation- In an additional study characterizing the effect of ERSR preconditioning against retinal endothelial inflammation, XBP1 was described to play a critical role in blunting pathophysiological responses to the inflammatory cytokine tumor necrosis factor-alpha (TNFα).[384] Specifically, the upregulation of spliced XBP1, afforded by low dose treatment of TM, inhibited both TNFα mediated IκB kinase/NFκB activity and prevented the expression of downstream inflammatory markers (e.g. soluble intercellular adhesion molecule-1 and vascular adhesion molecule). These cytoprotective effects could be mimicked in cells overexpressing spliced XBP1 with the transfection of adenovirus encoding spliced XBP1 and annulled using an XBP1 silencing RNA. In agreement with previous studies, increased IRE1α expression induced by TNFα treatment was buffered to basal levels in cells overexpressing XBP1.[365,391] The authors proposed the buffering capacity of XBP1 might be due to an XBP1-dependent increase in GRP78 that ameliorates ER stress and IRE1α activation. Additional studies however, have demonstrated ERSR preconditioning-dependent cytoprotection against inflammation independent of increased GRP78.[392] Thus, further studies are necessary to delineate the exact mechanism of ER stress induced XBP1-dependent inhibition of inflammatory responses to TNFα treatment.

Hypoxia- Like ER stress preconditioning facilitated cellular protection against inflammatory processes, IRE1α-dependent XBP1 activation is required for cellular protection against hypoxic insults. While it is understood that ERSR pathways respond to hypoxia, the mechanism of ERSR regulated hypoxic sensitivity was relatively unknown until recently.[393] Utilizing loss of function mutant alleles in various UPR genes, Mao and Crowder demonstrated the necessity of functioning IRE1α, XBP1 and ATF6 for appropriate ERSR mediated preconditioning against lethal hypoxic insults. In this study, null mutations for two GRP78 homologs had no effect, positive or negative, on TM preconditioning. Taken together, ER stress preconditioning mediated cytoprotection is a multifactorial process. The direct molecular signaling pathways responsible for priming cells seems to be dependent of the modality of applied ER stress and specific to the cell type undergoing preconditioning. While it has been shown to be highly beneficial in protecting cells against various forms of injury, future studies are required for the optimization of the technique prior to utilize as a therapeutic approach.

Remote Preconditioning of the Endoplasmic Reticulum Stress Response

Interestingly, the beneficial adaptations of prophylactic stress-mediated UPR-preconditioning is not restricted to the tissue being conditioned. Remote preconditioning is defined by brief episodes of stress applied to a discrete tissue or organ that result in global cytoprotection against future lethal stresses.[394] Similar to preconditioning, many of the first important studies characterizing the positive cytoprotective effects of remote preconditioning were performed in the context of myocardial ischemia/reperfusion injury.[395,396] It has since been demonstrated that remote preconditioning is not limited to ischemia/reperfusion injury or cytoprotection of myocardial tissue alone. In the context of the kidney, remote UPR preconditioning, via systemically administered pretreatments of TM or Thaps, ameliorated the consequences of a chemically induced form of glomerulonephritis in rats.[397] In addition, remote systemic ER stress preconditioning has been shown to suppress translation of UPR apoptotic effector proteins ATF4 and GADD153, inhibiting TM-mediated apoptosis in splenic macrophages, renal tubule cells and hepatocytes, preventing hepatosteatosis and renal dysfunction.[398] Based on these data, it is important to note for future studies that the UPR is a dynamic signaling network capable of exhibiting cell- and stimulus-specific responses.

Thesis Aims

This thesis consists of three primary aims. Our laboratory has previously demonstrated that the pregnant uterus facilitates uterine quiescence through UPR mediated activation of non-apoptotic myometrial CASP3.[350] It is unknown however, how CASP3 is maintained in a non-apoptotic state to maintain myometrial quiescence. In Aim 1 we looked to characterize the capacity of *in vitro* preconditioning of the ERSR to facilitate the maintenance of non-apoptotic CASP3. We hypothesize preconditioning the ERSR in uterine myocytes increases the ability of the myometrium to adapt to apoptotic stimuli through modulation of pro-survival and inflammatory responses, thus allowing for the maintenance of non-apoptotic CASP3. In Aim 2, we utilized our pregnant mouse model of ERSR induced preterm birth, previously described by Kyathnahalli and colleagues, to examine the *in vivo* effects of endogenous ERSR preconditioning on the regulation of myometrial adaptation to gestationally-induced uterine stressors.[350] We hypothesize that an inappropriately preconditioned uterus or a uterus that is unable to host an adaptive preconditioning response is more likely to undergo premature uterine contraction and subsequently preterm birth. In Aim 3 we tested the hypothesis that circulating factors, secreted from uterine myocyte in an ER-stress dependent manner, facilitate the transmission and activation of a potentially adaptive/preconditioning-like extracellular ERSR in a paracrine and endocrine manner. We further speculate that these secreted factors may act as potential biomarkers for uterine myocyte refractoriness.

CHAPTER 2

Introduction

In the context of the pregnant uterine myocyte, our laboratory has demonstrated active CASP3 is highly abundant across gestation and is critical for the regulation of uterine myocyte quiescence.[350,399] Specifically, we have shown that active CASP3 within the uterine compartment targets and degrades multiple components of the contractile architecture, such as α actin, γ actin and connexin 43 rendering the myocyte quiescent.[350] Classically, CASP3 activity has been linked to the execution of cellular apoptosis through proteolytic cleavage of DNA repair moles, such as poly (ADP-ribose) polymerase (PARP), resulting in intra-nucleosomal cleavage and fragmentation of DNA.[400] Interestingly, at no point during gestation does the myometrium succumb to apoptosis, suggesting CASP3 is maintained in a non-apoptotic state within the pregnant uterus while fulfilling its tocolytic action. Subsequently, it is known that non-apoptotic CASP3 action is essential for inhibiting myometrial contractility, however the mechanisms necessary for the maintenance of CASP3 in a non-apoptotic state within the pregnant myometrium remain completely elusive. It has been demonstrated in previous studies that stress-dependent preconditioning can maintain CASP3 in a functioning non-apoptotic state. Subsequently, we hypothesize that preconditioning the uterine myocyte UPR with minor prophylactic stress events will facilitate the maintenance of non-apoptotic CASP3 following a damaging bolus.

CASP3 can be activated through 1 of 3 signaling cascades: the extrinsic pathway, the intrinsic pathway or the UPR, as referred to in Chapter 1 Apoptosis.[401,402] In the pregnant mouse myometrium, there is UPR-dependent activation of GADD153 and CASP12 around E6-8, with concomitant increases in XBP1s.[403] As a result of

GADD153/CASP12 signaling, there is a robust activation of CASP3, as seen by a surge in its active cleavage fragments (14 and 17kD) at approximately E8-10.[403] Active CASP3 is then observed in high levels throughout early and mid-gestation, declining at approximately E17 prior to the onset of labor.[403] Intriguingly, PARP cleavage, an indicator of CASP3 mediated apoptosis, is minimal in the myometrium at the time heightened CASP3 activity.[404] Furthermore, there is no DNA fragmentation or positive TUNEL staining within the myometrium at any point in gestation, validating that active CASP3 is maintained in a non-apoptotic state during pregnancy. In other muscle systems such as the bladder, heart and diaphragm, non-apoptotic CASP3 has been described to have anti-contractile function.[405-407] While CASP3 targets were not identified in the smooth muscle of the bladder, CASP3 was found to target structural proteins in both the heart and diaphragm. In cardiac tissue specifically, both α-actin and α-actinin, components of the cardiac contractile architecture were directly cleaved by CASP3 and then further degraded. Similarly, we showed in the absence of apoptotic consequences non-apoptotic uterine CASP3 protease activity inhibits myometrial contraction in a tocolytic manner through the targeted cleavage and degradation of multiple components of the contractile architecture, i.e. connexin 43, α-actin and γ-actin.[350,399] While the tocolytic function of CASP3 is imminently important and has been highly characterized, it remains widely unknown how the uterus capacitates active CASP3 in a non-apoptotic state during pregnancy.

A growing body of evidence now suggests that non-apoptotic CASP3 action can be maintained through the process of cellular preconditioning.[408-410] The process of preconditioning biological systems against pathophysiological events can be observed in multiple forms is strongly conserved in evolution, as discussed in Chapter 1

Preconditioning.[411] In the context of neuronal ischemic preconditioning, active CASP3 expression has been reportedly high in neuronal cells that do not undergo delayed neuronal cell death following ischemic injury.[410] In this case, ischemic preconditioning was found to increase the expression and effectiveness of pro-survival inhibit-of-apoptosis family members, cIAP. In a separate study examining the effects of preconditioning with minor periods of oxidative stress and low ATP on neuronal excitotoxicity, McLaughlin and colleagues demonstrated a sharp increase in non-apoptotic CASP3 activity during the period of neuronal preconditioning. Further, when pan-caspase inhibitors were employed during the period of preconditioning the previously observed cytoprotective effects of KNC-dependent ROS generation were abrogated and cells exhibited increased apoptosis like that of the non-preconditioned neuronal population. In both of these studies, the preconditioning stimuli (hypoxia, oxidative stress and low ATP) that preserve non-apoptotic CASP3 activity have each been demonstrated to in turn activate the cellular UPR.[412] Subsequently, we propose directly preconditioning the UPR within the uterine myocyte may be a potential mechanism whereby active CASP3 can be kept in a non-apoptotic state.

In this current study we thus examined the cytoprotective effects of preconditioning the UPR in uterine myocytes, utilizing hTERT-HM cells. To test the hypothesis that UPR preconditioning facilitates the maintenance of non-apoptotic CASP3 and promotes cell viability, minor concentrations of TM and Thaps were applied prior to the exposure of a known cytotoxic dose in hTERT-HM cells. Herein we have identified preconditioning of the uterine UPR as the protective mechanism that facilitates the maintenance of non-apoptotic CASP3 within the pregnant uterine myocyte. These studies clearly demonstrated *in vitro* UPR preconditioning facilitates augmented uterine myocyte cell viability preventing apoptotic consequences of CASP3 action in the presence of elevated levels of CASP3 activation. Additionally, we have demonstrated UPR preconditioning decreases stress-dependent inflammatory responses in the human uterine myocyte.

Materials and Methods

Cell Culture

For the *in vitro* cell culture model system, we utilized hTERT-HM cells.[413] In detail, human myometrial cells were collected from the anterior wall of the uterine fundus in women of reproductive age undergoing a hysterectomy. The catalytic subunit of telomerase was then expressed in the myometrial cells via retroviral infection. In these studies, hTERT-HM cells were cultured in Dulbecco modified Eagle/F12 low glucose media (DMEM-F12) (Invitrogen Carlsbad, CA), supplemented with 10% fetal bovine serum (vol/vol) (Invitrogen) and antibiotic/antimycotic (10,000 U/ml; Invitrogen), and incubated at 37°C with 95% air and 5% CO_2.

Tunicamycin and Thapsigargin Treatments

For all *in vitro* experiments, TM was suspended in 20μl 10M sodium hydroxide and brought to a final concentration of either 0.1μg/ml or 1.0μg/ml in DMEM-12 media with 10% FBS and antibiotic/antimycotic. Thaps (Sigma-Aldrich, St. Louis, MO; Cat#T9033) was dissolved directly in cell culture media and brought to a final concentration of 10nM or 250nM. For TM preconditioned (P) and non-preconditioned (NP) treatments hTERT-HM were given a 24hr treatment of 0.1μg/ml TM or vehicle, respectively, 0-48hrs prior to a secondary treatment of 5.0μg/ml TM. Similarly, for Thaps preconditioned (P) and non-preconditioned (NP) treatments hTERT-HM cells were given a 24hr treatment of 10nM TH or vehicle, respectively, 48hrs prior to a secondary treatment of 250nM Thaps. In both conditions, media was replaced 1hr after the secondary treatment was given and cells and media were collected 47hrs later.

Cytosol and Nuclear Protein Fractionation from Cells

Cytoplasmic and nuclear protein fractions from hTERT-HM cells were prepared as previously mentioned. Initially, cells were rinsed in ice-cold PBS and centrifuged at 956 X *g*. The pellet was re-suspended and evenly homogenized in ice-cold NE1 buffer (10mM Hepes pH 7.5, 10mM $MgCl_2$, 5mM KCl, 0.1% Triton X-100 with 1X EDTA-free protease/phosphatase inhibitor mini tablet). The homogenate was then centrifuged at 2655 X *g*, the supernatant was retained as the cytoplasmic protein fraction and the pellet was washed in NE1 buffer and suspended in ice-cold NE2 buffer [20mM Hepes pH 7.9, 500mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA pH 8.0, 25% (vol/vol) glycerol with 1X EDTA-free protease/phosphatase inhibitor mini tablet]. The homogenate was vortexed for 30sec every 5min and after 1hr, centrifuged at 10,621 X *g*. The supernatant was then retained as the nuclear fraction. Protein estimation was performed using a bicinchoninic acid (BCA) assay, equal amounts of protein were loaded for immunoblotting, PDI and NCOA3 were utilized as loading controls for the cytoplasmic and nuclear fractions, respectively.

Immunoblotting and Densitometric Analysis

Equal amounts of protein were separated via electrophoresis on NuPAGE 4-12% gradient precast polyacrylamide gels (Life Technologies, Carlsbad, CA). Proteins were transferred onto Hybond-P PVDF membranes (Millipore, Billerica, MA) and blocked for 1hr at room temperature in 5% non-fat milk prepared in Tris Buffered Saline with 0.1%Tween-20 (vol/vol). Membranes were incubated with primary antibodies overnight at 4°C. Primary antibody concentrations were as follows: GRP78 (1:1000; Cat#3177), Cl CASP3 (1:250; Cat#9664), GADD153 (1:500; Cat#5554), Cl PARP (1:1000; Cat#9541), AFT4 (1:500; Cat#11815), p-eIF2α (1:500; Cat#3398), NFκB (1:1000; Cat#8242), XIAP

(1:250; Cat#2042) and PDI (1:5000; Cat#3501) were obtained from Cell Signaling Technologies; XBP1s (1:500; Cat#37152) was obtained from Abcam; ATF6 (1:500; Cat#24169-1-AP) was obtained from Proteintech; MCL-1 (1:1000; Cat#sc-819) was obtained from Santa Cruz Biotechnology, and NCOA3 (1:5000; Cat#PA1-845) was obtained from ThermoScientific. Following primary incubation, immunoreactivity was detected using horseradish peroxidase-conjugated secondary antibodies and visualized using an enhanced-chemiluminescence detection system (ThermoScientific, Rockford, IL). Immunoreactive band density was then quantified using ImageJ software.

Enzyme-Linked Immunosorbent Assay (ELISA)

Media samples were loaded into Amicon Ultra Centrifugal Filters (Millipore, cat#UFC500396) and centrifuged for 30 minutes at 14,000 x g to concentrate media approximately 10X. The level of human tumor necrosis factor-alpha (TNFα) was then measured in 10X concentrated media using an ELISA. Specifically, the MSD Multi-Spot TNFα ELISA (Meso Scale Diagnostics, Rockville, MD, Cat#K151QWD) was performed according to the manufacturer's instructions and results were read via the Meso Scale Discovery 1300 microplate reader. Each sample measurement was read in duplicate and the computed averages were taken based on the calculated standard curve.

Statistical Analysis

All data represent at least three individual experiments performed in triplicate. For the direct comparison of three or more conditions a one-way analysis of variance was performed, with multiple comparisons analyzed via Newmans-Keuls multiple comparisons test. When directly comparing two conditions a two-tailed student-t test was performed. All comparisons were considered significant with p-values less than 0.05.

Results

Appropriate In Vitro UPR-Preconditioning Renders CASP3 Non-Apoptotic in Human Uterine Myocytes

Activation of the UPR, CASP3 and apoptotic indices were examined by immunoblotting in control, preconditioned (0.1μg/ml, 24hrs TM) and non-preconditioned (vehicle, 24hrs) hTERT-HM cells, given a 0, 4, 24 and 48hr recovery period prior to a subsequent known cytotoxic dose of TM (5.0μg/ml, 1hr) (Figure 1).[385] A robust activation of the UPR was observed in the levels of GRP78 and CASP3 in both the TM preconditioned and non-preconditioned cells compared to the vehicle control (Figure 1A, B and C) for each recovery period. Examination of apoptotic indices, as quantified by cleavage of the nuclear DNA repair molecule Poly ADP ribose polymerase (Cl PARP), demonstrated that CASP3 activation and PARP cleavage levels were equivalent at all recovery time points examined except at the 48hr recovery time frame. With an allotted 48hr recovery period, the uterine myocytes of the preconditioned and non-preconditioned cells displayed equal levels of CASP3 cleavage, however remarkably the preconditioned cells had a 4 fold decrease in PARP cleavage compared to non-preconditioned cells suggesting that preconditioned cells given a 48hr recovery period between the preconditioning and cytotoxic stimuli have significantly reduced levels of apoptosis and that the observed active CASP3 is non-apoptotic in nature. Decreased cell viability of the non-preconditioned cells (NP) in comparison to control (C) and preconditioned (P) myocytes was further validated using a trypan blue assay (Figure. 2). These results demonstrate that 1) preconditioning the UPR provides

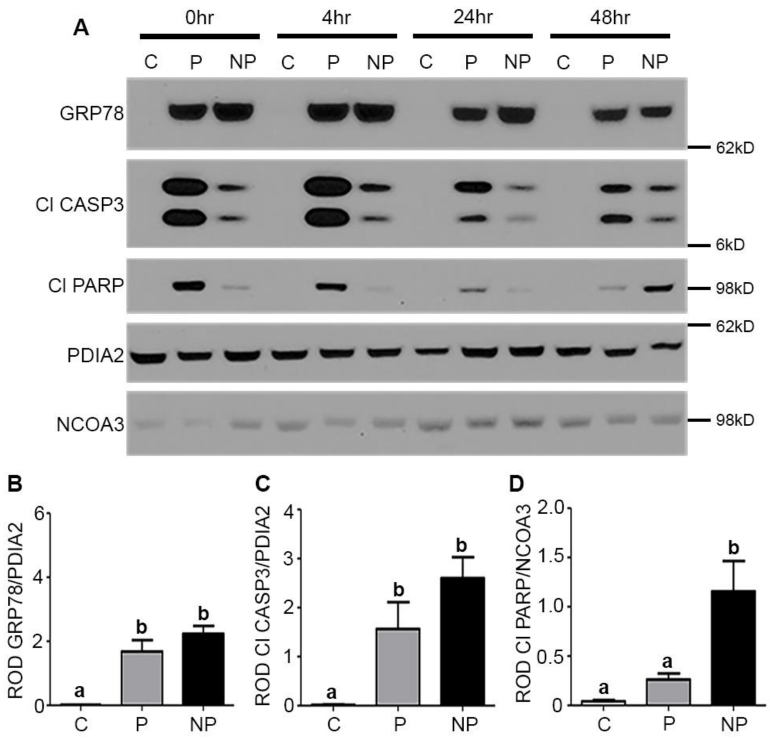


Figure 1. UPR preconditioning renders the hTERT-HM uterine myocyte CASP3 non-apoptotic. **(A)** Elevated levels of cytoplasmic GRP78 and Cl CASP3, and nuclear Cl PARP are observed in preconditioned *(P)* and non-preconditioned *(NP)* uterine myocytes as compared to controls *(C)* (n=3 per condition), when exposed to a cytotoxic dose of TM 0, 4, 24 and 48 hrs post TM preconditioning. At 48hrs to recovery there is equal activation of GRP78 **(B)** and Cl CASP3 **(C)** in both P and NP uterine myocytes. In contrast, Cl PARP **(D)** is significantly decreased in the P versus NP cells. PDIA2 and NCOA3 are utilized as cytoplasmic and nuclear protein loading controls. A representative blot from this experiment is shown. Statistical comparisons were performed using one-way ANOVA, and subsequent Newman-Keuls multiple-comparison tests. Data labeled with different letters are significantly different from each other (p<0.05).

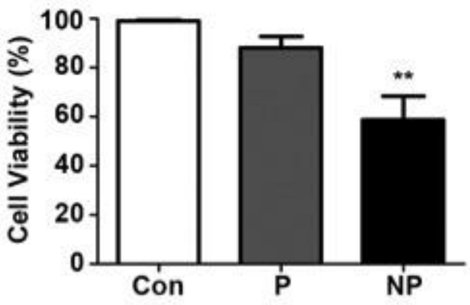


Figure 2. UPR preconditioning increases cell viability of the hTERT-HM uterine myocyte in the presence of active non-apoptotic CASP3. Decreased cell viability was observed in non-preconditioned *(NP)* uterine myocytes as compared to controls *(C)* and preconditioned myocytes *(P)* (n=3 per condition), when exposed to a cytotoxic dose of TM 48 hrs post TM preconditioning as measured using a trypan blue assay. Statistical comparisons were performed using a one-way ANOVA, and subsequent Newman-Keuls multiple-comparison tests. $*p\leq0.05$ and $**p\leq0.01$ compared with controls.

resistance to the apoptotic consequences of CASP3 activation (Figures 1D) and 2) UPR preconditioning-mediated cytoprotection is dependent on the amount of recovery time between the preconditioning stimuli (0.1μg/ml, 24hrs) and the subsequent damaging/lethal stress (5.0μg/ml, 1hr) TM stimulus. Additionally, the preconditioning dose of TM used was tested and found to activate the UPR, as seen by increased levels of GRP78, without inducing apoptosis shown by lack of GADD153 and CASP3 activation, or PARP cleavage (Figure 3).

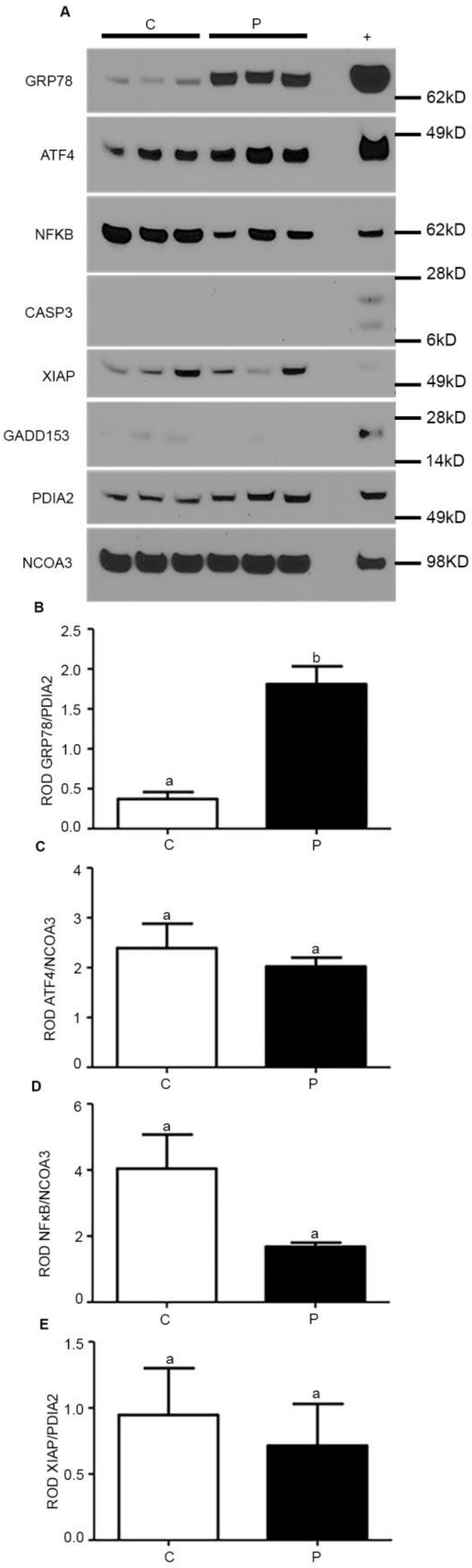


Figure 3. Preconditioning dose of TM has negligible impact on UPR, inflammatory, pro and anti-apoptotic indices in the hTERT-HM uterine myocyte. GRP78, ATF4, NFκB, CASP3, XIAP, and GADD135 levels were measured in vehicle treated *(C)* uterine myocytes and preconditioned *(P)* myocytes exposed to a minor UPR stress (TM, 0.1μg/ml, 24hrs). GRP78 levels were modified significantly whereas all others remained unchanged, indicating the lack of downstream consequences of the preconditioning stress alone *in vitro*. A representative blot from each experiment is shown. PDIA2 and NCOA3 are utilized as our cytoplasmic and nuclear protein loading controls. Statistical comparisons were done using a two-tailed student t-tests. Data labeled with different letters are significantly different from each other (p<0.05).

Cytoprotection Afforded by *In Vitro* UPR-Preconditioning in Human Uterine Myocytes is Independent of the Preconditioning Modality Employed

To validate that the observed anti-apoptotic effects of preconditioning were not modality-dependent, we repeated our preconditioning protocol using Thaps. Subsequently, hTERT-HM cells were preconditioned (10nM, 24hrs Thaps), given a 48hr recovery and compared to non-preconditioned (vehicle) cells following the administration of a known cytotoxic dose of Thaps (250nM, 1hr) (Figure 4). Again, we observed activation of the UPR as increased levels of GRP78 and CASP3 in both the Thaps preconditioned and non-preconditioned cells compared to the vehicle control (Figure 4A, B and C). Importantly, in a manner similar to the TM protocol, Thaps-preconditioning reduced PARP cleavage by 2 fold (Figure 4D) in the presence of a 25-fold increase in CASP3 activation (Figure 4C) at the 48hr recovery time point post

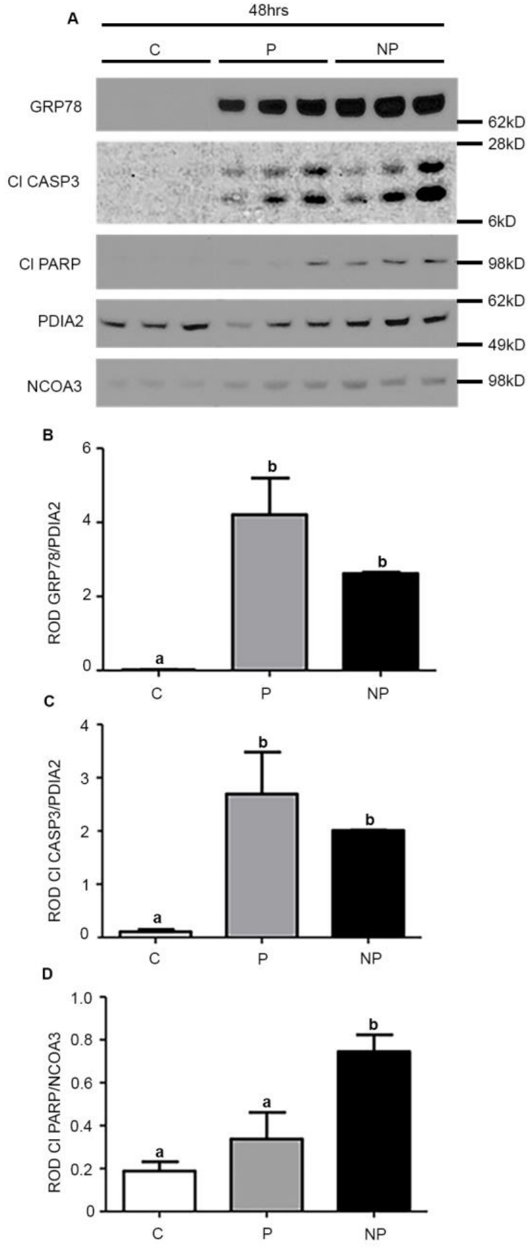


Figure 4. Thaps mediated UPR preconditioning renders the hTERT-HM uterine myocyte CASP3 non-apoptotic. **(A)** Elevated levels of cytoplasmic GRP78, nuclear CL CASP3 and CL PARP are observed in preconditioned *(P)* and non-preconditioned *(NP)* uterine myocytes as compared to controls *(C)* (n=3 per condition), when exposed to a known cytotoxic dose of Thaps (250nM, 1hr), 48 hrs post Thaps preconditioning (10nM, 24hrs). **(B)** At 48hrs to recovery there is equal activation of GRP78 and (**C)** Cl CASP3 in both P and NP uterine myocytes. (**D)** In contrast, Cl PARP is significantly decreased in the P versus NP cells. PDIA2 and NCOA3 are utilized as our cytoplasmic and nuclear protein loading controls. A representative blot from this experiment is shown. Statistical comparisons were performed using a one-way ANOVA, and subsequent Newman-Keuls multiple-comparison tests. Data labeled with different letters are significantly different from each other (p<0.05).

preconditioning, suggesting the cytoprotective effect of preconditioning the UPR is not modality-dependent.

Preconditioning the UPR Inhibits Inflammation in the Human Uterine Myocyte *In Vitro*

To define the mediators facilitating resistance to the apoptotic consequences of CASP3, NFκB activation in the nuclear compartment of the uterine myocyte was examined in control (C), preconditioned (P) and non-preconditioned (NP) cells exposed to TM or Thaps or vehicle treatment. Cells were collected 0.25, 2, 4 and 24hrs post administration of the cytotoxic bolus and compared to vehicle-treated controls. As seen in Figure 5A and B, non-preconditioned cells display a robust 5.5 fold activation of NFκB 2hrs post administration of the subsequent damaging stress whereas NFκB activation remains barely detectable in non-preconditioned cells at all time points examined post the lethal stress (0.25, 2, 4, 24hrs). Enzyme linked immunosorbent assays (ELISA) performed on control preconditioned and non-preconditioned hTERT-HM cells collected 48hrs post TM bolus revealed TNFα secretion was suppressed 0.5 fold in the preconditioned cells whereas, non-preconditioned cells (Figure 5C) demonstrated a 0.5 fold increase in levels compared to non-treated controls. Similar results were found when cells were preconditioned and stressed with Thaps (Figure 5D). NFκB activation and TNFα secretion was increased 19 fold 2hrs post and 4 fold 48hrs post exposure to the cytotoxic stress (Figure 5E and F) respectively, within non-preconditioned cells and remained inactive within the preconditioned cells.

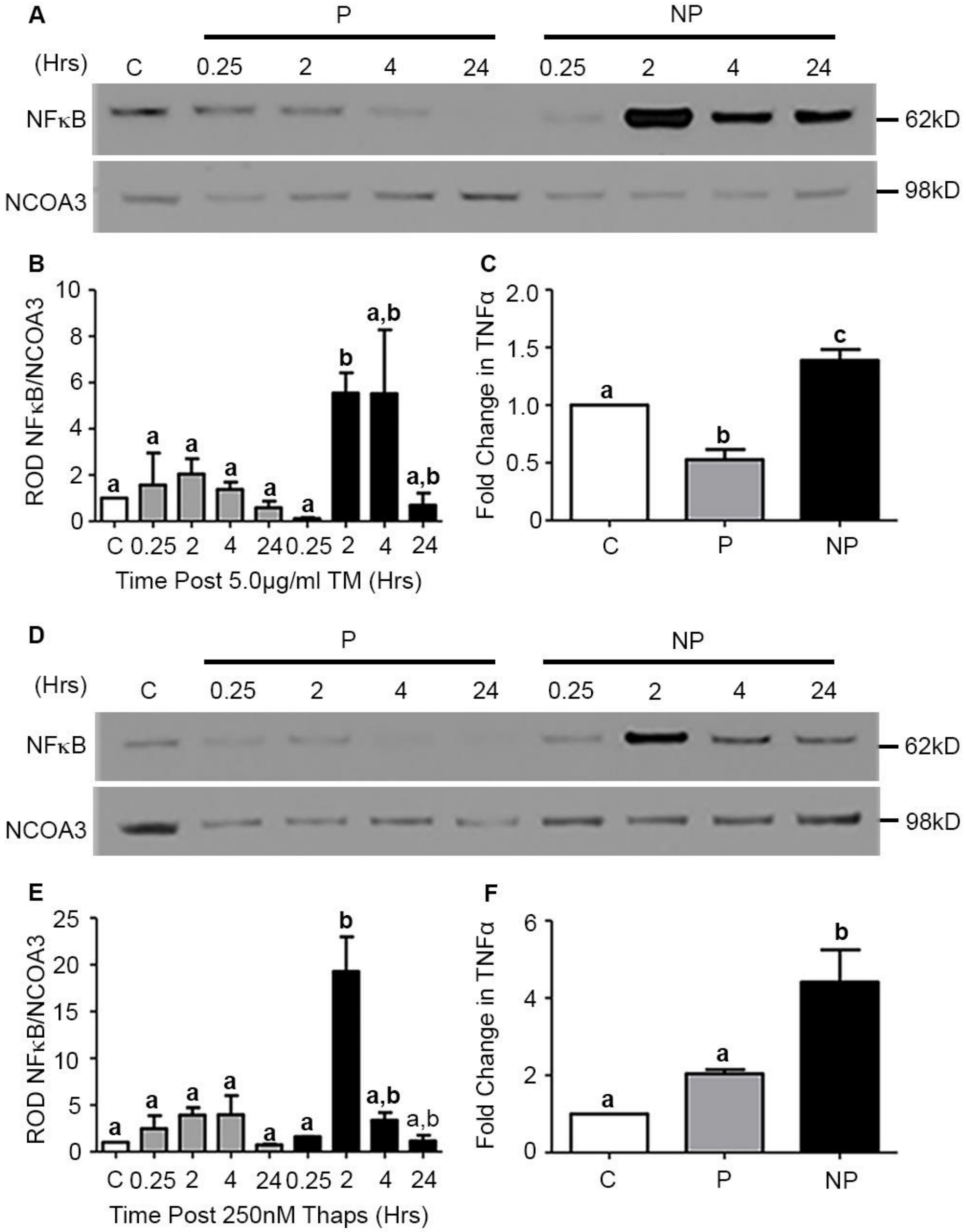


Figure 5. UPR preconditioning ablates NFκB activation in the hTERT-HM uterine myocyte. (**A, B, D, E)** Activation of NFκB was significantly increased in both TM and Thaps non-preconditioned *(NP)* cells and reduced to barely detectable levels in preconditioned *(P)* cells 2hrs post administration of a cytotoxic dose of TM/Thaps. (**C, F)** TNFα secretion was also reduced in P versus NP cells. A representative blot from each experiment is shown. NCOA3 is utilized as nuclear protein loading control. Statistical comparisons were performed using one-way ANOVA, and subsequent Newman-Keuls multiple-comparison tests. Data labeled with different letters are significantly different from each other ($p<0.05$).

Preconditioning Downregulates UPR Activated Apoptotic Signaling *In Vitro*

We quantified stress-mediated activation of the UPR pro-survival (spliced XBP1 (XBP1s) and phospho-elongation initiation factor 2α (p-eIF2α)) and pro-apoptotic (activating transcription factor 4 (ATF4) and dna damage inducible transcript 3 (GADD153)) signaling pathways by immunoblotting immediately following the application of the TM or Thaps bolus (0.25, 2, 4, or 24hrs post bolus) in control, preconditioned and non-preconditioned hTERT-HM cells (Figure 6A and F). p-eIF2 α levels remain unchanged between preconditioned and non-preconditioned cells, TM or Thaps (Figure 6B and G). XBP-1s levels were suppressed 4hrs post bolus in TM-preconditioned cells (Figure 6C), whereas no change in expression is observed between Thaps-preconditioned and non-preconditioned cells (Figure 6H). Pro-apoptotic signaling pathways in contrast, were significantly downregulated in both TM and Thaps preconditioned versus non-preconditioned cells. A 2 fold decrease in ATF4 at 24hrs (Figure 6D) and a 7 and 5 fold reduction of GADD153 at 4 and 24hrs respectively (Figure 6E) was observed in TM preconditioned cells. Similarly, a 0.5 fold reduction in ATF4 at 24hrs (Figure 6I) and a 2 fold reduction in GADD153 at 2, 4 and 24hrs (Fig. 6J) was observed in Thaps-preconditioned compared to non-preconditioned hTERT-HM cells. Anti-apoptotic factors XIAP and MCL1 were preferentially maintained in TM preconditioned cells (Figure 7).

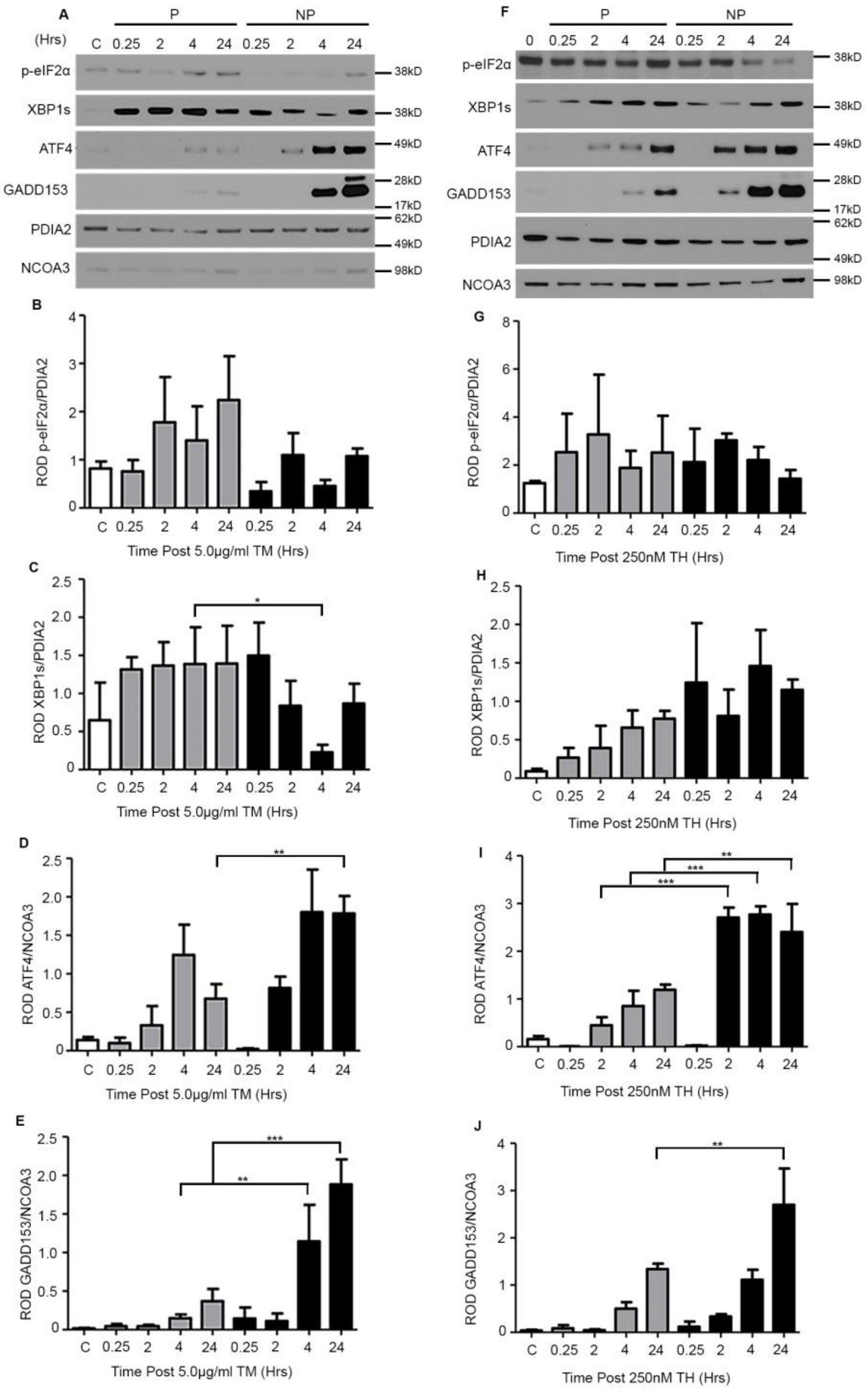


Figure 6. UPR preconditioning differentially regulates activation of the pro and anti apoptotic arms of the UPR in the hTERT-HM uterine myocyte **(A-J)**. TM **(A)** or Thaps **(F)** mediated preconditioning blocked activation of the pro-apoptotic arms of the UPR with ATF4 **(D, I)** and GADD153 **(E, J)** and TM preconditioning maintained activation of the anti-apoptotic arm of the UPR with XBP1s **(C)** significantly upregulated in preconditioned *(P)* versus non-preconditioned *(NP)* cells post administration of a cytotoxic dose of TM/Thaps. No changes in XBP1s **(H)** upon Thaps treatment, and p-eIF2α **(B, G)** upon TM and Thaps treatment. PDIA2 and NCOA3 are utilized as cytoplasmic and nuclear protein loading controls. A representative blot from each experiment is shown. Statistical comparisons were performed using one-way ANOVA, and subsequent Newman-Keuls multiple-comparison tests. *p<0.05, **p<0.01 and ***p<0.001 compared with controls.

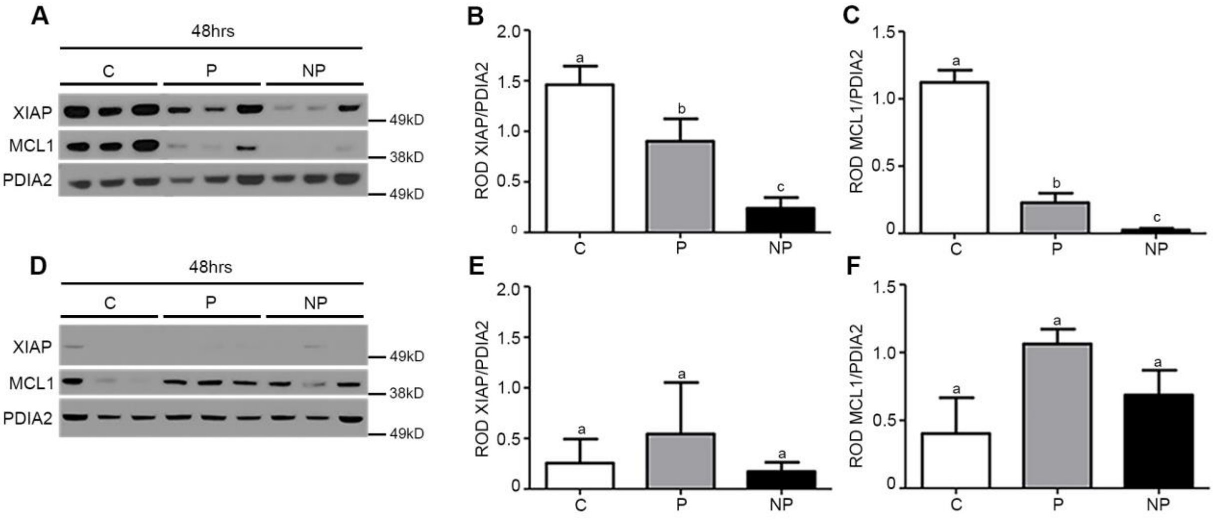


Figure 7. Increased maintenance of pro-survival molecules with TM mediated UPR preconditioning in the human uterine myocyte**. (A)** Pro-survival molecules XIAP and Mcl-1 were analyzed 48hrs post TM-bolus in non-preconditioned *(NP),* TM-preconditioned cells given a 48hr recovery period *(P)* and vehicle treated controls *(C)*. (**B) and (C)** Both Mcl-1 and XIAP were significantly elevated in TM-preconditioned cells when compared to non-preconditioned myocytes. (**D)** The experiment was repeated using Thaps as a preconditioning and bolus stimuli. **(E)** and **(F)** Neither Mcl-1 or XIAP were significantly different between Thaps-preconditioned and non-preconditioned myocytes. A representative blot from this experiment is shown. PDIA2 is utilized as our cytoplasmic loading control. Statistical comparisons were done using a one-tailed student t-tests. Data labeled with different letters are significantly different from each other ($p<0.05$).

Discussion

We have previously demonstrated the critical role that the UPR plays in CASP3 activation within the uterine compartment during pregnancy.[350] As recent studies have demonstrated preconditioning events, such as ischemia, that lead to the activation of the UPR can maintain active CASP3 in a non-apoptotic state following subsequent damaging stress, we hypothesis that the act of preconditioning the uterine UPR during pregnancy is essential in protecting the pregnant myometrium against a CASP3 mediated apoptotic fate. This is important because the loss of non-apoptotic CASP3 tocolytic action within the pregnant mouse myometrium initiates the onset of preterm birth.[350] Here we utilized a preconditioning protocol in which minor amounts of ER stress were given to hTERT-HM cells prior to the exposure of a large damaging stress to test the hypothesis UPR preconditioning facilitates the maintenance of non-apoptotic CASP3 in myometrial cells.

We report for the first time that preconditioning the UPR inhibits uterine myocyte apoptosis in the presence of highly abundant levels of active CASP3. To note, UPR preconditioning afforded cytoprotection that was independent of the stress modality employed and acted to suppress downstream stress-dependent apoptotic pathways (ATF4 and GADD153) and inflammatory responses (NFκB and TNFα release) with a dependency on the recovery time given between the preconditioning stress and the subsequent cytotoxic bolus.

In many studies the activation of CASP3 is used as a cellular marker for the induction of apoptosis (see Chapter 1 Apoptosis for more detail). In the context of the pregnant uterus, we have previously demonstrated that active CASP3 is not participating in the initiation of apoptosis and that instead it is functioning in a non-apoptotic state to inhibit muscle contractility, as demonstrated in other tissue types such as the heart, diaphragm and bladder. In this study, we argue that preconditioning the UPR is one mechanism in which the myocyte can capacitate CASP3 activity in the absence of cellular apoptosis, allowing for the maintenance of uterine quiescence in a non-apoptotic CASP3 dependent manner. Prolonged or severe ER stress can mediate both mitochondrial-dependent and independent apoptosis, and consequently ERSR signaling is implicated in many diseases associated with cellular dysfunction and cytotoxicity.[414,415] The initial signaling responses however, via PERK/eIF2α, IRE1 and ATF6 do not induce cell death and instead activate a subset of genes that aid in the restoration of cellular homeostasis, as described in Chapter 1 Activation of the Unfolded Protein Response. To do so, UPR-activated genes increase chaperone protein expression e.g. GRP78, attenuate protein translation and increase ER associated degradation of unfolded proteins.[310,314,416] Multiple *in vitro* studies have proven that preconditioning the endoplasmic reticulum stress response with minor insults of stress stimulates a protective adaptive UPR promoting resistance to the apoptotic processes associated with subsequent, more damaging stresses.[385,388,417] Many and varied perturbations such as stress mediated inflammation, hypoxia and cytotoxins have been demonstrated to successfully induce a preconditioning afforded cytoprotection.[382,385,418] The most basic of these approaches is to activate the UPR by chemically inducing an accumulation of unfolded proteins within the ER. Two small molecules commonly used are Thaps and TM. Thaps, originally identified as a tumor promoting sequiterpene lactone, dysregulates calcium (Ca^{2+}) homeostasis by binding and inhibiting the ATP-sensitive Ca^{2+} pump within the endoplasmic reticulum, causing an increase in free cytosolic Ca^{2+}.[419] Because Ca^{2+} binding is an important function of multiple ER resident chaperone proteins, decreased ER Ca^{2+} stores cause an accumulation of unfolded proteins in the ER and reduced protein synthesis.[420-422] In contrast, Tm inhibits N-linked glycosylation that is necessary for the recognition of unfolded proteins by ER chaperones, such as GRP78 and calnexin.[423] Our *in vitro* data reveals preconditioning of the uterine myocyte UPR with stress (TM or Thaps) promoted non-apoptotic CASP3 activation during periods of cellular stress (Figures1 and 4). As can be observed in Figure 1, despite equally elevated levels of CASP3 activation (Figure 1C) in preconditioned and non-preconditioned cells 48hrs post receiving a bolus (Figure 1), the preconditioned cells demonstrated a newly acquired resistance to CASP3 mediated apoptotic cell death as indicated by decreased PARP cleavage (Figure 1.D). These data show preconditioning the UPR in the uterine myocyte maintains non-apoptotic CASP3, suggesting *in vivo* preconditioning may be the mechanism in which myometrial CASP3 is maintained in a non-apoptotic state to fulfill its tocolytic action, inhibiting labor.

Increased resistance to apoptotic cell death in the preconditioned myocytes was most likely due to the presence of elevated levels of cellular GRP78 prior to delivery of the bolus. As seen in Figure 3, the adaptive arms of the ERSR were activated from the preconditioning stimuli, whereas pro-apoptotic indices remained the same as controls. In the event of ER stress and increased accumulation of unfolded proteins, prophylactic increases in the concentration of GRP78 are advantageous for maintaining ER homeostasis. In studies examining the cardioprotective effects of stress preconditioning, pre-induction of GRP78 has been identified as a key mechanism for affording cytoprotection. In support of this claim, Yuan and colleagues demonstrated inhibition of GRP78 expression using anti-sense oligonucleotides ablated cardioprotection afforded by late hypoxic preconditioning.[424] In our preconditioning paradigm, cytoprotection was not afforded unless the cells were given a 48hr recovery period between the initial stimulus and the bolus. This is like other preconditioning paradigms where a recovery period is required for facilitating cytoprotection, such as in ischemic preconditioning. This suggests prophylactic GRP78 is not the sole mechanism of afforded cell viability as GRP78 levels are substantially increased 24hrs prior to the initial preconditioning stimulus.[374] Other studies have implicated GRP78 as being only partially responsible for preconditioning-mediated cytoprotection. One study by Harama and colleagues demonstrated ER stress preconditioning prevented lipopolysaccharide/TNFα induced inflammatory responses while decreasing full length GRP78 expression in a time dependent manner.[392]

In addition to an increase in prophylactic GRP78, we also witnessed suppressed activation of the UPR-mediated apoptotic-signaling pathway, ATF4-GADD153 (Figure 6). As previously mentioned, the first response to stress through UPR signaling is to initiate pro-survival processes, such as p-eIF2α mediated transcriptional inhibition, to promote the return to luminal homeostasis. In case of severe or prolonged stress however, internal ribosomal entry site-dependent transcription of ATF4 occurs and the secondary apoptotic UPR signaling pathway is activated.[425] One of the main mechanisms preconditioning has been demonstrated to increase cell viability is through the depression of apoptotic UPR signaling.[398,426] While testing the effectiveness of LPS-preconditioning in the prevention of renal dysfunction and hepatosteatosis, Woo and colleagues demonstrated pre-activation of toll-like receptors 3 and 4 prior to TM-induced ER stress inhibited GADD153 expression and reduced apoptosis in splenic macrophage, renal tubule cells and hepatocytes. Importantly, pre-activation of the toll-like receptors did not alter the pro-survival pathways. In our study, we similarly found TM and Thaps-preconditioning reduced expression levels of ATF4 and GADD153 without altering pro-survival signaling transducers (p-eIF2α and XBP1s) (Figure 6), suggesting one mechanism in which preconditioning may be preventing CASP3-dependent apoptosis is through inhibition of pro-apoptotic signaling pathways.

Another pathway significantly linked to UPR-dependent apoptosis is NFκB-mediated inflammatory signaling, as discussed in Chapter 1 The UPR and Inflammation. Growing evidence suggests, that the coupling of the ERSR and inflammation is important in the pathogenesis of multiple diseases. In atherosclerosis, the development of plaque lesions on the endothelial lining of blood vessels has been linked to activation of the UPR and inflammation. With the loading of free cholesterol into ER membranes in circulating macrophages, UPR signaling is thought, in part to mediate activation of inflammatory transcription factors NFκB and janus kinase.[427] Further, it has been demonstrated that the induction of GADD153, ATF4 and XBP1s is necessary to produce IL-6 and potentially other chemokines such as IL-8, CXC-chemokine ligand 2 and 3.[428] Similar to atherosclerosis, the cross talk between the ERSR and inflammation is prominent in human inflammatory bowel disease. Interestingly, Blumberg and colleagues demonstrated the stress of rapid proliferation in paneth cells is sufficient to instigate a pro-inflammatory response and that in these same cells the absence of appropriate UPR signaling there is a significant induction in apoptotic cell death.[391] Interestingly, it has been demonstrated that preconditioning the UPR can mitigate UPR-mediated activation of inflammatory signaling pathways and subsequent apoptosis, please see Chapter 1 Preconditioning for more details. For example, a study by Rao and colleagues recently showed preconditioning mice with low-doses of LPS prior to ischemia/reperfusion injury in the liver 1) inhibited NFκB and downstream inflammatory signaling proteins TNFα and IL-6, 2) blocked GADD153/CASP3 dependent apoptosis and 3) promoted anti-inflammatory signaling of IL-10.[426] In our studies we also observe that preconditioning the UPR in uterine myocytes blocked the activation of NFκB and consequently decreased the secretion of its downstream target the inflammatory mediator TNFα following the delivery of the TM bolus (Figure 2B and C). In the context of pregnancy, the inhibition of premature NFκB signaling and the resulting downstream inflammatory mediators within the myometrium is extremely important as heightened inflammation initiates many processes necessary for the induction of labor, as discussed in Chapter 1 Parturition.

Taken together these data demonstrate that preconditioning the UPR plays a critical role in maintaining the uterine myocyte in a CAPS3 positive, non-apoptotic, anti-inflammatory, pro-survival state *in vitro*. These findings highlight a potential mechanism whereby CASP3 can fulfill its tocolytic function, while avoiding apoptosis in the uterine myocyte. Further, they suggest *in vivo* preconditioning may also be important in maintaining quiescence through the inhibition of contractile associated inflammatory signaling pathways, e.g. NFκB and TNFα. From these studies, it would next be important to examine the function of preconditioning *in vivo* in the pregnant uterus and its potential role in the regulation of myometrial quiescence.

CHAPTER 3

Introduction

In this chapter we will discuss the functional relevance of *in vivo* preconditioning in the maintenance of uterine quiescence. As previously mentioned, our laboratory has established that the preservation of non-apoptotic CASP3 activity in the myometrium is an integral part of the maintenance of uterine quiescence. Further, I have shown *in vitro* that CASP3 activity in the uterine myocyte can be maintained in a non-apoptotic state through preconditioning of the UPR. Current literature has established that throughout a normal gestation the uterine compartment is exposed to and must tolerate a variety of cellular stresses, i.e. hypoxia, hyperplasia, hypertrophy, hormone fluctuation and mechanical stretch, to reach term. Therefore, we hypothesize that *in vivo* transient incremental ER stress insults experienced by the uterus during gestation act to capacitate the myometrium to withstand additional subsequent stressors while maintaining the tocolytic action of non-apoptotic CASP3, thus preventing premature uterine contractility.

Beginning with implantation and continuing until labor, the myometrium experiences various modalities of stress that have been demonstrated in other organ systems to illicit an ERSR. One of the simplest forms of ER stress that may be contributing to this process is a gestationally regulated increase in uterine myocyte protein synthesis. In the pregnant rat, immunoblotting for proliferating cell nuclear antigen in conjunction with bromodeoxyuridine incorporation assays have shown that significant myometrial hyperplasia occurs between gestation days 6-14 and tapers off by E15-16 to accommodate the growing fetus.[429] While there is no direct evidence linking myometrial proliferation to activation of the ERSR, increased protein synthesis leading to an accumulation of unfolded protein has been repeatedly demonstrated to upregulate the

ERSR.[430] During mid-gestation uterine myocytes transition into a hypertrophic state and begin to stretch and increase in size, to further accommodate the growing fetus.[429] Unrelated studies, examining hypertrophic zones of the epiphyseal plate in models of metaphyseal chondrodysplasia, have demonstrated hypertrophy-dependent activation of each canonical ER stress sensors (IRE1α, PERK, and ATF6) further lending evidence that myometrial hypertrophy throughout early and mid-gestation causes activation of the UPR.[431] With increased fetal growth, the myometrium also experiences elevated mechanical stretch and subsequent bouts of transient hypoxia.[432] Both of which have the potential to initiate the ERSR. Finally, prior to the onset of labor the uterine compartment experiences a surge in reactive oxygen species and inflammation, which have also been demonstrated to activate an ERSR. While it is remains elusive which stress insults contribute to the activation of the UPR *in utero*, it is clear that the myometrium is experiencing stress as the ERSR markers XBP1, GADD153, GRP78, and CASP3 are all upregulated at different time points of gestation.[403]

Herein we tested the hypothesis that appropriate *in vivo* preconditioning of the uterine UPR during pregnancy facilitates the maintenance of non-apoptotic CASP3-dependent tocolysis and thus is essential for the regulation of gestational length. To do so, we utilized a novel pregnant mouse model where downstream stress-mediated UPR preconditioning effects were ablated by heightening tolerance to the gestational stresses through the administration of the chemical chaperone phenyl butyric acid (PBA). We observed increased apoptotic CASP3 action in the stressed-sub-preconditioned uterine compartment, resulting in the onset of preterm birth in over 50% of the mice, whereas 83% of endogenously preconditioned mice delivered at term when exposed to the same exogenous stress. Importantly we have discovered the downstream consequences of apoptotic CASP3 action in the uterine compartment to be activation of the inflammatory and prostaglandin-signaling cascades normally associated with the onset of term labor. Overall these studies these findings represent a paradigm shift in our understanding of the regulation of the timing of labor, revealing the critical role endogenous uterine preconditioning plays in promoting the maintenance of uterine quiescence to term by preventing the premature activation of apoptotic CASP3 within the endometrium, inflammatory signaling and thus the onset of luteolysis.

Materials and Methods

Animals

The Institutional Animal Care and Use Committee of Wayne State University approved all animal studies. Timed pregnant female CD-1 mice (6-8wks; gestation day 9) (Charles River Laboratories, Wilmington, MA) were housed in AALAC-accredited facilities according to IACUC guidelines. Accordingly, mice were given a standard pellet diet and water ad libitum.

Tunicamycin and Phenyl Butyric Acid Treatments

PBA was directly dissolved into phosphate buffered saline (PBS) at pH 8.0 (Santa Cruz Biotechnology, Dallas, TX; sc-200652). TM (Calbiochem, San Diego, CA; Cat#654380) was initially dissolved in 20μl 10M sodium hydroxide and then suspended in PBS, pH 8.0. Sub-preconditioned pregnant CD-1 female mice (E10-15) were administered twice-daily intraperitoneal injections (i.p) of 50mg/kg PBA, while preconditioned controls were administered PBS. At E16, stressed mice were administered 0.2mg/kg TM i.p, while controls were given volume matched PBS. Following TM injections, the length of gestation was then monitored and compared between a subset of sub-preconditioned and endogenously preconditioned mice. Uteri, ovaries and serum were harvested at E17 in the additional mice.

Cytosol and Nuclear Protein Fractionation from Tissues

Cytoplasmic and nuclear protein extracts were prepared from frozen mouse tissues by pulverizing the tissues in liquid nitrogen and homogenizing them in ice-cold NE1 buffer (10mM Hepes pH 7.5, 10mM $MgCl_2$, 5mM KCl, 0.1% Triton X-100 with 1X EDTA-free protease/phosphatase inhibitor mini tablet). The homogenate was then centrifuged at 2655 X *g*, the supernatant was retained as the cytoplasmic protein fraction and the pellet was washed in NE1 buffer and suspended in ice-cold NE2 buffer [20mM Hepes pH 7.9, 500mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA pH 8.0, 25% (vol/vol) glycerol with 1X EDTA-free protease/phosphatase inhibitor mini tablet]. The homogenate was vortexed for 30sec every 5min and after 1hr, centrifuged at 10,621 X *g*. The supernatant was then retained as the nuclear fraction. Protein estimation was performed using a BCA assay, equal amounts of protein were loaded for immunoblotting and PDI and NCOA3 were utilized as loading controls for the cytoplasmic and nuclear fractions, respectively.

Immunoblotting and Densitometric Analysis

Equal amounts of protein were separated via electrophoresis on NuPAGE 4-12% gradient precast polyacrylamide gels (Life Technologies, Carlsbad, CA). Proteins were transferred onto Hybond-P PVDF membranes (Millipore, Billerica, MA) and blocked for 1hr at room temperature in 5% non-fat milk prepared in Tris Buffered Saline with 0.1%Tween-20 (vol/vol). Membranes were incubated with primary antibodies overnight at 4°C. Primary antibody concentrations were as follows: GRP78 (1:1000; Cat#3177), Cl CASP3 (1:250; Cat#9664), Cl PARP (1:1000; Cat#9541), pNFκB (1:500; Cat#3033), COX-1 (1:1000; Cat#4841), PDI (1:5000; Cat#3501) and GAPDH (1:1000; Cat#5174) were obtained from Cell Signaling Technologies; iPLA2 (1:1000; Cat#07-169-1) was obtained from Millipore; HSD3B2 (1:1000; Cat#80500) was obtained from Abcam; and NCOA3 (1:5000; Cat#PA1-845) was obtained from ThermoScientific. Following primary incubation, immunoreactivity was detected using horseradish peroxidase-conjugated secondary antibodies and visualized using an enhanced-chemiluminescence detection system (ThermoScientific, Rockford, IL). Immunoreactive band density was then quantified using ImageJ software.

Enzyme-Linked Immunosorbent Assay (ELISA)

The level of progesterone (P4) was then measured in pregnant mouse serum using an ELISA. Specifically, the P4 ELISA Kit (Alpha Diagnostic International, San Antonio, TX, Cat#1955) was performed according to the manufacturer’s instructions and results were read via the Molecular Devices, SpectraMax M2 microplate reader. Each sample measurement was read in duplicate and the computed averages were taken based on the calculated standard curve.

Terminal Deoxynucleotidyl Transferase dUTP Nicked-End Labeling Assay

Tissues collected at E17, imbedded in optimal cutting temperature compound (Sakura Finetek USA Inc, Torrance, CA) were sectioned (10µm thick), mounted onto Superfrost Plus Micro Slides, and stored at -20°C. Sections were removed from storage and fixed in 4% paraformaldehyde for 15 minutes. Additionally, sectioned paraffin wax imbedded tissues were de-paraffinized and rehydrated and treated with 10µg/ml Proteinase K for 15min at 37°C. Analysis of apoptosis in all tissues was quantified using the *In Situ* Cell Death Detection Kit, AP (Roche, Indianapolis, IN, Cat#11684809910) according to the manufacturer’s instructions.

Small Molecule Liquid Chromatography-Mass Spectrometry Analysis

Dissected uterine tissues separated into endometrial and myometrial compartments, flash frozen in liquid nitrogen, were removed from -80°C storage and tissue weights were immediately recorded. Samples were then suspended in 1ml cold PBS pH 7.4, homogenized via bead homogenization, and centrifuged at 10,621 x g for 10 minutes. Supernatants were removed, and protein concentrations were determined using a BCA assay. Equal volume of protein (850µl) was then spiked with 5ng of internal standards suspended in 15% methanol dissolved in water (150µl), mixed thoroughly and purified using a C18 solid-phase cartridges. Prior to applying the sample, the cartridges were first washed with 1ml of 100% methanol followed by 1ml of 15% methanol. After the addition of the sample, tubes were rinsed twice with 1ml of PBS and the rinse was passed through the cartridges. Subsequently, the cartridges were rinsed with 2ml of hexane, vacuum dried for 30sec and proteins were eluted with 1ml of methanol containing 0.1% formic acid. All samples were evaporated to dryness with a gentle stream of nitrogen at 40°C, residues were re-suspended in 30µl methanol and stored at -20°C until LC-MS analysis. Prior to analysis, each sample was further diluted with 30µl 25mM aqueous ammonium acetate. Specific methods utilized for liquid chromatography mass spectrometry can be referenced in Yoon Park et al. 2014.

Immunofluorescence

Tissues collected at E17, embedded in optimal cutting temperature compound (OCT) (Sakura Finetek USA Inc, Torrance, CA) were sectioned (10µm thick), mounted onto Superfrost Plus Micro Slides, and stored at -20°C. Sections removed from storage were fixed in 4% paraformaldehyde for 2 minutes. Fixed sections were incubated with primary antibody overnight at 4°C and examined for primary immunoreactivity using a conjugated secondary antibody. The primary and secondary antibody concentrations were as follows: F4/80+ (1:250, Abcam, Cat#ab6640) diluted in PBS and detected by secondary goat anti-rat antibody conjugated to Alexa Fluor 488 (1:500, Abcam, Cat#150157), NFκB (1:400, Cell Signaling Technologies, Cat#8242) diluted in PBS and detected by secondary donkey anti-rabbit antibody conjugated to Cy3 (1:500, Jackson Immunoresearch, Cat#711-165-152).

Statistical Analysis

All data represent at least three individual experiments performed in triplicate. For the direct comparison of three or more conditions a one-way analysis of variance was performed, with multiple comparisons analyzed via Newmans-Keuls multiple comparisons test. When directly comparing two conditions a student-t test was performed. All comparisons were considered significant with p-values less than 0.05.

Results

Pregnant Mice Display Increased Incidence of Preterm Birth when Exposed to a Minor Exogenous Stress

We examined the timing of labor following a minor stress (0.2mg/kg TM) on gestation day 16 in a sub-preconditioned (TM+PBA) and an endogenously preconditioned (TM) population of timed pregnant mice (n=7 and n=6, respectively). The effects of PBA and vehicle (Con) alone were also examined (n=3 for both groups). Using live-video recording, we observed that 57% of TM+PBA mice (4/7) delivered preterm, with an average delivery time of 30hrs post TM administration (Table 1). In contrast, the mice that experienced normal endogenous gestational stressors prior to the delivery of a minor stress (TM) had a preterm birth rate of 17% (Table 1). No effects were observed in the timing of birth from the mice administered PBA alone (3/3), similar to Con mice (3/3) which delivered at term on E19 (Table 1). All animals that delivered at term resulted in live pups.

Table 1. The Effects of *In Vivo* Preconditioning on Uterine Quiescence

	Term Birth	Preterm Birth	Percent Preterm
Control	3	0	0%
PBA	3	0	0%
TM+PBA	4	3	57%
TM	1	5	17%

Preconditioning Suppresses Pregnant Uterine Inflammatory Signaling *In Vivo*

We examined components of the inflammatory signaling cascade in the uteri collected from Con, PBA, TM+PBA and TM treated pregnant mice at E17 prior to the onset of term and preterm birth. Uterine NFκB activation, macrophage infiltration, COX-1 and COX-2 levels were examined (Figure 8). Premature uterine activation of NFκB occurs in the stressed-sub-preconditioned (TM+PBA) mice prior to the onset of labor; a 2.7 fold increase in p65 nuclear translocation was observed when compared to control animals (Figure 8A). Immunohistochemistry validated the observed increased NFκB activation in stressed-sub-preconditioned mice and revealed the increase in activity occurred within both the myometrial and endometrial compartments (Figure 9A and B, respectively). This is demonstrated by enhanced nuclear translocation of NFκB within the stressed-sub-preconditioned mice compared to controls. While COX-2 levels remained undetectable, COX-1 levels significantly increased over 3 fold in the stressed-sub-preconditioned uteri (TM+PBA) (Figure 8B) in comparison to Con uteri. Macrophage infiltration of the uterine tissue of the Con, PBA, TM+PBA and TM treated mice was examined by F4/80 immunofluorescence analysis and a greater than 10 fold increase in the number of macrophages was observed in the TM+PBA uteri that consequently undergo preterm birth in comparison to endogenously preconditioned uteri (TM and Con) and PBA controls (Figure 8C).

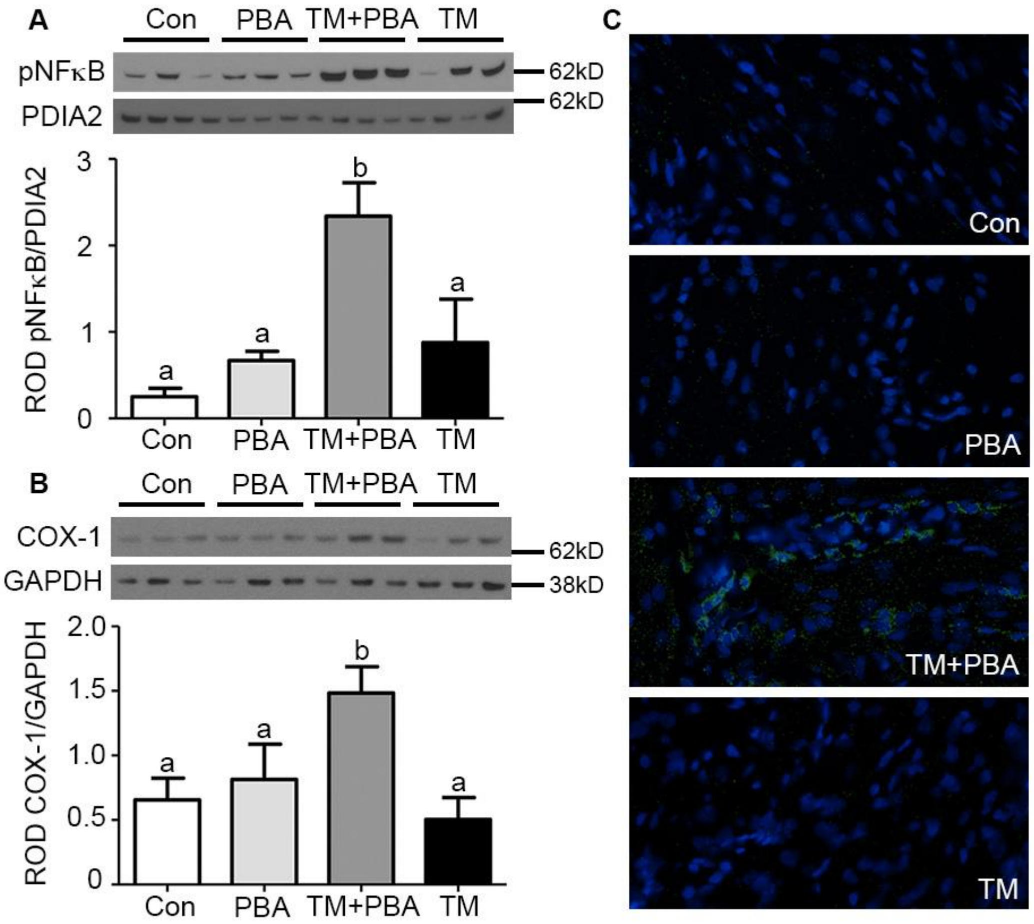


Figure 8. Endogenous preconditioning prevents premature activation of uterine inflammation in the pregnant mouse. Uteri collected from vehicle treated (*Con*), sub-preconditioned (*PBA*), exogenously stressed sub-preconditioned (*TM+PBA*) and exogenously stressed preconditioned (*TM*) mice on E17 prior to the onset of preterm or term birth were examined for **(A)** NFκB, **(B)** COX and **(C)** macrophage infiltration. Increased NFκB activation, COX1 expression and elevated levels of macrophage infiltration were isolated to the *TM+PBA* uteri. PDIA2 and GAPDH are utilized as cytoplasmic loading controls. A representative blot or immunohistochemical image from each experiment is shown. Statistical comparisons were performed using one-way ANOVA, and subsequent Newman-Keuls multiple-comparison tests. Data labeled with different letters are significantly different from each other (p<0.05).

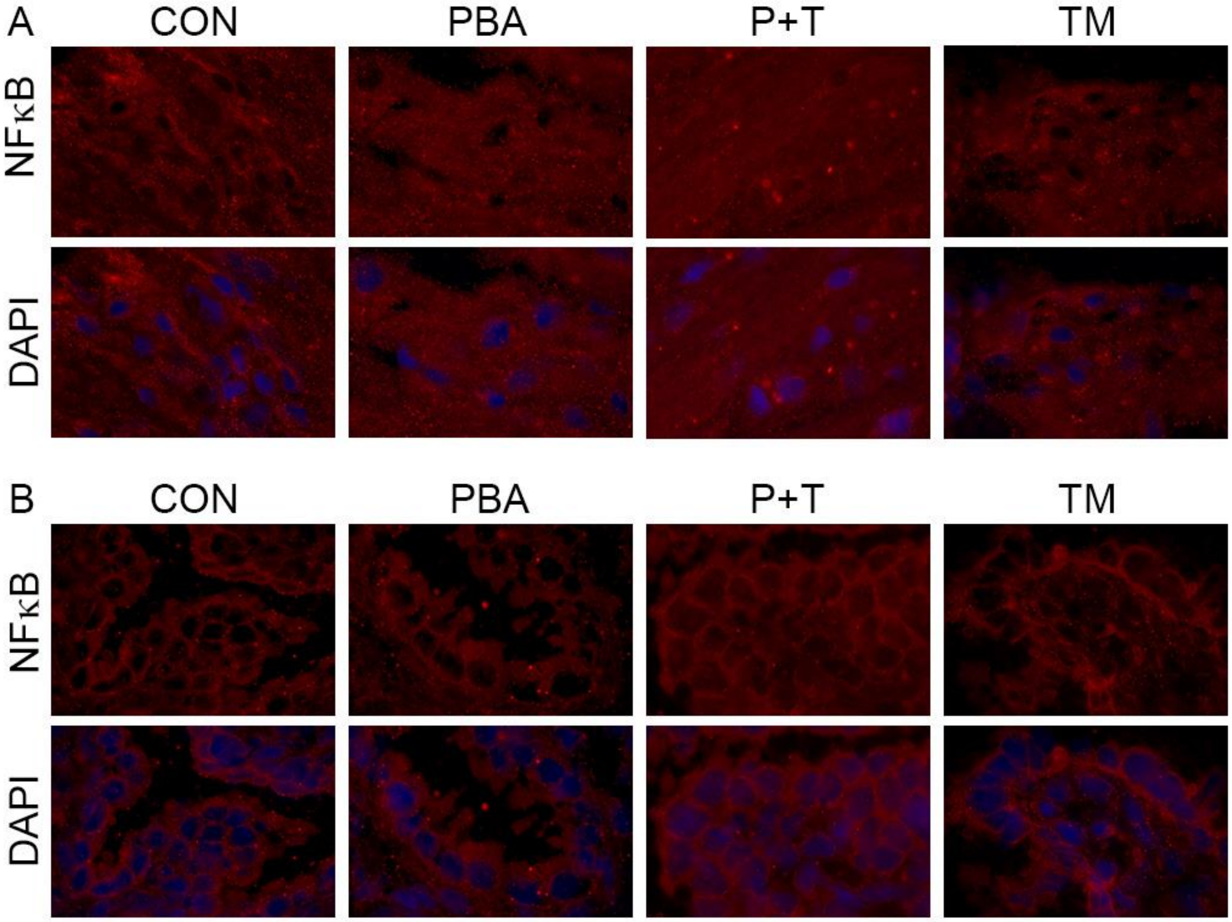


Figure 9. Endogenous preconditioning prevents premature activation of NFκB in the myometrial and endometrial compartments of the pregnant mouse. Uteri collected from vehicle treated (*Con*), sub-preconditioned (*PBA*), exogenously stressed sub-preconditioned (*TM+PBA*) and exogenously stressed preconditioned (*TM*) mice on E17 prior to the onset of preterm or term birth were examined for activation of NFκB in the **(A)** myometrium and **(B)** endometrial compartments via immunohistochemistry. Heightened NFκB activation was observed in both the **(A)** myometrium and **(B)** endometrium of the *TM+PBA* uteri.

Preconditioning Suppresses Apoptotic CASP3 Activity in the Pregnant Uterus *In Vivo*

Uteri isolated from Con, PBA, TM+PBA and TM mice were examined prior to the onset of term or preterm labor at E17 by immunoblotting for CASP3 activation. Levels of CASP3 activation were not significantly changed between the 4 groups examined (Figure 10A). However, the stressed-sub-preconditioned uteri (TM+PBA) demonstrate increased incidence of apoptotic CASP3 activation as indicated by a 4.6 fold increase in the levels of uterine PARP cleavage (Figure 10B) when compared to endogenously preconditioned

(TM and Con) and PBA controls uteri. Positive terminal deoxynucleotidyl transferase dUTP nicked-end labeling (TUNEL) staining in the sub-preconditioned uteri isolated from the endometrial compartment, but not endogenously preconditioned (TM and Con) and PBA controls uteri validated these results (Figure 10C).

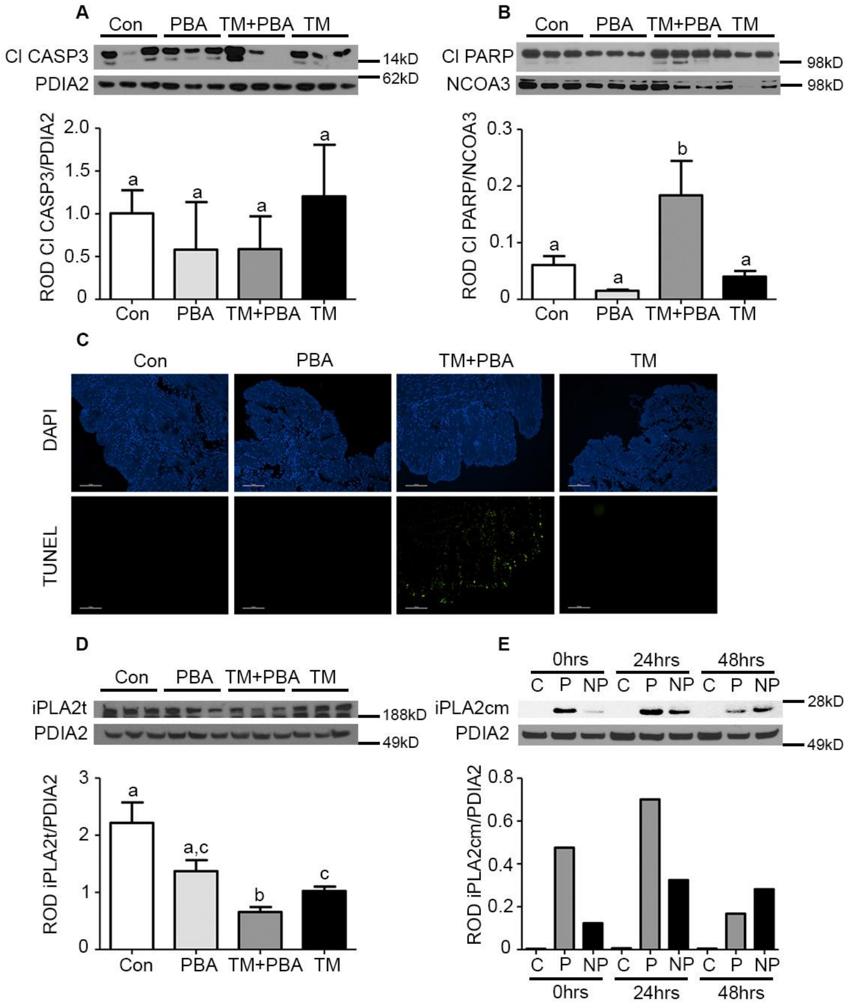


Figure 10. Endogenous preconditioning facilitates the maintenance of non-apoptotic CASP3 and suppresses iPLA2 activation in the pregnant mouse uterus. Uteri collected from vehicle treated (*Con*), sub-preconditioned (*PBA*), exogenously stressed sub-preconditioned (*TM+PBA*) and exogenously stressed preconditioned (*TM*) mice on E17 prior to the onset of preterm and term birth were examined for **(A)** active Cl CASP3. **(B)** Cl PARP and **(C)** TUNEL staining were used as a measure of apoptotic cell death. iPLA2t levels as an indirect measure of iPLA2 activation **(D)**. Cl CASP3 levels remained unchanged across all 4 groups examined however increased Cl PARP and TUNEL activity and decreased levels of the inactive iPLA2t were isolated to the TM+PBA treated mice. **(E)** In the hTERT-HM cells the cleaved active monomeric form of iPLA2 (iPLA2cm) was elevated in a relative manner to the levels of apoptotic CASP3 present in the preconditioned and non-preconditioned cells (Fig. 1A). A representative blot or image from each experiment is shown. PDIA2 and NCOA3 are utilized as cytoplasmic and nuclear protein loading controls. Statistical comparisons were performed using one-way ANOVA, and subsequent Newman-Keuls multiple-comparison tests. Data labeled with different letters are significantly different from each other ($p<0.05$).

As CASP3 has been demonstrated to activate calcium-independent phospholipase A2 (iPLA2), we further examined iPLA2 expression in the presence or absence of preconditioning *in vivo* and *in vitro* via immunoblotting (Figure 10). We observed a 2 fold decline in the homotetromeric non-active form of iPLA2 (Figure 10D) in the apoptotic CASP3 positive (Figure 10A and B) TM+PBA uteri in comparison to the Con and PBA treated uteri. In the hTERT-HM we were able to detect the cleaved active form of iPLA2 and observed a 2 fold increase (Figure 10E) isolated to the non-preconditioned cells which also display elevated levels of apoptotic CASP3 as indicated by the pattern of PARP cleavage in Figure 1A.

Preconditioning Prevents Premature Prostaglandin (PG) Production

Uteri isolated from endogenously preconditioned (Con), sub-preconditioned (PBA), stressed-sub-preconditioned (TM+PBA) and stressed-endogenously preconditioned (TM) containing both the endometrial and myometrial compartment were mice examined at E17 for prostaglandin production utilizing targeted-small molecule liquid chromatography and tandem mass spectrometry. Significantly elevated levels of PGE2, PGE1 and PGD3 were isolated to the sub-preconditioned mice exposed to a minor exogenous stress (TM+PBA) (Figure 11A, B and D). Furthermore, downstream by-products of arachidonic acid metabolism, e.g. 11-HETE and 13-HODE, were also significantly elevated in sub-preconditioned mice compared to preconditioned controls (Figure 12). As apoptotic CASP3, as measured by TUNEL, and presumably iPLA2 activity, is contained within the endometrial compartment, we next further examined prostaglandin production specifically within endometrial and myometrial tissue collected from TM+PBA and Con mice on E17. Results demonstrated elevated prostaglandin synthesis (i.e. PGE2, PGD3 and PGA2) within the endometrium of sub-preconditioned stressed mice (TM+PBA) compared to the myometrial compartment (Figure 13). These differences do not appear between the control mice, suggesting CASP3 must be in an apoptotic state to induce iPLA2-dependent increases in prostaglandin production.

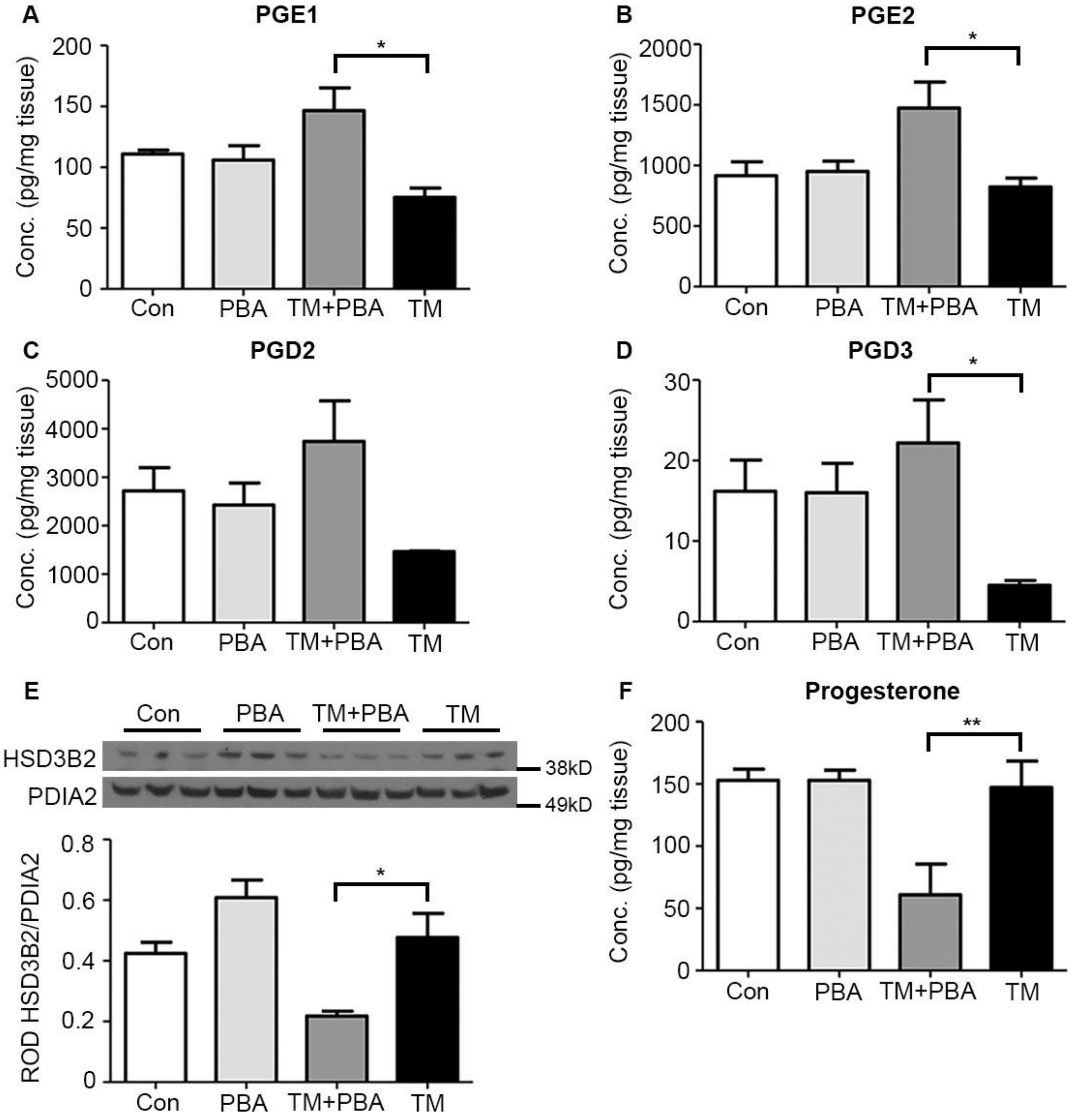


Figure 11. Preconditioning facilitates the suppression of prostaglandin synthesis thereby preventing premature luteolysis and P4 withdrawal. Uteri collected from vehicle treated (*Con*), sub-preconditioned (*PBA*), exogenously stressed sub-preconditioned (*TM+PBA*) and exogenously stressed preconditioned (*TM*) mice on E17 prior to the onset of preterm and term birth were examined for prostaglandin production. Significantly elevated levels of **(A)** PGE1, **(B)** PGE2 and **(D)** PGD3 were isolated to TM+PBA uteri. **(E)** Ovarian HSD3B2 and **(F)** serum P4 levels were significantly decreased in the TM+PBA treated mice. A representative blot from each experiment is shown. PDIA2 is utilized as cytoplasmic protein loading control. Statistical comparisons were performed using one-way ANOVA, subsequent Newman-Keuls multiple-comparison tests and student-t test. *p≤0.05 and **p≤0.01 compared with controls.

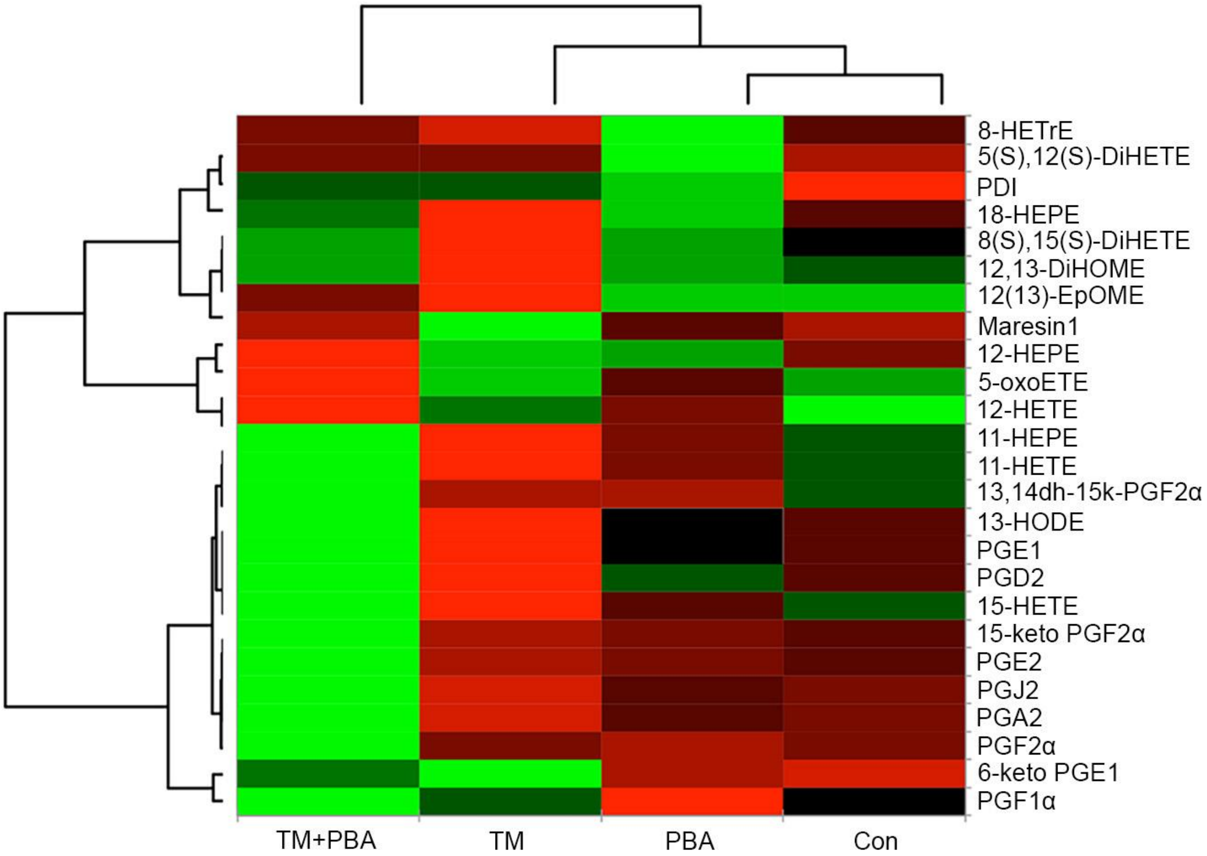


Figure 12. UPR Preconditioning *in vivo* suppresses local uterine prostaglandin production. Uteri collected from vehicle treated (Con), sub-preconditioned (PBA), exogenously stressed sub-preconditioned (TM+PBA) and vehicle treated (TM) mice on E17 prior to the onset of preterm and term birth, were examined for prostaglandin levels. Significantly elevated levels of PGE1, PGE2, PGD3 were isolated to the sub-preconditioned mice exposed to a minor exogenous stress (TM+PBA). Further, downstream products of arachidonic acid metabolism were also elevated in stressed-sub-preconditioned mice compared to preconditioned control.

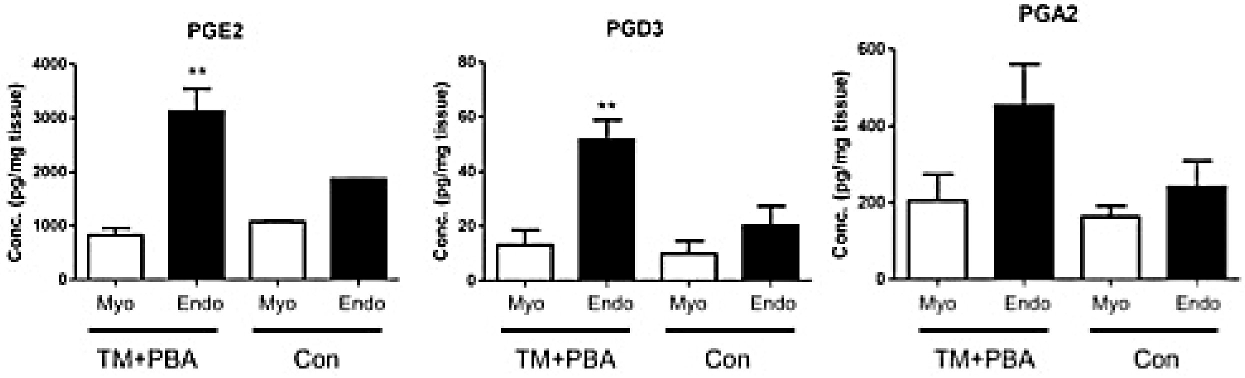


Figure 13. Premature apoptotic CASP3 in the endometrium increases prostaglandin synthesis. Myometrial (Myo) and endometrial (Endo) tissue collected separately from exogenously stressed sub-preconditioned (*TM+PBA*) and control (Con) mice on E17 prior to the onset of preterm and term birth were examined for prostaglandin production. Concentrations of PGE2 and PGD3 were significantly elevated in the Endo isolated from TM+PBA uteri. Statistical comparisons were performed between Myo and Endo samples using a student-t test. *p≤0.05 and **p≤0.01 compared with controls.

Preconditioning Prevents Premature Luteolysis and P4 Withdrawal

Ovaries collected from Con, PBA, TM+PBA and TM treated pregnant mice on E17 were analyzed for HSD3B2, which declines with the onset of luteolysis, as discussed in Chapter 1 Luteolysis and Progesterone Decline in Lower Mammalian Species. As seen in Figure 11E, there was a significant reduction (2.2 fold) in HSD3B2 expression in the ovaries of the TM+PBA mice in comparison to Con, PBA and TM ovaries. To validate premature luteolysis in TM+PBA pregnant mice, serum collected on E17 from each cohort of mice was analyzed with ELISA for circulating P4 levels. As observed in the TM+PBA mice that undergo preterm delivery (Table 1), there was a significant decline (2.4 fold) in circulating P4 levels in comparison to Con, PBA and TM treated mice (Figure 11F).

Discussion

Our laboratory, as well as others has proven biological preconditioning to be viable mechanism for the maintenance of non-apoptotic CASP3. As CASP3 tocolysis plays an integral role in inhibiting myometrial contractility in the absence of apoptosis, we hypothesize that endogenous cellular stress throughout the course of a normal pregnancy is acting in a preconditioning-like manner to support its non-apoptotic action. In this aim we demonstrate that appropriate preconditioning of the uterine UPR during pregnancy promotes prolonged uterine myocyte quiescence through the suppression of inflammation and apoptotic CASP3. In turn, we demonstrate that apoptotic CASP3 activation in the sub-preconditioned stressed mice mediates activation of two interdependent signaling pathways, the inflammatory and iPLA2-prostaglandin signaling cascade leading to a significant increase in the proportion of mice that delivered preterm. Overall this work shows that appropriate UPR preconditioning within the pregnant uterus prevents early onset activation of the normal signaling cascades associated with normal term labor, e.g. apoptotic CASP3-inducted iPLA2/prostaglandin signaling, to inhibit premature luteolysis and preterm labor. Thus, we propose pregnant women may be placed at increased risk for precocious apoptotic CASP3 activation and a heightened incidence of preterm birth in the absence of appropriate preconditioning.

Our previous *in vitro* studies revealed UPR preconditioning facilitates the maintenance of non-apoptotic CASP3 in uterine myocytes. To test whether stress across gestation preconditions the uterine myocyte to capacitate the tocolytic function of CASP3 in its non-apoptotic state, we removed the downstream action of endogenous preconditioning stimuli by alleviating the ER protein load with reoccurring treatments the chemical chaperone PBA. Herein we found the absence of appropriate UPR preconditioning across gestation placed stressed-sub-preconditioned pregnant mice (TM+PBA) at an increased risk of preterm birth (57%) in comparison to mice that experienced normal endogenous preconditioning prior to the delivery of a minor exogenous stress (TM), which displayed a preterm birth rate of only 17% (Table 1). As anticipated, these results suggest the preconditioning-action of endogenous pregnancy related stress regulates gestational length and is necessary for the maintenance of uterine quiescence. As non-apoptotic CASP3 action has previously been demonstrated to maintain quiescence, we looked to characterize the state of CASP3 in our sub-preconditioned (TM+PBA) and preconditioned (TM) stressed mice. Analysis of CASP3-dependent apoptosis and activity in the pregnant uterus reveal preconditioning protects the endogenously stressed uterine compartment from undergoing a precocious apoptotic CASP3 mediated cell death. While uterine CASP3 activation remained unmodified across treatments (Figure 10A), the stressed-sub-preconditioned mice (TM+PBA) displayed increased uterine apoptotic CASP3 as indicated by both increased PARP cleavage

(Figure 10B) and elevated TUNEL staining, like that observed in laboring tissue on E19. Importantly, apoptotic CASP3 action was observed primarily within the endometrial compartment (Figure 10C), similar to our previous studies demonstrating complete avoidance of myometrial apoptosis prior to labor and post-partum.[433]

As the endometrial compartment is non-apoptotic prior to the induction of labor, as seen in Con, PBA and TM stressed mice we next looked to identify the role of apoptotic CASP3 found within the endometrium of stressed-sub-preconditioned mice. It has previously been established that CASP3 activity is a critical upstream component in prostaglandin production through cleavage and activation of iPLA2 allowing the release of free arachidonic acid to be converted into prostaglandins in a COX1/2 and NFκB dependent manner, as discussed previously in Chapter 1 Luteolysis and Progesterone Decline in Lower Mammalian Species and observed in Figure 10.[434,435] Specifically, iPLA2 exists in three separate states 1) an inactive tetrameric form that is approximately 350kD, 2) an active 72kD monomeric form and 2) a highly active truncated form weighing roughly 25kD.[436] Within the active monomeric form there are multiple putative CASP3 cleave motifs, in which CASP3 targets and cleaves, resulting in truncated iPLA2 that has increased activity compared to monomeric iPLA2.[436] Interestingly, a study by Huang and colleagues in breast cancer cells (4T1) demonstrated that only apoptotic CASP3 action was sufficient for the activation of iPLA2-induced prostaglandin production.[348] If cells were not actively undergoing apoptosis or lacking iPLA2 no changes were observed in prostaglandin signaling. The production of uterine prostaglandins is an extremely important process in the induction of labor, as prostaglandin signaling 1) stimulates luteolysis resulting in the decline in circulating progesterone and 2) increases intracellular calcium levels in the myocyte necessary for uterine contraction, as described in Chapter

1 Smooth Muscle Contraction. Interestingly, this study reveals that apoptotic CASP3 activity is required for appropriate initiation of prostaglandin synthesis while non-apoptotic CASP3 though active is unable to do so. Upon apoptotic CASP3 activation (TM+PBA) we observed decreased levels of the inactive uterine iPLA2 *in vivo* (Figure 10C, D). *In vitro* the cleaved active form of iPLA2 was readily detectable (Figure 10E) and found to be significantly upregulated in the presence of apoptotic CASP3 and reduced in the presence of non-apoptotic CASP3 (48hr P versus NP Figure 1A). As iPLA2 functions to increase the concentration of free arachidonic acid, these data suggest activation of apoptotic CASP3 should increase prostaglandin synthesis. Subsequently, we observed concomitant to increased apoptotic CASP3 action within the endometrium of stress-sub-preconditioned mice, increased prostaglandin signaling within the endometrial compartment compared to the non-apoptotic CASP3 positive myometrial compartment (Figure 13).

Typically, increased uterine inflammatory and prostaglandin signaling herald the end of gestation and are normally associated with the onset of labor in both human and mouse.[437] Specifically, NFκB-dependent activation of COX1/2 leads to increased prostaglandin synthesis and thus enhanced uterotonic sensitivity. In preterm and term pregnancies alike, activation of NFκB is observed prior to the onset of labor.[438] Our previous *in vitro* data suggest that the lack of UPR preconditioning allows for precious activation of inflammatory signaling (Figure 5). Further, multiple studies examining inflammation-derived diseases have demonstrated that preconditioning can abrogate stress-induced inflammatory signaling. In a study analyzing the use of heat preconditioning for the protection of kidney tubules against ischemia /reperfusion injury in mice, preconditioning was found to significantly blunt the activation of NFκB through the inhibition of IκB kinase and inhibit tubular cell apoptosis.[439] Similarly, Zhang and colleagues demonstrate ER stress preconditioning reduces TNFα-dependent vascular leakage in the mouse retina. Based on *in vitro* studies, they postulate the mechanism of TNFα inhibition is likely inhibition of NFκB activation. In this study we observed that appropriate endogenous preconditioning helps maintain the uterus' capacity to suppress inflammatory signaling cascades late in gestation. Specifically, in Figure 8A both the myometrium and endometrium of the TM+PBA mice demonstrated significantly elevated levels of NFκB activation compared to the uteri of preconditioned controls (Figures 8A and 9). As the result of increased NFκB activation, uteri from sub-preconditioned stressed mice were also found to have increased COX1 expression and macrophage infiltration, demonstrating an overall heightened inflammation (Figure 8B and C). Subsequently, heightened inflammation and augmented NFκB-dependent increases in COX1 expression resulted in increased prostaglandin production, allowing for the conversion of free arachidonic acid into PGG2 and subsequently PGH2 in stressed-sub-preconditioned mice (TM+PBA mice) (Figure 12). Importantly, precocious prostaglandin production (Figure 11) within the TM+PBA mice was found to trigger premature luteolysis as evidenced by decreased ovarian HSD3B2 (Figure 11E).[440] Consequently, luteolysis decreased circulating P_4 levels (Figure 11F), which lead to the onset of preterm birth within the stressed-sub-preconditioned (TM+PBA) mice (Table 1).

Overall these data demonstrate that normal endogenous uterine preconditioning, acting to suppress premature apoptotic CASP3 activation and inflammation, is for the first time placed upstream of regulating the timing of normal parturition by actively preventing the well-established endogenous uterotonic signaling cascades such as prostaglandin synthesis, luteolysis and consequently P4 withdrawal that herald the onset of normal term labor in the pregnant mouse. These findings are critical for the future advancement of tocolytic therapies, as they provide a solid foundation for the mechanism in which non-apoptotic CASP3 can fulfill its tocolytic function in the uterine myocyte in the absence of myometrial apoptosis and how apoptotic CASP3 is associated with the onset of normal signaling cascades that result in term labor, which may be being altered in the context of preterm birth.

CHAPTER 4

Introduction

One mechanism in which effective remote preconditioning occurs is through the dissemination of a protective signal with the secretion of a unique paracrine or endocrine secretome from cells experiencing the original prophylactic stress.[396] It is well characterized that cells of many varieties respond to stress by the secretion of a unique secretome.[441-443] In the pregnant mouse model, we have demonstrated that preconditioning-like stress events that activate the UPR and are experienced by the pregnant uterus across gestation are critical for maintaining non-apoptotic CASP3-dependent tocolysis and inhibition of local uterine inflammation. Separate studies examining the function of the UPR-generated secretome have found secretory factors such as GRP78 can inhibit systemic inflammation though the modulation of circulating peripheral mononuclear cells. Thus, we propose preconditioning-like events result in the generation of a novel preconditioned uterine secretome, which acts to propagate the tocolytic phenotype in an endocrine and paracrine manner. In this aim we looked to 1) characterize the stress-mediated myometrial secretome and 2) elucidate the physiological function of UPR-mediated protein secretion during normal and pathological pregnancies.

Preeclampsia is a common cardiovascular disease distinguished by the onset gestational hypertension that affects approximately 3-8% of pregnancies and remains one of the leading cause of maternal death in the United States.[444] Women affected by preeclampsia are at risk for intracerebral hemorrhage, placental abruption, and intrauterine death.[445] Additionally, babies born from preeclamptic pregnancies have an increased chance of intrauterine growth retardation.[445] Clinically, the major diagnostic symptom associated with preeclampsia in humans is an increased mean arterial pressure (MAP) greater than 140/90mmHg, as seen with high peripheral vascular resistance and decreased cardiac output.[446] Along with increased MAP, women with preeclampsia also frequently present with increased proteinuria, peripheral edema and liver dysfunction.[446,447] It has been long speculated that preeclampsia is initiated by placental stress-mediated inflammation leading to endothelial dysfunctional, which further results in improper spiral artery invasion into the uterus, increased maternal peripheral resistance and a further decline in perfusion pressure of the placenta.[448,449] More specifically, placental ischemia in preeclamptic pregnancies increases the activation of the UPR within the placenta of preeclamptic compared to normal pregnancies.[450,451] In conjunction with increased levels of oxidative stress, also found in the placenta during preeclampsia pregnancies, ER stress augments the production and secretion of placental inflammatory cytokines, such as TNFα, IL-1β, IL-6 and IL-10, 2).[452] Placental cytokines subsequently increase 1) systemic activation of granulocytes and monocytes, 3) circulating reactive oxygen species 3) and diminished concentrations of circulating vascular endothelial growth factor which is all thought to contribute to maternal endothelial damage.[449] Moreover, an overall shift in the inflammatory cytokines within the extracellular milieu drives polarization of decidual macrophages into an M1 or inflammatory state further promoting trophoblast cell death and endothelial dysfunction.[453] While there are limited studies examining the role of ER stress in endothelial damage in the context of preeclampsia, it has been extensively studied and identify as an important component in atherogenesis.[454,455] In turn endothelial dysfunction results in inappropriate spiral artery remodeling and thus poor placentation during the first half of pregnancy and manifests as the outward clinical symptoms listed above during the second half of the pregnancy.[456,457]

Additionally, the proteomic profile of urine from women with preeclampsia identified differential secretion of many proteins associated with UPR protein compared to normal controls.[458] Interestingly, women who smoke prior to and during their pregnancies 1) have a decreased risk of developing preeclampsia and 2) tend to have less severe symptoms when developing preeclampsia.[472] As smoking acts as a transient hypoxic stress, primarily to the lungs, we suspect smoking may be acting in a remote preconditioning-like manner to increase the tolerance of the maternal vasculature and potentially the placenta to increased ER stress, as seen with preeclampsia. Overall, preeclampsia can be characterized by precocious activation of the ERSR within placenta that result in heightened systemic inflammation and subsequently the onset of endothelial dysfunction and reduced placental perfusion.

The secretion of a signature set of proteins following cellular insults, such as inflammatory cytokines or ER stress, is a primitive form of cell-to-cell communication that has been demonstrated to provide prophylactic cellular adaption in a paracrine and endocrine manner. Defining discrete clusters of proteins secreted from cells during specific normal and physiological states has become a useful for the discovery of circulating biomarkers, as well as the development of novel therapeutics.[441,459] A simple was to characterize a novel secretome for the first time is to utilize *in vitro* cell culture together with high-throughput protein quantification methods like liquid chromatography tandem mass spectrometry (LC/MS/MS). One caveat with this method however, is that fetal bovine serum which is typically necessary to sustain cell viability in culture, contains an abundant level of innate proteins that can act to mask the unknown secretome or inhibit the identification of proteins being secreted from the cell type of interest. To resolve this issue protein labeling techniques such as stable isotope amino acid labeling in culture

(SILAC), allow for selective targeted protein identification.[460] As many amino acids have more than one isotope present, it is possible when using SILAC to directly quantify and compare differences in secreted protein concentrations between two or more distinct protein sets.[461] Utilizing SILAC techniques, Gronburg and colleague distinguished a unique set of 145 proteins secreted from cancerous pancreatic ductal cells, including several that had never been associated with pancreatic cancer, that may be effective as potential biomarkers for clinicians and early pancreatic cancer screens.[442] Of interest, a similar study defining the stress-generated secretome in pancreatic islet cells, found the secretion of multiple proteins that aid in sustaining adaptive UPR-signaling responses.[443]

The propagation of systemic adaptive signaling responses during the process of remote preconditioning provides protective cellular responses to a secondary remote tissue.[394,396] While the mechanisms of remote preconditioning have not been fully elucidated, multiple studies suggest the secretion of humoral factors (i.e. proteins, peptides, ssRNA or DNA) from the cells being directly conditioned can interact with and mount intracellular signaling responses in secondary cells/tissues.[330,462] Importantly, many times the activation of intracellular signaling responses in remote tissues has been proven to be cytoprotective against subsequent stresses. One of the earliest studies demonstrated this, by Przyklenk and colleagues found effluent taken from ischemic myocardial tissues was effective in remotely preconditioning naïve myocardial against subsequent damaging ischemic events.[463] The use of remote ischemic preconditioning has since been greatly expanded upon, proving to be effective in protecting additional parenchymal tissues as well, including but not limited to the kidneys, lungs and ovaries.[464] In addition to remote ischemic preconditioning, recent studies show targeted UPR preconditioning is also effective in affording cytoprotection to remote secondary tissues, as previously discussed in Chapter 1 Remote Preconditioning of the Endoplasmic Reticulum Stress Response. Overall, these studies strongly suggest that low dose stress can successfully propagate a circulatory signal that induces systemic cellular adaptation.

In this aim we examined the ability of myometrial cells undergoing active ER stress to transmit a distinct UPR-derived secretome and test whether the propagation of the secretome has any paracrine and/or endocrine function, particularly in modulating remote cellular UPR and systemic inflammation. Initially, the secretome from TM-treated hTERT-HM cells that were labeled with stable isotope amino acid labeling in culture (SILAC), was examined via liquid chromatography tandem mass spectrometry and compared to the secretome of non-stressed cells. LC/MS/MS identified over 90 validated secreted proteins, which were bone-fide components of the UPR activated uterine myocyte secretome. Of interest, the secretion of GRP78 was substantially increased by stress and this was validated using ELISA. For preliminary analysis of the bioactivity of the stress-generated secretome, conditioned media (stress and vehicle conditioned) was incubated with a secondary set of naïve hTERT-HM cells and activation of the UPR in the naïve hTERT-HM cells was quantified. Results revealed that the UPR mediated secretome actively propagated activation of the UPR in the naïve untreated uterine myocytes, as observed by increased GRP78, GADD153 and active CASP3. Conclusively, as the pathology of preeclampsia has been tightly linked to systemic inflammation stemming from exaggerated ER stress within the placenta, we examined UPR proteins within the serum of women with and without preeclampsia. As cigarette smoking prior to and during pregnancy has been identified as protective against developing preeclampsia, we further analyzed differences between serum from women that participated in or refrained from cigarette smoking, with or without preeclampsia. Preliminary results suggest serum

GRP78 and GADD153 levels are altered in a stress-dependent manner and that increased circulating GRP78 may promote maternal anti-inflammatory signaling to decrease the risk of preeclampsia. Consequently, we propose that endocrine and/or paracrine transmission and propagation of the uterine myocyte UPR allows for local uterine myocyte tissue type fidelity and systemic conditioning of the vasculature and immune response during pregnancy.

Methods

Cell Culture

For the *in vitro* cell culture model system we utilized hTERT-HM cells.[413] hTERT-HM cells were cultured in Dulbecco modified Eagle/F12 low glucose media (DMEM-F12) (Invitrogen Carlsbad, CA), supplemented with 10% fetal bovine serum (vol/vol) (Invitrogen) and antibiotic/antimycotic (10,000 U/ml; Invitrogen), and incubated at 37°C with 95% air and 5% CO_2.

Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC)

hTERT-HM cells were grown in DMEM/F12 (Invitrogen) media with 10% (vol/vol) dialyzed fetal bovine serum (Invitrogen) in preparation for SILAC and supplemented with antibiotic/antimycotic (10,000U/ml; Invitrogen) at 37°C in a 5% CO_2 incubator. Cells were then passaged every three days, after reaching 70-80% confluency, for a total of 6 passages in the SILAC media with heavy labeled arginine and lysine or SILAC media with light labeled arginine and lysine (Thermo Scientific, Cat# 1862636). Specifically, heavy SILAC media was prepared by combining 500mls DMEM/F12 media with 10% dialyzed FBS, antibiotic/antimycotic (10,000U/ml), 50mg of $^{13}C_6$ L-Lysine-2HCl and 50mg of $^{13}C_6$ L-Arginine-HCl. Whereas, light SILAC media was prepared by combining 500mls DMEM/F12 media with 10% dialyzed FBS, antibiotic/antimycotic (10,000U/ml), 50mg of

L-Lysine-2HCl and 50mg of L-Arginine-HCl. Following 6 passages in SILAC media, it is assumed that incorporation of heavy L-lysine and heavy L-arginine should be greater than 95%.[465] Subsequently, the incorporation of the heavy amino acids into newly synthesized peptides leads to 6Da mass shift compared to non-labeled peptides, easily picked up with LC/MS/MS proteomic analysis.[460]

Tunicamycin Treatments and Media Conditioning

For all *in vitro* experiments, TM was suspended in 20μl 10M sodium hydroxide and brought to a final concentration of 1.0μg/ml in DMEM-12 media with 10% FBS and antibiotic/antimycotic. To analyze the UPR-generated secretome, heavy-labeled and light-labeled SILAC treated hTERT-HM cells, were treated with 5.0μg/ml TM for 24hrs, washed three times and incubated with fresh media. After 24hrs of conditioning, the fresh media (TM-CM) containing SILAC-labeled proteins was removed and analyzed via LC/MS/MS. Additionally, control media collected after 24hr incubations with heavy-labeled and light-labeled SILAC treated hTERT-HM cells, was analyzed via LC/MS/MS.

To analyze the function of the UPR-generated secretome, hTERT-HM cells were treated with 5.0μg/ml TM or vehicle (volume matched sodium hydroxide media) for 24hrs, washed three times and incubated with fresh media. After 24hrs of conditioning, the fresh media (TM-CM or vehicle conditioned control media) was collected for analysis or placed on second set of naïve hTERT-HM cells. The second set of naïve hTERT-HM cells were collected after 48hrs of incubation with the TM-CM or vehicle conditioned control media.

To validate UPR propagation was not induced by TM-contamination, hTERT-HM cells were treated with 5.0μg/ml TM for 0, 24 or 48hrs. However, in this experiment the TM was prepared in media containing 0, 5 or 10% FBS. After TM treatment, hTERT-HM were washed three times like normal and incubated with fresh media. After 24hrs of conditioning, the fresh media was placed on a second set of naïve hTERT-HM cells. The second set of naïve hTERT-HM cells were collected after 48hrs of incubation with the 0, 1 or 24hr TM-CM.

Cytosol and Nuclear Protein Fractionation from Cells

Cytoplasmic and nuclear protein fractions from hTERT-HM cells were prepared as previously mentioned. Initially, cells were rinsed in ice-cold PBS and centrifuged at 956 X *g*. The pellet was re-suspended and evenly homogenized in ice-cold NE1 buffer (10mM Hepes pH 7.5, 10mM $MgCl_2$, 5mM KCl, 0.1% Triton X-100 with 1X EDTA-free protease/phosphatase inhibitor mini tablet). The homogenate was then centrifuged at 2655 X *g*, the supernatant was retained as the cytoplasmic protein fraction and the pellet was washed in NE1 buffer and suspended in ice-cold NE2 buffer [20mM Hepes pH 7.9, 500mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA pH 8.0, 25% (vol/vol) glycerol with 1X EDTA-free protease/phosphatase inhibitor mini tablet]. The homogenate was vortexed for 30sec every 5min and after 1hr, centrifuged at 10,621 X *g*. The supernatant was then retained as the nuclear fraction. Protein estimation was performed using a BCA assay, equal amounts of protein were loaded for immunoblotting and PDI and NCOA3 were utilized as loading controls for the cytoplasmic and nuclear fractions, respectively.

Immunoblotting and Densitometric Analysis

Equal amounts of protein were separated via electrophoresis on NuPAGE 4-12% gradient precast polyacrylamide gels (Life Technologies, Carlsbad, CA). Proteins were transferred onto Hybond-P PVDF membranes (Millipore, Billerica, MA) and blocked for 1hr at room temperature in 5% non-fat milk prepared in Tris Buffered Saline with 0.1%Tween-20 (vol/vol). Membranes were incubated with primary antibodies overnight at 4°C. Primary antibody concentrations were as follows: GRP78 (1:1000; Cat#3177), Cl

CASP3 (1:250; Cat#9664), GADD153 (1:500; Cat#5554), Cl PARP (1:1000; Cat#9541), AFT4 (1:500; Cat#11815), p-eIF2α (1:500; Cat#3398), p65 (1:1000; Cat#8242), XIAP (1:250; Cat#2042), PDI (1:5000; Cat#3501) and GAPDH (1:1000; Cat#5174) were obtained from Cell Signaling Technologies; XBP1s (1:500; Cat#37152) was obtained from Abcam; ATF6 (1:500; Cat#24169-1-AP) was obtained from Proteintech; MCL-1 (1:1000; Cat#sc-819) was obtained from Santa Cruz Biotechnology, and NCOA3 (1:5000; Cat#PA1-845) was obtained from ThermoScientific. Following primary incubation, immunoreactivity was detected using horseradish peroxidase-conjugated secondary antibodies and visualized using an enhanced-chemiluminescence detection system (ThermoScientific, Rockford, IL). Immunoreactive band density was then quantified using ImageJ software.

Liquid Chromatography Tandem Mass Spectrometry

All SILAC labeled media samples underwent 40% ethanol precipitation for 2hrs, followed by 10,000g centrifugation to remove albumin. The heavy and light SILAC labeled precipitates were then washed, resuspended in 2% lithium dodecasulfate and subjected to high-energy sonification. Protein concentrations were determined utilizing the bicinchoninic acid method. Equal amounts of protein from SILAC heavy and light lysates were combined, reduced with dithiothreitol and then alkylated with iodoacetamide. SILAC samples were initially separated with SDS-PAGE on 10% polyacrylamide gels. Each sample lane was divided into 21 fractions and excised from the gel. The protein gels were trypsin digested overnight, proteins were eluted from the gel and then solubilized in 0.1% formic acid. Subsequent samples underwent reverse phase liquid chromatography using an Easy nLC ultra-high-pressure liquid chromatography system (Thermo). Collected effluent fractions were then ionized with an ADVANCED Ion Source (Michrom) and introduced into an LTQ-XL linear ion trap mass spectrometer. Peptide concentrations were analyzed with Proteome Discover using the Mascot search algorithm and further analyzed using MaxQuant.

Enzyme-Linked Immunosorbent Assay (ELISA)

The level of GRP78 and GADD153 were measured in serum taken from pregnant women who with or without clinically diagnosed preeclampsia, who did or did not participate smoke cigarettes while pregnant using an ELISA. Specifically, the GRP78/BiP ELISA Kit (Enzo Life Sciences Inc., Farmingdale, NY, Cat# ADI-900-214) and the Human DDIT3 ELISA Kit (LifeSpan Biosciences Inc., Seattle, WA, Cat#LS-F11284) were performed according to the manufacturer's instructions and results were read via the Molecular Devices, SpectraMax M2 microplate reader. Each sample measurement was read in duplicate and the computed averages were taken based on the calculated standard curve.

Statistical Analysis

All data represent at least three individual experiments performed in triplicate. For the direct comparison of three or more conditions a one-way analysis of variance was performed, with multiple comparisons analyzed via Newmans-Keuls multiple comparisons test. When directly comparing two conditions a student-t test was performed. All comparisons were considered significant with p-values less than 0.05.

Results

hTERT-HM Cells Generate a Unique Secretome in Response to TM-Dependent Activation of the UPR

To define the myometrial secretome generated by the activation of the UPR, hTERT-HM cells first that underwent 6 passages of SILAC treatment to incorporate heavy-labeled and light-labeled amino acids (Lysine and Arginine), allowing for targeted detection of heavy-labeled peptides with LC/MS/MS proteomic analysis, in proteins exclusively secreted from the cell and not innate to the FBS used for the maintenance of cell growth within the media (Figure 14A). SILAC heavy-labeled and light-labeled hTERT-HM cells were then exposed to TM (5.0μg/ml) for one hour, washed thoroughly with fresh media to remove TM contamination and then incubated with fresh media for an additional 24hrs. The media that had been incubated with TM-treated cells for 24hrs was then removed, labeled as TM stressed-conditioned media (TM-CM) and analyzed via LC/MS/MS for cell-secreted SILAC-labeled proteins. Additionally, control media collected after a 24hr incubation with either naïve SILAC heavy-labeled and light-labeled hTERT-

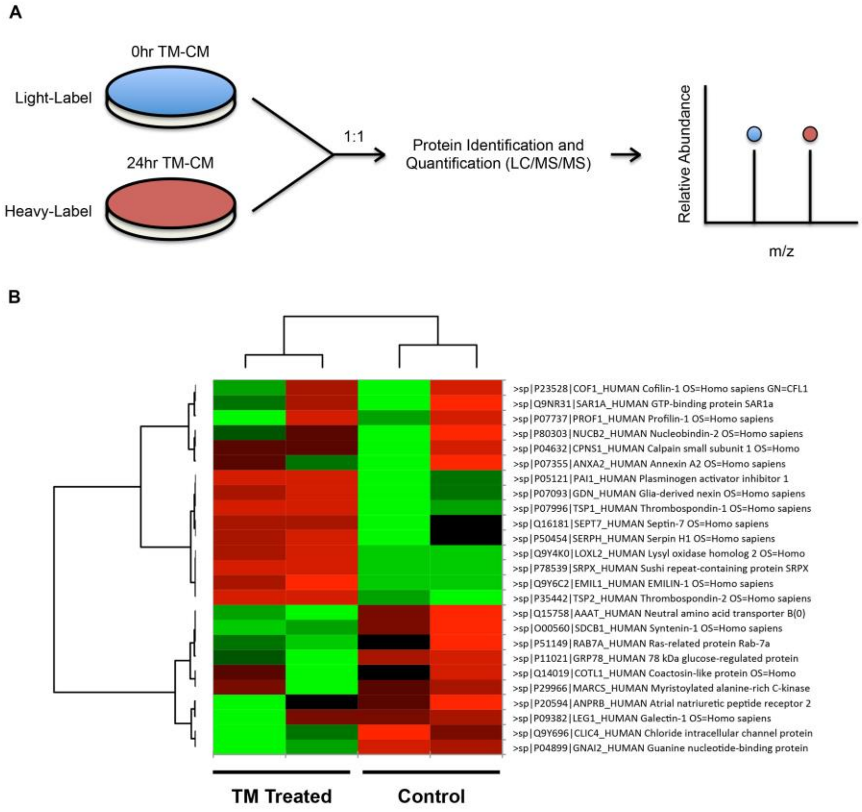


Figure 14. SILAC-dependent identification and quantification of TM-induced protein secretion in hTERT-HM cells. **(A)** Quantitative mass spectrometry workflow in which 24hr TM-CM was collected from heavy-labeled hTERT-HM cells and 0hr TM-CM (control media) was collected from light-labeled hTERT-HM cells, mixed at a 1 to 1 ratio and analyzed using LC/MS/MS. **(B)** Heat map comparisons of differential protein expression in 24hr TM-CM treated hTERT-HM cells and 0hr TM-CM treated hTERT-HM cells. Green is representative of higher relative protein expression, whereas red indicates lower relative protein expression.

HM cells, that had not been previously exposed to TM, was also analyzed via LC/MS/MS for cell-secreted SILAC-labeled proteins. LC/MS/MS identified 96 heavy-labeled secreted proteins validated in from both TM-CM media and control media, 53 of which were found to be upregulated or downregulated by a fold of two or more with TM-treatment (Figure 14B). UPR activation up-regulated secreted proteins that are largely associated with adaptation to pregnancy, anti-inflammatory action and smooth muscle tocolysis (Table 2). The most upregulated protein is GRP78 (27 fold), has the ability in a cell-free capacity to be an act in a pro-survival anti-inflammatory manner, as previously discussed in section Extracellular Functions of the Unfolded Protein Response. Stress-dependent secretion of GRP78 from the uterine myocyte was further validated in cell culture, utilizing an ELISA (Figure 15). Accordingly, UPR action also down-regulates secreted proteins that are largely associated with pro-apoptotic signaling and pro-inflammatory response (Table 3). For example, biglycan, fibronectin and versican core protein were all down-regulated by at least 9 fold and have been identified as pro-inflammatory mediators, which will be discussed in more detail in Chapter 2 Discussion. Cell viability assays performed on TM-treated hTERT-HM cells using Trypan Blue staining revealed negligible membrane permeability at the time in which the TM-CM was collected, demonstrating the secretome analyzed was not due to TM-mediated cell lysis (Figure 16).

Table 2. Proteins with Increased Stress-Induced Secretion

Swiss-Prot accession no.	Description	Control	TM treated	Fold change
P11021	78 kDa glucose-regulated protein	5.47E+07	2.19E+06	24.95
P04899	Guanine nucleotide-binding protein G(i) subunit α-2	1.63E+06	2.13E+05	7.67
P23396	40S ribosomal protein	1.91E+06	4.77E+05	3.99
P29966	Myristoylated alanine-rich C-kinase substrate	6.14E+06	1.61E+06	3.81
P62081	40S ribosomal protein S7	4.70E+05	1.30E+05	3.61
O00560	Syntenin-1	1.08E+06	3.07E+05	3.52
P20594	Atrial natriuretic peptide receptor	2.46E+07	7.70E+06	3.20
P62987	Ubiquitin-60S ribosomal protein	1.41E+07	4.45E+06	3.16
Q99880	Histone H2B type 1-L	9.29E+06	3.09E+06	3.01
Q562R1	β-actin-like protein 2	1.24E+07	4.23E+06	2.94
P09382	Galectin-1	1.46E+07	5.17E+06	2.83
Q15758	Neutral amino acid transporter B(0)	3.04E+06	1.10E+06	2.75
P51149	Ras-related protein	8.03E+05	3.52E+05	2.28
Q9Y696	Chloride intracellular channel protein 4	7.05E+05	3.21E+05	2.20
Q14019	Coactosin-like protein	6.13E+05	2.94E+05	2.09
P35579	Myosin-9	9.43E+05	4.75E+05	1.98
P60033	CD81 antigen	7.54E+06	3.93E+06	1.92
P68363	Tubulin α-1B chain	7.31E+06	4.39E+06	1.67
P62805	Histone H4	6.25E+06	3.88E+06	1.61
P61923	Coatomer subunit ζ-1	2.19E+05	1.38E+05	1.59

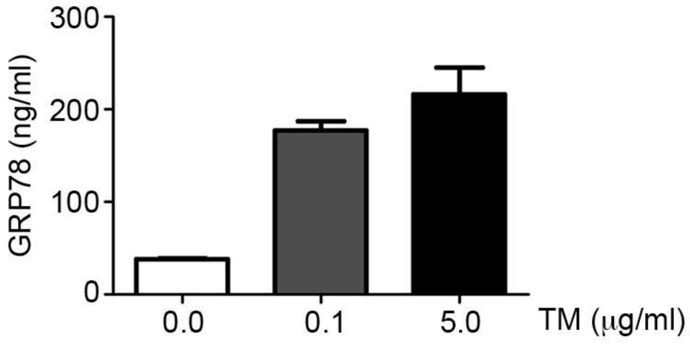


Figure 15. GRP78 is actively secreted from uterine myocytes in a stress-dependent manner. hTERT-HM cells were treated with 0, 0.1 or 5.0μg/ml TM for 24hrs, washed three times and then incubated with fresh media for 24hrs (TM-CM). GRP78 concentrations were then analyzed in 0, 0.1 and 5.0 μg/ml TM-CM with an ELISA and found to increase in a stress-dependent manner. Statistical comparisons were performed using one-way ANOVA, subsequent Newman-Keuls multiple-comparison tests and student-t test. *$p \leq 0.05$ and **$p \leq 0.01$ compared with controls.

Table 3. Proteins with Decreased Stress-Induced Secretion

Swiss-Prot accession no.	Description	Control	TM treated	Fold change
Q9Y613	FH1/FH2 domain-containing protein	5.55E+05	4.25E+07	76.58
P07996	Thrombospondin-1	9.37E+06	6.15E+08	65.64
P35442	Thrombospondin-2	6.54E+06	4.20E+08	64.22
P02452	Collagen alpha-1(I) chain	1.77E+07	1.04E+09	58.76
P02751	Fibronectin	8.45E+07	3.04E+09	35.98
P21810	Biglycan	7.12E+06	2.45E+08	34.41
P12110	Collagen alpha-2(VI) chain	2.83E+06	5.98E+07	21.13
Q76M96	Coiled-coil domain-containing protein 80	7.61E+05	1.39E+07	18.27
P07093	Glia-derived nexin	2.36E+06	3.37E+07	14.28
P08123	Collagen α-2(I) chain	1.86E+07	2.42E+08	13.01
P20908	Collagen α-1(V) chain	2.82E+06	3.55E+07	12.59
P12109	Collagen α-1(VI) chain	6.99E+06	8.01E+07	11.46
P24593	Insulin-like growth factor-binding protein	1.08E+06	1.19E+07	11.02
P13611	Versican core protein	1.36E+06	1.29E+07	9.49
P05121	Plasminogen activator inhibitor 1	4.27E+07	3.62E+08	8.48
Q15113	Procollagen C-endopeptidase enhancer 1	5.94E+06	5.03E+07	8.47
P13639	Elongation factor 2	6.12E+05	4.72E+06	7.71
P23284	Peptidyl-prolyl cis-trans isomerase B	4.16E+05	2.76E+06	6.63
P05388	60S acidic ribosomal protein	1.01E+06	5.18E+06	5.13
Q9Y6C2	EMILIN-1	4.84E+06	2.37E+07	4.90

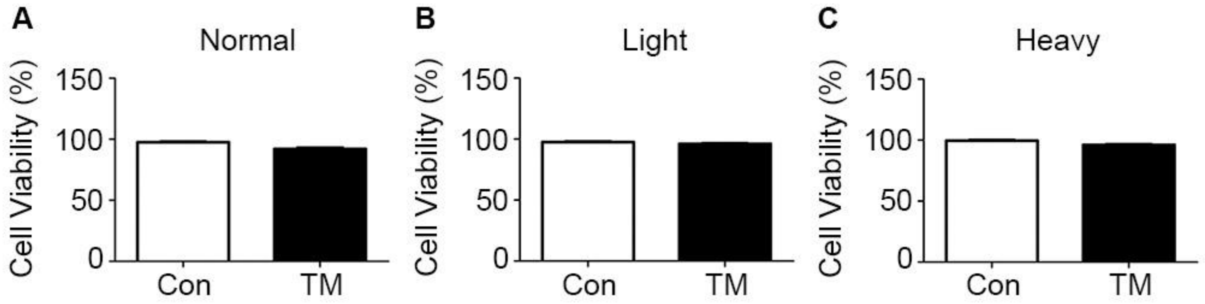


Figure 16. hTERT-HM cell plasma membranes are intact at the time of media collection. No significant differences in cell viability were observed between 0hr (control) and 24hr TM-treated normal, light and heavy-labeled hTERT-HM cells at the time of TM-CM collection. Statistical comparisons were performed using a one-way ANOVA, and subsequent Newman-Keuls multiple-comparison tests. *p≤0.05 and **p≤0.01 compared with controls.

Naïve hTERT-HM Cells Mount an UPR When Exposed to TM Stress-Conditioned Media

We next examined the ability of the stress-generated secretome to propagate the UPR by exposing naïve hTERT-HM cells to TM-CM for 48hrs and immunoblotting for activation of the UPR, CASP3 and apoptotic indices. Specifically, conditioned media was incubated for 24hrs with hTERT-HM cells that had been directly treated with TM (5.0μg/ml) for 0, 1, 4 or 24hrs and washed three times, collected and further incubated with naïve hTERT-HM cells that had never experienced stress. Our results demonstrate that naïve uterine myocytes exposed to 1, 4 and 24hr TM-CM mount an UPR, as seen by increased expression of GRP78, GADD153 and CASP3 in cells compared to TM-CM controls (Figure 17A). To validate that activation of the UPR is in fact driven by the

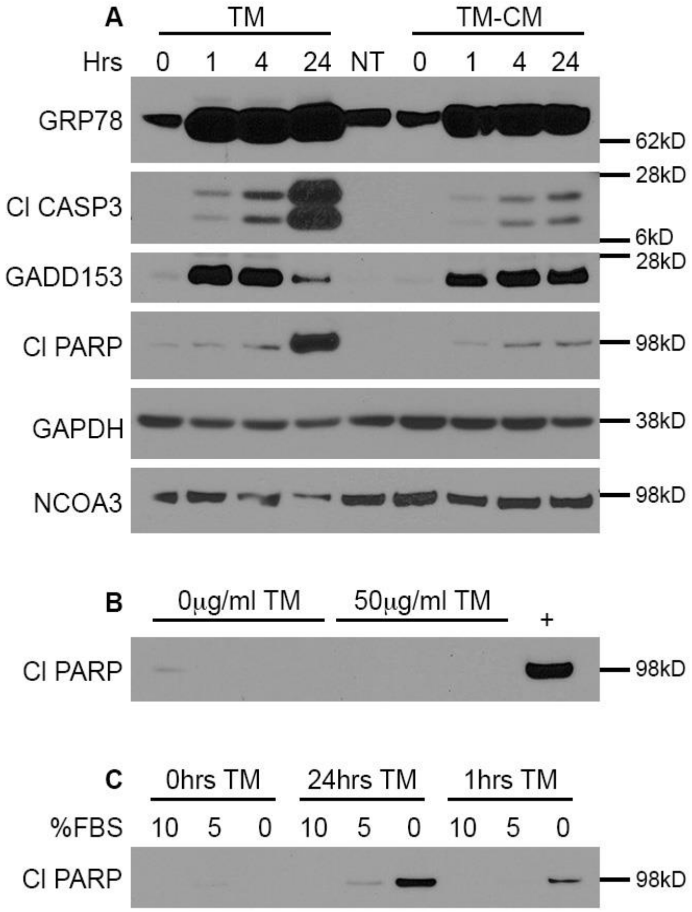


Figure 17. TM-dependent activation of the myometrial UPR generates a unique stress-specific secretome. **(A)** The induction of the UPR, CASP3 and apoptotic indices were analyzed in hTERT-HM cells directly treated with 5.0μg/ml TM for 0, 1, 4 or 24hrs (TM), naïve hTERT-HM cells (NT) or naïve hTERT-HM cells incubated with TM-CM (0, 1, 4, and 24hr). Activation of UPR markers GRP78 and GADD153, CASP3 and Cl PARP was significantly increased in all TM and TM-CM treated myocytes compared to 0hr TM and NT controls. Further, augmentation of UPR, Cl CASP3 and Cl PARP in TM-CM treated hTERTs positively correlated to the initial exposer time of TM. **(B)** No Cl PARP expression was observed in hTERT-HM cells treated with 0 or 0.05μg/ml TM for 234hrs. **(C)** PARP cleavage in hTERT-HM cells treated with 5.0μg/ml TM for 0, 1 or 24hrs in the presence of 0, 5 or 10% FBS was positively correlated to the exposer time of TM and further increased with the depletion of FBS, in a dose-dependent manner. PDIA2 and NCOA3 are utilized as cytoplasmic and nuclear protein loading controls. A representative blot from each experiment is shown.

stress-generated secretome and not TM contamination, we next analyzed the TM concentration in 1 and 24hr TM-CM via liquid chromatography tandem mass spectrometry (LC/MS/MS) and found an average of approximately 0.05μg/ml of TM per ml of CM. Subsequently, naïve hTERT-HM cells were directly treated with 0.05μg/ml TM for 48hrs and examined for apoptotic indices. The absence of Cl PARP in TM treated hTERT-HM cells (Figure 17B), suggests the previously observed transmission of the UPR in naïve hTERT-HM cells was secretome mediated and not due to TM contamination. To further validate this, we added an additional chemical-free stress (FBS depletion) during the same period of TM treatment prior to TM-CM media conditioning. Subsequently, any changes observed in the activation of the UPR in naïve hTERT-HM cells would be due to increased stress in the TM treated cells and not TM contamination. We observed chemical free stress-dependent increases in Cl PARP in naïve hTERT-HM cells treated with TM-CM from hTERT-HM cells treated 5.0μg/ml TM for 1 or 24hrs with media depleted of FBS in various concentrations (0, 5 and 10% FBS) (Figure 17C). As no chemicals were used to increase the stress in TM treated hTERT-HM cells used to condition the TM-CM, we are confident the induction in the UPR in TM-CM treated naïve cells is derived from the stress-generated secretome.

Smoking Promotes a Systemic Anti-Inflammatory Profile in Pregnant Women and Without Preeclampsia

To characterize the effects of a “preconditioning-like” transient stress stimulus during pregnancy on markers of the UPR in the circulation, we analyzed serum GRP78 and GADD153 concentrations in women with normal and preeclamptic pregnancies that participated or refrained from cigarette smoking while pregnant. As previously identified, we found GRP78 levels to be higher in women without preeclampsia versus women with preeclampsia (Figure 18A). Interestingly, we found a trend of increased serum GRP78 in women who smoked cigarettes compared to non-smokers, in both preeclamptic and normal pregnancies. Further, serum GADD153 was decreased with smoking in normal and preeclamptic pregnancies, with overall normal pregnancies showing reduced serum GADD153 compared to women with preeclampsia (Figure 18B). In each cohort, GRP78 concentrations were inversely proportional to serum GADD153.

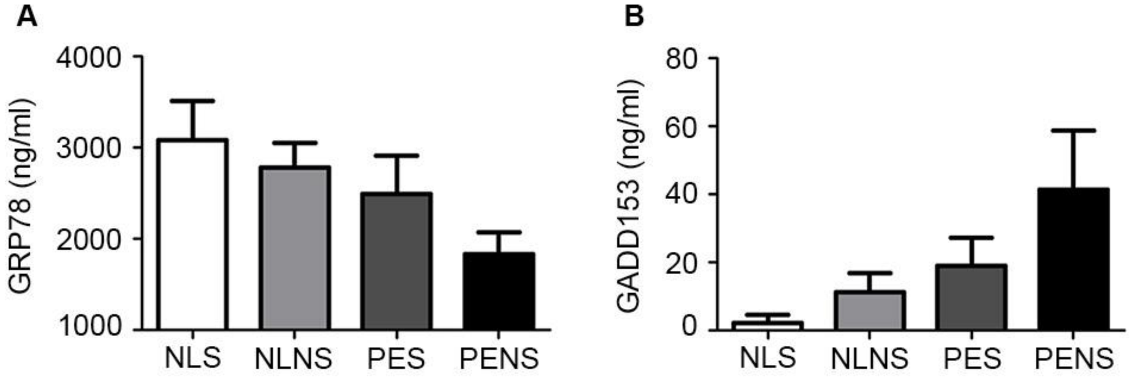


Figure 18. Serum GRP78 and GADD153 concentrations from normal and preeclamptic pregnancies in women who participated or refrained from cigarette smoking. Serum samples collected from non-laboring smoking (NLS), non-laboring non-smoking (NLNL), preeclamptic smoking (PES) and preeclamptic non-smoking (PENS) during the 3rd trimester, were analyzed with GRP78 and GADD153 ELISAs. **(A)** GRP78 was reduced in preeclamptic pregnancies with a further smoking-dependent decrease, **(B)** while GADD153 augmented in preeclamptic pregnancies with a further smoking-dependent increase.

Discussion

In this study we 1) characterized the stress-dependent secretome generated by the uterine myocyte and 2) examined its physiological function within the uterine compartment and circulation. With SILAC labeled hTERT-HM cells we were able to confidently define the UPR-mediated secretome, which consisted of over 90 validated proteins using LC/MS/MS. Further, we described novel signal transduction of the UPR from the stress cells to the naïve cells as a result of extracellular factors secreted into TM-CM. Importantly, while examining the role of the UPR secretome in pregnant women we found the secretion of GRP78 was decreased with clinically diagnosed preeclampsia but was partially restored with the introduction of cigarette smoking, which is thought to generate transient remote preconditioning-like systemic stress. Taken together these data confirm our hypothesis that the generation and transmission of the uterine secretome is in part a UPR regulated process that has the potential to propagate UPR-derived tocolysis and abrogate systemic inflammatory processes, which can result in preterm labor.

Initially, we proposed local uterine myocyte UPR stressors act to stimulate the secretion of a uterine secretome throughout the course of gestation. Here we were able to identify for the first time, a discrete set of proteins secreted from the uterine myocyte in stress-dependent manner (Figure 13). As expected many of the proteins identified have been demonstrated to modify the ER stress and inflammatory responses (Tables 2 and 3).[466,467] Numerous proteins with TM-dependent decreased secretion, e.g. versican core protein, fibronectin and biglycan were found to participate in the propagation of inflammation.[468-470] During pregnancy, abrogation of these circulating pro-inflammatory proteins would be critical in the maintenance of uterine quiescence. As previously discussed in Chapter 1 Initiation of Uterine Activation, heightened inflammation in the pregnant uterus and maternal circulation contributes to the positive feedback pathway in which active NFκB increases expression of CAPs necessary for the induction of labor. In this case, the inability of stress to abrogate the secretion of pro-inflammatory proteins such as versican core protein or fibronectin may prematurely increase maternal inflammatory signaling, resulting in preterm labor. In contrast, atrial natriuretic peptide (ANP) receptor 2, galectin-1 and GRP78 each had increased secretion in response to TM-induced stress. Importantly, ANP acting through the ANP receptor 2 can act as a tocolytic agent, while galectin-1 and GRP78 have both been demonstrated to possess anti-inflammatory properties.[471] ANP/ANP receptor 2-dependent increases in cyclic guanine monophosphate is important for inhibiting MLCK and inducing muscle relaxation in smooth muscle tissues, such as the aorta.[472] While the importance of ANP/ANP receptor 2 activity has not been studied in the context of myometrial relaxation, it has been shown that ANP receptors are present in relatively high abundance within the pregnant human myometrium and decidua.[473] Together these data suggest, stress-dependent increases in ANP receptor 2 from the myometrium may be important in modulating uterine quiescence. Of additional interest, galectin-1 and GRP78 both play a role in regulating the ER stress and inflammatory responses. Specifically, the knockout of galectin-1 impaired appropriate ERSR signaling, while appropriate galectin-1 signaling inhibits NFκB-mediated inflammation and promotes immune tolerance during pregnancy.[466,474] Conclusively, we propose that enhanced GRP78 secretion upon activation of the UPR (Figure 14) plays a significant role inhibiting myometrial contractility to maintain quiescence as it has previously been shown to promote anti-inflammatory signaling responses. Please refer to Chapter 1 Extracellular Functions of the Unfolded Protein Response for more detail.

After confirming the presence of a novel UPR-generated secretome from the uterine myocyte, we speculated that these specific secreted factors might have the capacity to transmit and propagate the tocolytic-preconditioned phenotype to adjacent naive cells. In other studies, it has been demonstrated that mice injected with conditioned media isolated from stressed tumor cells, mounted an ER stress response within the liver which did occur when media was conditioned with unstressed cells.[330] In our experiments, these results were recapitulated when we demonstrated stressed conditioned media (TM-CM) but not unstressed control media activated the UPR in naïve hTERT-HM cells (Figure 16). Similarly, cell-cell interactions mediated by soluble secreted factors have frequently been defined in the extracellular matrix of tumors, where this phenomenon has been implicated in modulating tumor cell progression.[475] Cancer associated fibroblasts have been demonstrated to secrete factors that promote the multipotent mesenchymal stem cells to differentiate into cancer associated fibroblast contributing further to tumor development. Size fractionation and mass spectrometry analysis identified conclusively that GRP78 was the factor promoting the transition from mesenchymal stem cell to cancer-associated fibroblast.[476] Further the active translocation of GRP78 to the membrane and release into the extracellular space in tumors is directly correlated to tumor resistance and decreased apoptosis following the application of chemotherapeutic agents.[477,478] This is primarily thought to due to the activated plasma proteinase inhibitor α2-macroglobulin binding to GRP78 and inducing cell proliferation and survival via RAS-MAPK, PI 3-kinase/AKT, cAMP-dependent and UPR signaling.[479-481] These data clearly indicate an exciting potential mechanism whereby the stressed pregnant uterus gives rise to a uterine secretome which is secreted into the circulation to provide a systemic alert or update to circulating immune cells, other somatic cell types or remote organs of the need to adapt, accommodate or protect themselves against a possible stress events.

In other words, we propose that a uterine myocyte derived UPR secretome, in the circulation of pregnant women is necessary for systemic remote preconditioning of other maternal tissues during pregnancy, such as macrophages and vascular endothelial cells, to allow for improved resistance to and increased tolerance of normal gestational stresses every pregnancy experiences and promote the maintenance of quiescence. Subsequently, a wide range of normal gestational stressors that affect the uterine myocyte, e.g. hypoxia, hyperplasia, mechanical stretch would be critical in promoting preconditioning-mediated systemic adaptations and thus the propagation of tocolysis. While there is no direct evidence for this, studies have found pregnancy-dependent attenuation of pathophysiological cardiac function, which may be in part the result of remote UPR preconditioning experienced explicitly during pregnancy.[482] Specifically, in a mouse model of left ventricular pressure overload, pregnancy was found to mitigate pathological LV remodeling, pulmonary congestion and transverse aortic constriction-dependent gene expression.[482] It is also speculated that the hemodynamic changes experienced during pregnancy may reduce a women’s risk of cardiovascular disease later, which suggests pregnancy-dependent uterine UPR positively influences maternal systemic vascular function. Interestingly, having been pregnant also significantly reduces your risk of developing certain types of cancer.[483] For instance, an increase in the cumulative months of being pregnant correlates to a decrease in the risk of a woman developing epithelial ovarian cancer.[484] Overall, these data lend evidence to the idea that systemic UPR signaling from the myometrium are important in facilitating systemic adaptations that contribute to the maintenance of uterine quiescence.

One mechanism whereby the UPR secretome may modulate systemic conditioning is through the propagation of anti-inflammatory signaling. Extracellular GRP78 specifically, has been demonstrated in multiple studies, both *in vitro* and *in vivo*, to play an anti-inflammatory immunomodulatory role. Upon UPR activation GRP78 levels increase in the stressed cell and translocate to the surface with high amounts being released into the culture media.[319,324] Cells that secrete GRP78 into their extracellular environment were found to gain the ability to promote a pro-survival and anti-apoptotic phenotype displaying resistance to anti-angiogenic chemotherapeutic agents such as

Bortezomib.[319] *In vivo*, elevated extracellular cell-free GRP78 is found in the synovial fluid of patients with rheumatoid arthritis, saliva, serum and oviductal fluid.[320-323] It was determined in this context that GRP78 also performed anti-inflammatory and immunomodulatory functions. When exposed to cell free GRP78, human peripheral blood mononuclear cells displayed a dose dependent increase in the anti-inflammatory cytokines TNFα and IL-10 secretion. In stimulated PBMCs, the presence of extracellular GRP78 lowered the levels of IL-1 beta and increased the levels of interleukin 1 receptor antagonist. The concentration of soluble TNFRII levels, which act to suppress the pro-inflammatory activities of TNFα also increased, confirming the role of extracellular GRP78 as a propagator of an anti-inflammatory signaling cascade.[321] It has become apparent that extracellular GRP78 has remarkable anti-inflammatory and immunomodulatory properties. In animal models of collagen-induced arthritis, prophylactic administration of recombinant GRP78 one week before the initiation collagen immunization was sufficient to prevent the induction of collagen induced arthritis.[485] Administration of GRP78 at the onset of collagen-induced arthritis was also successful in suppressing the development of arthritis.[459] Further, suppression of collagen-induced arthritis was achieved by parenteral (gavage) administration of lentiviral vectors expressing GRP78. A single dose of exogenous GRP78 was sufficient to induce permanent remission of inflammation in collagen induced arthritis, suggesting not only does GRP78 mediate anti-inflammatory actions, but is also capable of driving resolution of inflammation, likely through immune cell differentiation.[459] The effects of serum GRP78 have been found to be at least partially dependent of IL-4, as the suppressive effects of GRP78 are abrogated in the IL4-/- mouse. These analyses suggest, that administration of exogenous extracellular GRP78 can allow for increased resistance to and active resolution of inflammatory challenges. In a similar manner, in our recent PNAS paper, we administered 4-phenyl butyric acid, a chemical chaperone that mimics GRP78 action, and found we could reverse ER stress induced preterm birth.[350]

While the protective effects of cigarette smoking do not outweigh its harmful consequences, women who participate in cigarette smoking compared to non-smokers have a reduced risk of developing preeclampsia.[486] It has been well established that smoking a cigarette is a transient stress that decreases the level of tissue oxygen for a short period of time following inhalation of nicotine.[487] Interestingly, smoking has been demonstrated to promote an anti-inflammatory milieu with the suppression of M1 polarized macrophage related inflammatory genes and the upregulation of M2 polarization programs.[488] In this study, we have identified that 1) transient stress promotes the active secretion of GRP78 from uterine myocytes (Table. 2 and Figure 14) and 2) women with preeclampsia tend to have lower levels of serum GRP78 compared to women who do not, but that GRP78 levels are restored to approximately normal levels in women that smoke (Figure 17), which suggests the transitory systemic hypoxic stress from smoking in pregnant women increases the secretion of GRP78. Subsequently, we propose smoking may act to remotely precondition the vascular endothelium through GRP78-mediated anti-inflammatory signaling, allowing pregnant smoking women who become preeclamptic to avoid endothelial dysfunction. Additionally, these data suggest the serum GRP78 may be a novel therapeutic approach for promoting endothelial sufficiency and/or a proficient biomarker for myometrial viability and disease severity in women with preeclampsia.

Conclusively, these data demonstrate that activation of the myometrial UPR generates and propagates a unique secretome that has the potential to transmit uterine tocolysis and suppress systemic inflammation, which can result in pregnancy disorders such as preeclampsia or preterm labor. Importantly, these data set the stage for the development of novel tocolytic strategies and potentially new biomarkers for advanced identification of women who are at risk for undergoing multiple pregnancy disorders.

CHAPTER 5

Conclusion and Synthesis

This collection of studies focuses on how active CASP3 is maintained within the pregnant uterine myocyte in a non-apoptotic state to fulfill its tocolytic function of inhibiting myometrial contractility for the preservation of uterine quiescence. Subsequently, we hypothesized that preconditioning the myometrial UPR would allow for the maintenance of non-apoptotic CASP3 activity and thus sustain uterine quiescence. To test this hypothesis all experiments were performed utilizing an immortalized human myometrial cell line *in vitro*, a timed-pregnant CD-1 mouse model or serum from pregnant women with or without preeclampsia who participated in or refrained from cigarette smoking. The main findings of this work are that appropriate uterine UPR preconditioning 1) maintains non-apoptotic CASP3 by mitigating apoptotic stress pathways and inflammation, 2) reduces the risk of preterm birth 3) inhibits the precocious transition of CASP3 into an apoptotic state within the endometrium where it participates in iPLA2/prostaglandin-dependent initiation of luteolysis, 4) produces and transmits a unique stress-dependent secretome and 5) promotes systemic adaptive signaling. From this data we suggest endogenous pregnant-dependent stress stimuli experienced across gestation act in a preconditioning-like manner to sustain the tocolytic action of non-apoptotic CASP3 within the pregnant uterus in the presence of ensuing stresses and promote an all-around adaptive environment through paracrine and endocrine propagation of a myometrial stress-derived secretome. Furthermore, from these results we speculate that women who are unable to host an appropriate preconditioning response to gestational stresses are at a significantly increased risk of undergoing spontaneous preterm.

If a woman walked into the emergency room today undergoing premature uterine contractions, there are absolutely no drugs available that could be administered to prevent premature birth from occurring. With three limited treatments: progesterone; cervical cerclage; and possibly cervical pessary, the ability to accurately predict and prevent preterm labor remains one of the most critical challenges facing modern obstetrics.[23,26,30] Cervical length measurement and a previous preterm birth are currently the strongest predictors for a subsequent preterm birth, however nulliparous women with no past obstetrical history remain at a heightened risk.[16] With such a large subset of women unidentifiable or unresponsive to the currently available treatments, two major questions remain 1) what cellular mechanisms lead to the initiation of premature uterine contractility and 2) what therapies can be developed to predict and inhibit spontaneous preterm labor in all women? Previous studies from our laboratory have demonstrated that the uterine UPR plays a large role in modulating myometrial quiescence and that exogenous progesterone, the most successful of preventative treatments, and progesterone receptor inhibitors modify the local uterine UPR drastically in favor of quiescence or labor, respectively. Specifically, we demonstrated using a CD-1 pregnant mouse model, that the balance between adaptive GRP78 and tocolytic non-apoptotic CASP3 is crucial for the maintenance of quiescence. Throughout early and mid-gestation we observed sufficient ER stress-dependent GADD153/CASP3 activity is needed to degrade and disable the myometrial contractile architecture (α and γ actin, CX43) to inhibit contraction.[399] Equally important, at the end of gestation increases in gestationally generated stress stimuli that upregulate adaptive UPR signaling causes GRP78-dependent resolution of ER stress and thus a decline in GADD153/CASP3 tocolytic action.[403] This was abundantly evident in previous studies from our lab where precocious doses of stress (TM, 1.0mg/kg, E15) administered to timed-pregnant mice prematurely augmented GRP78 action, which lead to diminished CASP3 tocolysis and subsequently preterm birth.[350] Interestingly, in this same study a smaller dose of stress (TM, 0.2mg/kg) acted to increase both GADD153/CASP3 activity and GRP78. As the induction of GRP78 was not large enough to completely resolve the corresponding ER stress, GADD153 and CASP3 activity remained intact decreasing the occurrence of preterm birth compared to animals injected with 1.0mg/kg TM. These data further highlight the importance of GADD153/CASP3 action in tocolysis and how the balance between GRP78 and CASP3 maintains quiescence. Subsequently, in the first chapter we hypothesized increasing the capacity of the uterine myocyte to tolerate extraneous stress may act as a buffer for maintaining the balance between active non-apoptotic CASP3 and adaptive GRP78 signaling, which *in vivo* is important for preventing premature contractility to maintain an appropriate gestational length. Starting at the level of the individual uterine myocyte (hTERT-HM), we demonstrate that indeed UPR preconditioning may be a plausible mechanism by which CASP3 activity is maintained in a non-apoptotic state in the presence of precocious cellular stress-challenges. As seen in Figure 1, hTERT-HM cells challenged with a cytotoxic dose of stress maintained active CASP3 in the absence of apoptosis and in the presence of abundant levels of GRP78. Based on these studies it is reasonable to speculate that spontaneous preterm birth in women may in part be due to the mismanagement of stress that dysregulates tocolytic and adaptive signaling in a way that CASP3 activity is prematurely diminished due to precocious activation of adaptive GRP78. Indirect evidence supporting this hypothesis is the fact that exercising during pregnancy has been demonstrated to decrease the risk of preterm birth.[489] Exercise, while providing exponential benefits, has clearly been identified as a transient stress to the body that challenges homeostasis at both a cellular and systemic level and may be acting in a preconditioning-like manner to reduce the risk of preterm birth.[490,491] While our *in vitro* data work did not directly examine the role of pregnancy-dependent stress stimuli in preconditioning the myocyte, it importantly provided a potential mechanism for how similar gestational stress-stimuli could be acting *in vivo* to maintain active non-apoptotic CASP3 and appropriately manages tocolytic and adaptive signaling responses.

Approximately 45% of all preterm births currently are spontaneous occurring and idiopathic in nature, occurring in the absence of maternal or fetal infection or premature preterm rupture of the membranes (PPROM).[17] Many of the discernable maternal risk factors associated with the onset of spontaneous preterm have been directly and indirectly associated with the onset of severe ER stress, i.e. increased mechanical stretch due to twin pregnancy or redox stress due to advanced maternal age.[492-495] Having previously demonstrated in Aim 1 that UPR preconditioning enables the maintenance of non-apoptotic CASP3 in the presence of exaggerated ER stress, we hypothesized appropriate UPR preconditioning from endogenous pregnancy-generated stress stimuli, e.g. hypoxia, hyperplasia, hormone fluctuation, hypertrophy and mechanical stretch, may be critical for the preservation of the tocolytic action of CASP3. When we tested this using our model of sub-preconditioned stressed mice (TM+PBA), inappropriate UPR preconditioning in the presence of even a minor stress was sufficient to increase the occurrence of preterm birth as predicted. Subsequently, this data suggests pregnant women who do not efficiently or are unable to host a preconditioning response to gestational stresses due to innate maternal factors such as advanced age or preexisting chronic stress would be at a heightened risk of preterm delivery, as already clinically observed. An example of this would be a pregnant woman with a preexisting chronic inflammatory disease such as diabetes or rheumatoid arthritis. In this context, we believe major chronic inflammation would act to disrupt appropriate uterine preconditioning, as it a continuous non-transient stress that would burden the myometrial ERSR instead of prompting it to facilitate adaptation. Accordingly, rheumatoid arthritis and diabetes milletus have been proven to increase the risk of preterm birth.[496,497] Of note, premature induction of labor in each of these cases can occur in the absence of maternal/fetal infection and PPROM, as seen in our sub-preconditioned stressed mice. Interestingly, it has been demonstrated that a large portion of patients (approximately 26%) of women that present with preterm labor and intact fetal membranes have intra-amniotic inflammation in the absence of microbial-associated infection.[498] In non-preconditioned cells and sub-preconditioned stressed mice, we similarly observed heightened levels of uterine and systemic inflammation, as seen by increased NFκB signaling and enhanced TNFα secretion, in the absence of appropriate preconditioning, like that of sterile inflammation in the amniotic fluid of some women that deliver preterm. These data suggest that appropriate UPR preconditioning is not only important for the maintenance of non-apoptotic CASP3-mediated tocolysis but is also necessary for inhibiting premature precocious induction of inflammation which is associated with the onset of labor. Subsequently, there are multiple pharmaceutical agents, currently used for the treatment of UPR-dependent diseases that could be repurposed for preconditioning therapies like those currently being utilized in the field of liver and cardiovascular ischemia/reperfusion.[499,500] TUDCA, in particular, has already been shown to alleviate extreme UPR stress in other pregnancy related disease and may be a promising agent for the restoring of a preconditioning-like uterine profile.[501] Overall, this work offers a novel mechanism, to explain why women undergo spontaneous preterm labor and what may increase a woman's susceptibility to premature labor. Further, it suggests that drugs that aid in the management of the UPR may be effective in alleviating the occurrence of preterm birth.

Prior to this work being done it was already well understood due to previous work from our group that non-apoptotic CASP3 was functioning in the uterus to inhibit myometrial contractility and with the addition of these studies we further understood that gestationally regulated UPR preconditioning contributes to the maintenance of non-apoptotic CASP3 activity and thus regulates gestational length. In contrast, our laboratory had previously observed the activation of apoptotic CASP3 within the endometrial compartment associated with the onset of term labor without having resolved its function. Knowing that 1) the endometrial compartment primarily participates in prostaglandin synthesis, which is an important process for the induction of labor and that 2) apoptotic CASP3 action activates iPLA2 to enhance prostaglandin signaling in breast tissue we examined the role of apoptotic endometrial CASP3 in prostaglandin synthesis. Like in breast tissue, our *in vitro* data revealed enhanced iPLA2 activation in response to apoptotic CASP3 action, but not non-apoptotic CASP3. *In vivo*, we indirectly demonstrated increased iPLA2 activity resulting in heightened prostaglandin synthesis only in the preterm laboring endometrium where inappropriate preconditioning lead to the transition of endometrial CASP3 from a non-apoptotic to apoptotic state. In the context of pregnancy these data add credence to the importance of appropriate uterine UPR preconditioning in the inhibition of contraction and suggests a new mechanism in the regulation of prostaglandin production for both term and preterm pregnancies. Central to the importance of these studies, is the possibility of designing new therapeutic interventions targeting this novel-signaling pathway. Compared to other PLA2 enzymes, the function of iPLA2 and its role in modulating disease has only recently been discovered. Subsequently, the development of iPLA2 inhibitors is relatively limited. There are however, a few trifluoromethyl ketones of fatty acids that act to reversibly inhibit Group VI iPLA2 enzymes, with the most important of these being bromoenol lactone (BEL).[502] In rat mesangial cells, BEL-dependent inhibition of iPLA2 significantly attenuated IL-1β-induced PGE2 production.[503] Interestingly, it has further been demonstrated that the BEL treatment to vascular smooth muscle, significantly decreases basal concentration of free arachidonic acid and inhibits smooth muscle contraction.[504] This study, however did not examine the subsequent effects of BEL on prostaglandin synthesis. Together, these preliminary studies characterizing the effects of iPLA2 inhibition on prostaglandin synthesis and muscle contractility are promising for the future development of myometrial tocolytic agents.

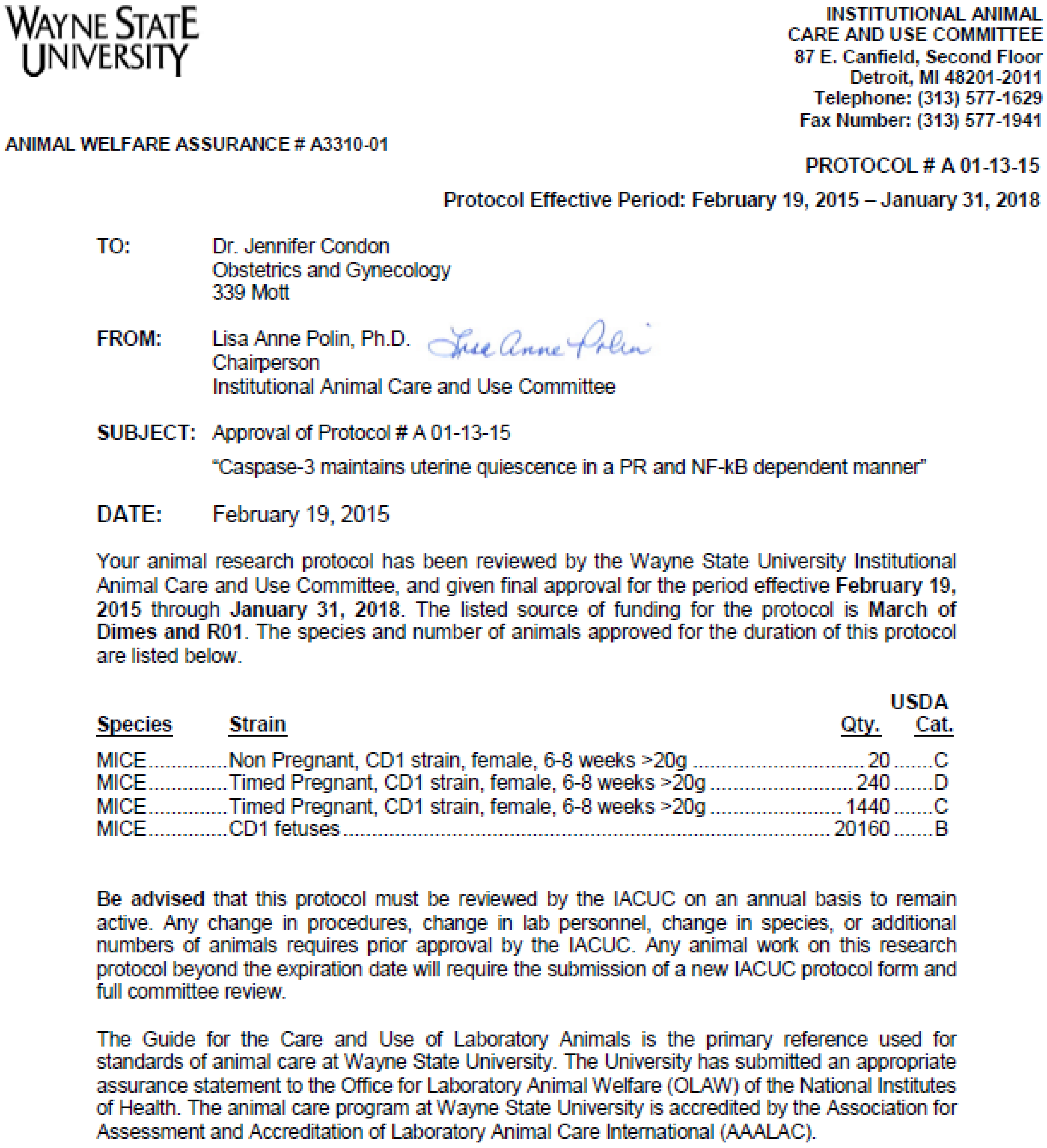
In the absence of effective tocolytic agents, the use of predictive biomarkers would greatly improve our understanding and treatment of preterm birth. The discovery and validation of clinical biomarkers that can act in a predictive or prognostic manner is an important area of research today. In oncology specifically, many biomarkers are being used to diagnose cancer and also predict the efficacy of chemotheraputic agents in the treatment of cancer.[505] Serum miR-21 for example is an onco-microRNA that is significantly elevated in patients with hormone-refractory prostate cancer.[506] In addition to acting as a predictive biomarker for the presence of hormone-refractory prostate cancer, the serum levels of miR-21 have been found to correlated with the tumor resistance to docetaxel-based chemotherapy.[507] Similarly, serum miR-200c was recently identified as a prognostic biomarker in patients with colorectal cancer that additionally effective in predicting metastasis.[508] Unfortunately, in the context of preterm birth there have been no biomarkers identified to date that effectively predict whether a woman will deliver preterm or not. As previously mentioned is Chapter 1 Preterm Birth, mid-trimester cervical length is the only accurate predictor of determining the future occurrence of preterm birth. In a literature review examining research from the last four decades, one study states 116 distinct biomarkers have been analyzed in hopes of identifying a strong predictive biomarker for the onset of preterm labor without success.[509] Within the 217 studies included, these biomarkers were assayed approximately 758 times to no avail.[509] A more recent review utilizing multivariate adaptive regression splines generated model, found multiple biomarkers when data was stratified based on race.[510] While stratification of biomarkers by race did highlight a limited number of proteins, such as TNFα, TNFR1 and TGF-β_1 within the serum, cord blood and amniotic fluid from African American and Caucasian women that were associated with preterm birth, no biomarkers were found to be predictive in nature. However, knowing that 1) many tissues respond to ER stress by propagating the secretion of a discrete collection of proteins unique to the stressed cell type and 2) gestationally-regulated myometrial ER stress stimuli are key for the maintenance of uterine quiescence and inhibition of preterm labor, we proposed targeting and characterizing the UPR-generated secretome from the uterine myocyte may lead to the discovery of a predictive biomarker of preterm birth. As seen in Aim 3 Figure 14, we successfully identified and quantified a novel UPR-generated secretome in hTERT-HM cells using a SILAC-targeted model of LC/MS/MS proteomic analysis. While many of the proteins identified were new in the scope of labor, some were known to be related to preterm birth and pregnancy. Plasminogen activating inhibitor 1, versican core protein, thrombospondin 1 and syntenin for example have all been demonstrated to participate in various processes throughout the course of gestation, suggesting the collection of proteins we characterized as the uterine myocyte secretome is most likely an accurate depiction of the proteins secreted from pregnant myometrium *in vivo* in response to gestationally regulated stresses.[511-514] Although, this analysis of the uterine myocyte UPR-derived secretome is preliminary, it is an incredibly important foundation for the future studies and the elucidation of a useful biomarker for the prediction and potential prognosis of preterm labor.

In addition to providing important potential biomarkers for the recognition of preterm birth, we further predict the extracellular uterine secretome resulting from gestationally regulated stress stimuli within the myometrium, participates in the maintenance of uterine quiescence. In Aim 3, our data shows a stress-generated secretome from a uterine myocyte has the capacity to propagate the activation of UPR in a paracrine manner to non-stress naïve hTERT-HM cells (Figure 17A). Having also demonstrated in Aim 1 that the pre-induction of the UPR prior to a lethal stress affords significant cytoprotection, it is easy speculate that UPR activation in naïve hTERT-HM following incubation with TM-CM (Figure 17A) could also afford cytoprotection against a subsequent stress. As previously mentioned in Chapter 1 Remote Preconditioning of the Endoplasmic Reticulum Stress Response, in the process of remote preconditioning the application of stress to a discrete tissue/organ can provide systemic adaptations and global cytoprotection against additional stresses. While we did not further stress the naïve hTERT-HM cells that mounted an UPR following incubation with TM-CM media, I would hypothesize those cells might demonstrate an increased resistance to stress-mediated apoptosis. This hypothesis is indirectly supported by our *in vivo* data, which demonstrates increased concentrations of GRP78, which was found to be differentially secreted in a stress-dependent manner, in the serum of pregnant women without preeclampsia compared to pregnant women with preeclampsia. These data suggest women unable to host an appropriate uterine response to stress resulting in reduced circulating GRP78, are at an increased risk of developing preeclampsia. Further, as preeclampsia has been identified as a pregnancy disorder associated with dysfunctional UPR signaling within the placenta and maternal endothelium we propose decreased signaling from the stressed uterus may disrupt normal remote preconditioning and peripheral adaptations necessary for the maintenance of a normal pregnancy. Subsequently, we hypothesize the dysregulation of balance between secretome-dependent maternal adaptation and uterine stress may play a role in the etiology of pregnancy-dependent complications, such as IUGR and tobacco smoke induced utero-placental hypoxia. In this context, we speculate the severity of stress evoked by these conditions and other uterine stressors correlated with preterm birth (e.g. twin pregnancy or preeclampsia) may alter the uterine-derived secretome and thus the prophylactic remote preconditioning it promotes, resulting in a maladaptive pro-labor phenotype. Interestingly, pregnant women that smoked had increased serum concentrations of GRP78 compared to those that did not, in both normal and preeclamptic pregnancies. As it is already known that pregnant women that smoke are at a reduced risk of developing preeclampsia, this data suggests UPR preconditioning, similar to that of smoking without the carcinogenic effects, may be a potential therapy for treating women who are at an increased risk of developing preeclampsia and other UPR-associated pregnancy disorders.

Conclusively, my hypothesis represents a paradigm shift in how the UPR controls cellular homeostasis in an autocrine, paracrine and endocrine manner during pregnancy and how dysfunctional regulation of this system may lead to deleterious pregnancy outcomes. Gaining a greater understanding of the mechanisms associated with the onset of preterm birth will ultimately allow for enhanced preterm birth diagnostics, novel tocolytic drug design and more accurate preventative intervention protocols.

APPENDIX A

IACUC Protocol Approval Letter



WAYNE STATE
UNIVERSITY

INSTITUTIONAL ANIMAL
CARE AND USE COMMITTEE
87 E. Canfield, Second Floor
Detroit, MI 48201-2011
Telephone: (313) 577-1629
Fax Number: (313) 577-1941

ANIMAL WELFARE ASSURANCE # A3310-01

PROTOCOL # A 01-13-15

Protocol Effective Period: February 19, 2015 – January 31, 2018

TO: Dr. Jennifer Condon
Obstetrics and Gynecology
339 Mott

FROM: Lisa Anne Polin, Ph.D. Lisa Anne Polin
Chairperson
Institutional Animal Care and Use Committee

SUBJECT: Approval of Protocol # A 01-13-15
"Caspase-3 maintains uterine quiescence in a PR and NF-kB dependent manner"

DATE: February 19, 2015

Your animal research protocol has been reviewed by the Wayne State University Institutional Animal Care and Use Committee, and given final approval for the period effective **February 19, 2015** through **January 31, 2018**. The listed source of funding for the protocol is **March of Dimes and R01**. The species and number of animals approved for the duration of this protocol are listed below.

Species	Strain	Qty.	USDA Cat.
MICE	Non Pregnant, CD1 strain, female, 6-8 weeks >20g	20	C
MICE	Timed Pregnant, CD1 strain, female, 6-8 weeks >20g	240	D
MICE	Timed Pregnant, CD1 strain, female, 6-8 weeks >20g	1440	C
MICE	CD1 fetuses	20160	B

Be advised that this protocol must be reviewed by the IACUC on an annual basis to remain active. Any change in procedures, change in lab personnel, change in species, or additional numbers of animals requires prior approval by the IACUC. Any animal work on this research protocol beyond the expiration date will require the submission of a new IACUC protocol form and full committee review.

The Guide for the Care and Use of Laboratory Animals is the primary reference used for standards of animal care at Wayne State University. The University has submitted an appropriate assurance statement to the Office for Laboratory Animal Welfare (OLAW) of the National Institutes of Health. The animal care program at Wayne State University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

APPENDIX B

IRB Approval Letter

WAYNE STATE UNIVERSITY

IRB Administration Office
87 East Canfield, Second Floor
Detroit, Michigan 48201
Phone: (313) 577-1628
FAX: (313) 993-7122
http://irb.wayne.edu

NOTICE OF FULL BOARD CONTINUATION APPROVAL

To: Sonia Hassan
Obstetrics/Gynecology
3990 John R

From: Lawrence R. Crane, M.D. or designee L. Crane, MD/MB
Chairman, Medical Institutional Review Board (M1)

Date: February 05, 2015

RE: IRB #: 040302M1F

Protocol Title: The Biochemistry of a Short Cervix

Funding Source: Sponsor: NATIONAL INSTITUTE OF CHILD HEALTH AND HUMAN DEV.
Sponsor: NATIONAL INSTITUTES OF HEALTH

Protocol #: 0511003176

Expiration Date: February 04, 2016

Risk Level / Category: Research involving greater than minimal risk presenting no prospect of direct benefit, but likely to yield generalizable knowledge about the participant's condition

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Continuation for the above-referenced protocol and items listed below (if applicable) were **APPROVED** following Full Board review by the Wayne State University Institutional Review Board (M1) for the period of 02/05/2015 through 02/04/2016. This approval does not replace any departmental or other approvals that may be required.

- Actively accruing participants
- Informed Consent - Pregnant Women at 14-24 Weeks' Gestation with HIPAA Authorization (revision dated 12/4/09)
- Informed Consent - Pregnant Women on Labor and Delivery with HIPAA Authorization (revision dated 12/4/09)
- Informed Consent - Non-Pregnant Women Undergoing a Hysterectomy with HIPAA Authorization (revision dated 12/4/09)
- Informed Consent Addendum with HIPAA Authorization (revision dated 12/4/09)

- Federal regulations require that all research be reviewed at least annually. You *may* receive a *"Continuation Renewal Reminder"* approximately two months prior to the expiration date; however, it is the Principal Investigator's responsibility to obtain review and continued approval ***before*** the expiration date. Data collected during a period of lapsed approval is unapproved research and can *never* be reported or published as research data.
- All changes or amendments to the above-referenced protocol require review and approval by the IRB **BEFORE** implementation.
- Adverse Reactions/Unexpected Events (AR/UE) must be submitted on the appropriate form within the timeframe specified in the IRB Administration Office Policy (http://www.irb.wayne.edu/policies-human-research.php).

NOTE:

1. Upon notification of an impending regulatory site visit, hold notification, and/or external audit the IRB Administration Office must be contacted immediately.
2. Forms should be downloaded from the IRB website at **each** use.

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ABSTRACT

APPROPRIATE PRECONDITIONING OF THE UTERINE ENDOPLASMIC RETICULUM STRESS RESPONSE INHIBITS PRETERM LABOR

by

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May 2018

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Introduction: In this study, we are testing the overarching hypothesis that preconditioning the myometrial UPR allows for the maintenance of non-apoptotic CASP3 activity and thus sustains uterine quiescence. We have previously demonstrated that the pregnant uterus facilitates uterine quiescence through UPR mediated activation of non-apoptotic CASP3, yet the mechanism in which CASP3 utilizes to avoid its apoptotic cell fate is unresolved. There is a growing body of evidence including our own that demonstrates remote and direct preconditioning with minor stresses propagates cytoprotective mechanisms that allow for the avoidance of apoptotic cell death upon exposure to a subsequent more damaging stress, through modulation of the UPR. In this study we demonstrate endogenous pregnant-dependent stress stimuli experienced across gestation act in a preconditioning-like manner to sustain the tocolytic action of non-apoptotic CASP3 within the pregnant uterus in the presence of ensuing stresses and promote an all-around adaptive environment through paracrine and endocrine propagation of a myometrial stress-derived secretome.

Methods: *In vitro* preconditioning: utilizing the hTERT-HM cell line, uterine myocytes were preconditioned with a minor UPR stress (0.1μg/ml TM) or vehicle and

exposed 48 hrs later to a lethal UPR stress (5μg/ml TM) (n=3). *In vivo* sub-preconditioning: we generated a sub-preconditioned pregnant mouse model (TM+PBA) by inhibiting the effect UPR mediated stress across gestation (50mg/kg PBA (i.p, E10-15)) or vehicle. Endogenous preconditioned and sub-preconditioned mice were exposed to a mild exogenous stress at E16. Time of delivery was noted. From both the *in vitro* and *in vivo* models apoptotic and inflammatory indices were examined. *In vitro* secretome analysis: SILAC labeled hTERT-HM cells underwent UPR activation by exposure to TM, 5.0μg/ml, 1hr or vehicle. Additionally, SILAC labeled proteins transmitted from the UPR activated myocyte into the media were analyzed via LC/MS/MS to define the UPR generated secretome. In a separate experiment the conditioned media was incubated with a secondary set of naïve hTERT-HM cells, which were examined for UPR activation 48hrs later.

Results: Preconditioning the hTERT-HM cell activated CASP3 in the absence of apoptotic consequences. Reduced NFκB activation and TNFα secretion were also observed. *In vivo*, the sub-preconditioned mouse experienced CASP3 activation in the uterine compartment, which transitioned into an apoptotic state within the endometrial compartment upon exposure to a mild exogenous stress. Furthermore endometrial apoptotic CASP3-dependent iPLA2 activation, increased NFκB activation and COX1 expression upregulated prostaglandin synthesis, which resulted in a progesterone withdrawal and subsequently a 57% preterm birth rate in the preconditioned mice in comparison to 14% in the endogenously preconditioned animals. Further activation of the UPR in hTERT-HM cells generates a unique stress-generated secretome made up of roughly 90 bone-fide proteins, which propagate systemic adaptive signaling.

Conclusion: We speculate that women who are unable to host an appropriate preconditioning response to gestational stresses are at a significantly increased risk of undergoing spontaneous preterm.

AUTOBIOGRAPHICAL STATEMENT

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Education

2009-13	**B.Sc.**	Biomedical Sciences	Grand Valley State University, MI, USA
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Peer-Reviewed Publications:

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