THE EXPRESSION OF P-SER16 HspB6 IN UTERINE SMOOTH MUSCLE DURING PREGNANCY

MANDY ELIZABETH PEACH

The Expression of P-Ser16 HspB6 in Uterine Smooth Muscle During Pregnancy.

By

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Abstract

The small heat shock protein HspB6 has been associated with smooth muscle relaxation when phosphorylated at amino acid serine-16 (P-Ser16). P-Ser16 HspB6 expression has not been previously determined in the myometrium during normal pregnancy and labour, periods of uterine quiescence and contraction, respectively. P-Ser16-HspB6 expression in the rat myometrium significantly increased during late pregnancy and labour (One-way ANOVA; $p<0.05$) and decreased significantly post-partum. At late pregnancy, expression of P-Ser16 HspB6 in a unilateral pregnancy model was increased in the gravid horn compared to the empty horn by immunoblot analysis. Immunoblot analysis also showed that 17β-Estradiol significantly increased P-Ser16 HspB6 expression in a 17β-Estradiol administration model compared to vehicle controls (Two-way ANOVA; $p<0.05$). Thus, 17β-Estradiol and uterine stretch positively regulate post-translational phosphorylation of HspB6. Finally, studies with the M-11 myometrial cell line demonstrated that HspB6 was localized in the cytoplasm, while P-Ser16 HspB6 was primarily localized in the nucleus under standard tissue culture conditions.

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Abbreviations

ACTH – corticotrophin
AKAP – A-kinase anchoring protein
ANOVA – analysis of variance
AP-1 – activator protein-1
ATP – adenosine triphosphate
BSA – bovine serum albumin
BM – basement membrane protein
BrdU –5'-bromo-2'-deoxyuridine
cAMP – cyclic adenosine monphosphate
CAP – contraction associated protein
cGMP – cyclic guanosine monophosphate
CBP – cAMP response element binding protein (CREB)-binding protein
CHAPS – 3-[(3-chloamidopropyl)dimethylammonio]-1-propanesulfonate
COL – collagen
COX-2 – cycloxgenase-2
CRH – corticotrophin-releasing hormone
CX43 – connexin 43
d – day
DAPI – 4',6-Diamidino-2-Phenylindole
DMEM – Dulbecco's modified Eagle's medium
DNA – deoxynucleic acid
EBP – estrogen binding protein
ECL – electrochemiluminescence
ECM – extracellular matrix protein
EDTA – ethylenediaminetetraacetic acid
EGF – epidermal growth factor
ER – estrogen receptor
ERK – extracellular signal regulated kinase
FA – focal adhesion
FAK – focal adhesion kinase
FN – fibronectin
G – gravid
5-HETE – 5-hydroxyeicosatetraenoic acid
HIFIα – hypoxia-induced factor 1α
HPA – hypothalamic-pituitary-adrenal axis
h – hour
HRP – horseradish peroxidase
Hsp – heat shock protein
IGF – insulin-like growth factor
IgG – immunoglobulin G
IRS – insulin recetor substrate
LAM – laminin

M-11 – human myometrial cell line
MAP – mitogen activated protein
MCLK – myosin light chain kinase
Min – minute
MMP – matrix metalloproteinases
MPR – membrane bound progesterone receptors
mRNA – messenger ribonucleic acid
mTOR – mammalian target of rapamycin
NG – non-gravid
NO – nitric oxide
NOS – nitric oxide synthase
NP – non-pregnant
nPR – nuclear progesterone receptors
OTR – oxytocin receptor
PBS – phosphate buffered saline
PCNA – proliferating cell nuclear agent
PFA – paraformaldehyde
PG – prostaglandin
PGI_2 – prostacyclin
PGIS – prostacyclin synthase
PKA – protein kinase A
PKC – protein kinase C
PKG – protein kinase G
PP – post-partum
PR – progesterone receptor
PR –A/B/C – progesterone receptor A/B/C
P-Ser16 HspB6 – phosphorylated HspB6
P-Ser15 HspB1 – phosphorylated HspB1
RIPA – radioimmunoprecipitation assay
RU486 – mifepristone
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
SRC – steroid receptor coactivator
SSC – saline sodium citrate buffer
TBST – tris-buffered saline-tween
TNF – tumor necrosis factor
TUNEL – deoxynucleotidyl transferase dUTP nick end labeling
ZBF – zinc-based fixative

Chapter One

Introduction

1.1 Preterm Labour

Human gestation, generally 37-42 weeks in duration, is controlled by a synergy between maternal, placental and fetal influences (Liggins et al., 1973; Thorburn et al., 1991). During this period these influences collaborate to promote and aid the tremendous growth and development of the fetus, ensuring fetal survival in the extrauterine environment. For parturition to be successful there has to be maturation of the fetal organ systems to ensure extrauterine survival. The uterus, which remained quiescent throughout gestation, must generate coordinated contractions and cervical dilation to allow passage of the fetus through the birth canal (Challis et al., 2000). However, maternal and fetal influences are not always harmonious and asynchrony between the two results in preterm birth (Challis et al., 2000).

Preterm labour, defined as labour occurring < 37 weeks of gestation, is a major obstetric problem in healthcare today. In 2005, preterm births accounted for as many as 12.7% of births in the United States (Sayres, 2010) and in 2003, 7.7% of births in Canada were preterm (Joseph et al., 2007). Over the past 20-30 years preterm birth remains to be the leading cause of neonatal mortality and morbidity in North America, responsible for approximately 70% of neonatal deaths and 75% of neonatal illness (Challis, 2001; Challis et al., 2001; Gibb & Challis, 2002; Challis et al., 2002). Infants born before 37 weeks have an increased incidence of blindness, deafness, cerebral palsy, neurological disorders and pulmonary disorders (Lye, 2001). The cost of caring for preterm and low weight

neonates is staggering. In 2007, it was estimated that the average hospital stay for a preterm infant was approximately 12.9 days, at a cost of $15,100 versus an average $600, 1.9 day stay for uncomplicated births (Russell et al., 2007). Between the risk to the neonate and the healthcare economic burden, there is strong rationale to prevent preterm birth.

Preterm birth can occur for a variety of reasons. Some preterm births are elective, however approximately 30% occur due to infection, and about 50% of preterm births occur for unknown reasons (Challis, 2001; Challis et al., 2001;Gibb & Challis, 2002; Challis et al., 2002). There are no effective diagnostic tools to accurately predict preterm birth, and no concrete scientific evidence pointing to precisely why preterm birth occurs. There are risk factors associated with preterm pregnancy such as a history of preterm birth, tobacco use, and shortened cervical length (Goldenberg et al., 2008); however, none of these factors can adequately help predict the occurrence or onset of premature labour. The current method for dealing with preterm birth is to provide the patient with a tocolytic, or labour repressant, that delays delivery for about 48 hours - long enough to administer corticosteroid to speed up fetal lung maturation. Unfortunately, this method has not been consistently shown to improve neonatal and perinatal outcomes (Berkman et al., 2003).

It is clear that to be able to develop adequate treatments to deal with preterm birth we first have to understand the physiological and biochemical mechanisms underlying normal labour. Only then can we begin to understand the cause of asynchrony between maternal and fetal influences in preterm birth.

1.2 Uterus: the Myometrium

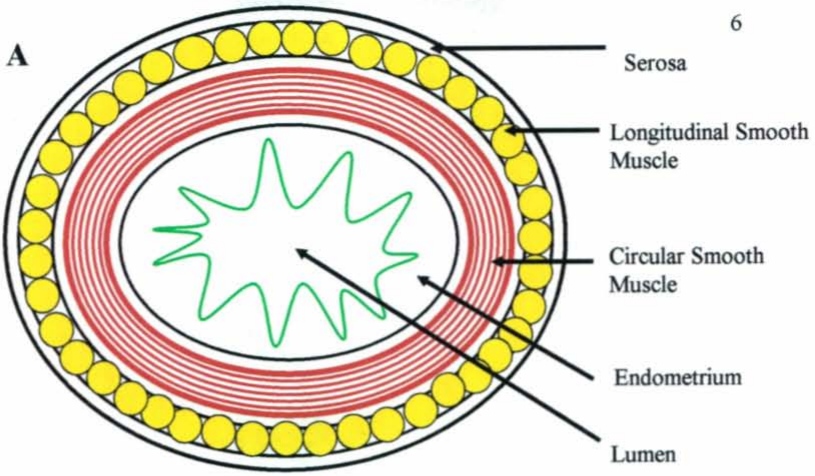
The uterus is a hollow reproductive organ unique to viviparous mammals. The human uterus is composed of three major tissue layers: the serosa - a thin outer connective tissue layer, the myometrium - a thick middle layer containing smooth muscle and the endometrium which is the innermost layer where implantation and placental formation occur (Silverthorne, 2010). The lumen of the uterus houses the fetus(es), placenta and amniotic fluid throughout the duration of pregnancy. To accommodate the immense growth of the fetus, the uterus is capable of undergoing a dramatic increase in size, weight and capacity throughout pregnancy. To put this in perspective, the nonpregnant human uterus weights 40 to 70 g and has a capacity of 10 mL, while a uterus at term weighs upwards of 1100-1200g and has an average capacity of 5 L (Monga & Sanborn, 2004). The uterus has two principal functions. Firstly, the uterus must harbor the intrauterine contents, while providing a protective, calm environment for the developing fetus. Secondly, upon activation, the myometrium must change rapidly to a contractile syncytium capable of generating strong cohesive waves of contraction to expel the fetus (Hertelendy & Zakar, 2004). These robust contractile waves are generated by poorly defined muscle layers in the human and two easily recognized muscle layers in the rodent myometrium. In the rat there is outer longitudinal layer and the inner circular layer. In the rat myometrium, both layers can be distinctly seen under a microscope with the longitudinal layer appearing as muscle bundles along the outer edge of the myometrium, and the circular layer located circumferentially next to the inner endometrium. Both muscle layers are nourished by the vascular plexus, an extensive network of blood vessels

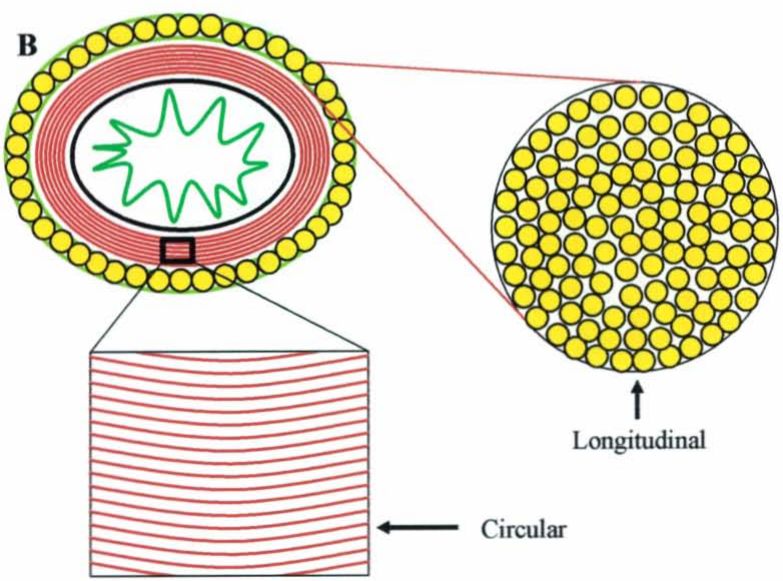
separating the two layers (Shynlova et al., 2005) (Figure 1.1). The myometrium itself accounts for greater than 60% of the uterine mass (Martin et al., 1973).

1.3 Myometrial Contraction

Smooth muscle cells are small cells tightly packed with myofilaments and dense bodies, which comprise the contractile machinery (Gabella, 1984).The contractions developed by the myometrium are generated through actomyosin crossbridging achieved via two fibrillar domains found in smooth muscle cells: a contractile domain and a cytoskeletal domain with specific associated proteins (Tessier et al., 2003). The contractile domain is made up of thin filaments (actin), actin associated proteins such as tropomyosin, caldesmon and calponin, and the myosin thick filaments (Tessier et al., 2003). In uterine smooth muscle cells, in particular, there is ~6 fold more actin than myosin (Word et al., 1993) similar to other types of smooth muscle cells. The cytoskeletal domain provides the cells with structural integrity and contains non-muscle actin and intermediate filament proteins such as desmin, vimentin and synemin (Tessier et al., 2003). These intermediate filaments may play an important role in force development as vimentin filaments insert into dense bodies, that anchor the actin filaments, as well as into plasma membrane desmosomes, which serve as intercellular junctions. When actin and myosin are activated during a contraction, the intermediate filaments may facilitate spatial reorganization of the machinery to optimize contractile force (Wang et al., 2006). Upon labour, the influx of calcium into myometrial cells via voltage-gated channels leads to the activation of myosin light chain kinase (MCLK) by the calcium-calmodulin complex. MLCK

Figure 1.1. (A) Diagrammatic representation of a cross section of the rat uterine horn (B) The two muscle layers can be easily differentiated when a uterine tissue section is observed under a microscope. The longitudinal muscle layer is composed of numerous muscle bundles which align along the long axis of the uterus, while the circumferentially oriented circular muscle layer surrounds the myometrium.


A
Serosa
Longitudinal Smooth Muscle
Circular Smooth Muscle
Endometrium
Lumen


B
Longitudinal
Circular

phosphorylates the light chain of myosin, triggering cycling of myosin cross-bridges along actin filaments, converting the chemical energy of ATP to mechanical energy required for contraction (Walsh, 1991; Rembold, 1992; Jiang & Stephens, 1994; Hertelendy & Zakar, 2004; Tang et al., 2005). The mechanism through which this force is transmitted within and between smooth muscle cells is not entirely clear; however, it is hypothesized that through intercellular mechanical couplings the contractile filaments may be organized into a transcellular mechanical syncytium. Therefore, the filaments lying parallel to the longitudinal axis of the muscle bundle and coincidental to the axis of force transmission can generate and transmit force (Kuo & Seow, 2004). This mechanical syncytium is thought to develop within the myometrium prior to and during labour via increased expression of integrins, focal adhesion (FA) turnover and remodeling of extracellular matrix proteins (Figure 1.2). Together these processes are hypothesized to facilitate proper smooth muscle cohesion in myometrial cells, thus activating the myometrium and facilitating sustained, coordinated and robust contractions required for labour (Williams et al., 2005).

1.4 Phases of Myometrial Differentiation

Throughout pregnancy the smooth muscle cells of the myometrium undergo dramatic phenotypic changes to prepare for labour. Each distinct phase of pregnancy is regulated by either mechanical (distension) influences, endocrine influences or both (Figure 1.3). The ultimate goal at term is the formation of a spontaneously active and excitable muscle which is responsive to agonists promoting uterine contraction (Williams et al., 2005). The work presented in this thesis is based upon the rat model of pregnancy. Therefore, further

Figure 1.2. Schematic representation of contractile filament architecture in a bundle of smooth muscle cells. Contraction in smooth muscle involves cyclic interaction of myosin and actin. Mechanical connections provided by dense plaques (or focal adhesions) on opposing cells allow transfer of the force from one cell to another and, thus, the formation of a functional syncytium. Adapted from Kuo and Seow, 2003.

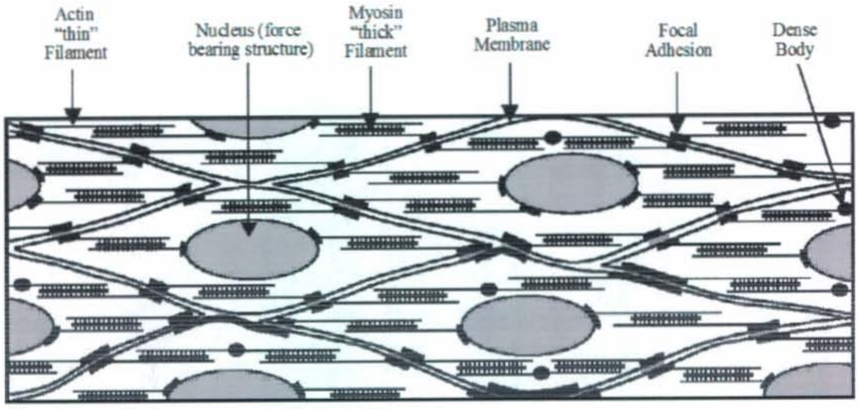
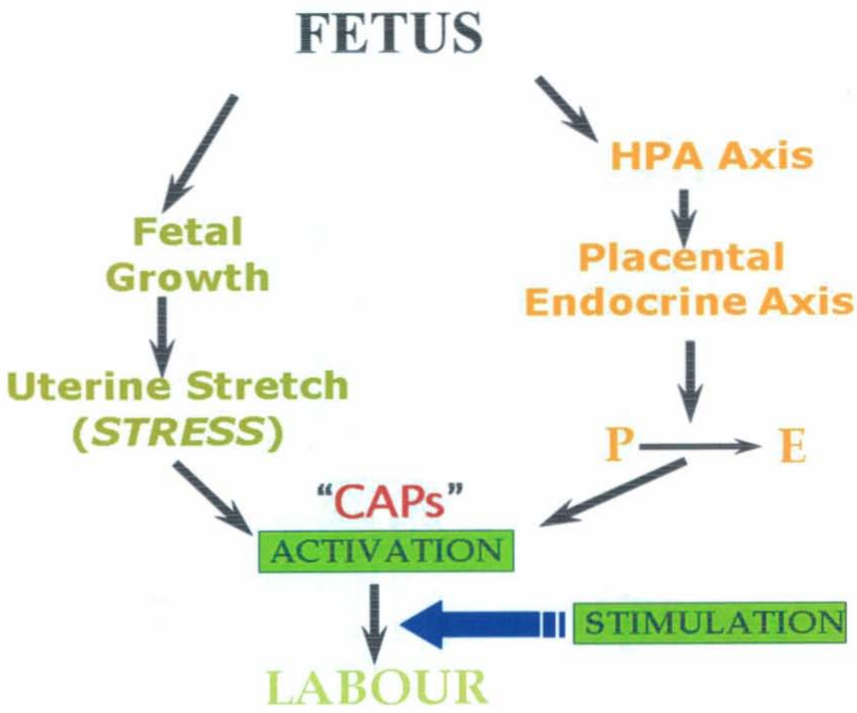

Actin
"thin"
Filament
Nucleus (force
bearing structure)
Myosin
"thick"
Filament
Plasma
Membrane
Focal
Adhesion
Dense
Body

Figure 1.3 The dual pathway by which the fetal genome contributes to the onset of labour via mechanical and endocrine signals. The fetal hypothalamic-pituitary-adrenal (HPA) axis leads to initiation of endocrine cascades that cause a shift, in most animals, in the progesterone (P): estrogen (E) ratio which favours estrogen just prior to labour. Mechanical stretch of the uterine muscle due to fetal growth leads to stretch-related increases in contraction associated proteins (CAPs). Together, both pathways activate the myometrium and enable it to become responsive to labour-associated agonists, resulting in labour.


FETUS
HPA Axis
Fetal Growth
Placental Endocrine Axis
Uterine Stretch (STRESS)
P
E
"CAPs"
ACTIVATION
STIMULATION
LABOUR

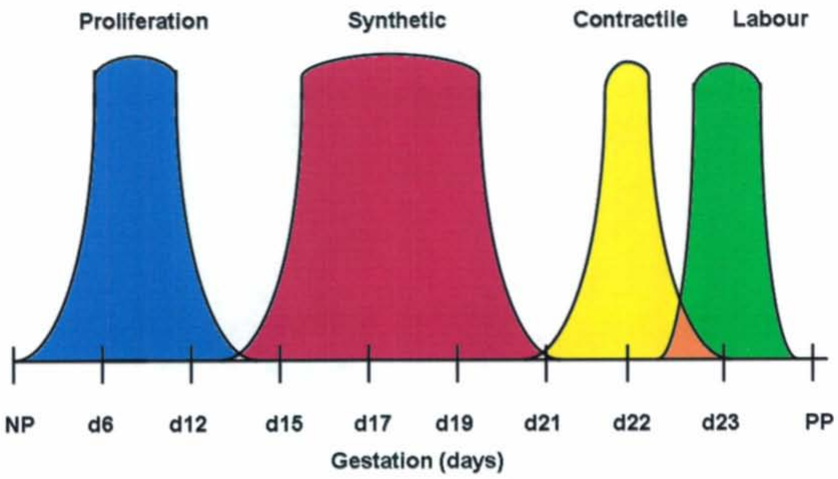
discussion will mainly centre on the rat myometrium and the phases of rat pregnancy (Figure 1.4).

1.4.1 Proliferation

The initial phase of pregnancy occurs from a non-pregnant state to approximately day 14 of gestation in the rat. This phase is characterized by a period of rapid cell proliferation, predominantly in the longitudinal muscle layer (Shynlova et al., 2009). In 2006, Shynlova and colleagues provided evidence of this when they found increased incorporation of 5-bromo-2'-deoxyuridine (BrdU), a nonradioactive analogue of thymidine and marker of individual cell proliferation, into uterine myocytes at days 6 and 12 of pregnancy. They also found increased expression of proliferating cell nuclear antigen (PCNA) between day 6 and day 15 of pregnancy. Such increases were found in both gravid and non-gravid horns of unilaterally pregnant rats, indicating that proliferation is primarily regulated by endocrine influence(s), rather than mechanical stretch (Shynlova et al., 2006). Such endocrine influence is often mediated by growth factor signaling.

Insulin-like growth factor-1 (IGF-1) is one factor expressed in most tissues of the body and upon activation of its receptor, it promotes cell proliferation and differentiation (Le Roith, 2003). Experiments in the non-pregnant rat indicated that estrogen-regulated growth factors such as IGF-1 and EGF (epidermal growth factor) were the regulators of myocyte proliferation (Lye et al., 2001). In the pregnant state, IGF-1 and its associated binding proteins were found to be upregulated in the myometrium during days 6-14 of rat gestation (Shynlova et al., 2007). IGF-1 is thus an important promoter of uterine cell

Figure 1.4 Phases of myometrial differentiation. Proliferative phase (blue) from NP to d14 characterized by dramatic increase in cell number. Synthetic phase (pink) from d14 to d21 consists of myometrial cell hypertrophy. Contractile phase (yellow) from d21 to approximately d23 is characterized by basement membrane synthesis. Lastly, the labour phase (green) is evident by the upregulation of contractile associated proteins (CAPs) that initiate labour. The transition between the contractile and labour phase (orange) indicates that the changes in differentiation are not distinct – the time of transition is approximate.


Proliferation
Synthetic
Contractile
Labour
NP
d6
d12
d15
d17
d19
d21
d22
d23
PP
Gestation (days)

hyperplasia, triggered by pregnancy-related sex steriods. Estrogen is also proposed to promote hyperplasia in the uterine epithelia and stroma by regulating mitosis during times of physiological growth, such as pregnancy (Jaffer et al., 2009). 17β-Estradiol more specifically was found to induce cellular proliferation in uterine smooth muscle cells by promoting G1 cell cycle progression (Yin et al., 2007). The mammalian target of rapamycin (mTOR) is a serine/threonine pathway known to regulate cell proliferation in many tissues via cell cycle progression and cell survival (Hay & Sonenberg, 2004). It is initially activated by estrogen circulation (Jaffer et al., 2009). Estrogen interacts with and activates IGF-1 which then binds to its associated receptors. This causes the phosphorylation of insulin receptor substrate (IRS) which in turn recruits phosphoinositide-3-kinase (PI3K). This recruitment causes a cascade of phosphorylation reactions such as the phosphorylation of Akt. Akt, also known as protein kinase B, is a serine/threonine kinase that positively regulates mTOR activity. This reaction, in addition to other phosphorylation events, ultimately leads to the activation of mTOR activity (Jaffer et al., 2009). Studies on the pregnant rat over gestation indicated that upstream regulators such as IRS-1, PI3K, Akt and downstream effectors S6K1 and 4EBP1 (estrogen binding protein) - which are all involved in activation of mTOR - were upregulated during the proliferative stage of pregnancy. Such findings reaffirm mTOR's likely involvement in hyperplasia of the myometrial cells (Jaffer et al., 2009).

Apoptosis, or the action of removing excess or dysfunctional cells during development or homeostasis, is another process that has been studied with relation to the proliferative stage of pregnancy. Apoptosis is known to occur during pregnancy in the

uterus across species such as the mouse (Mu et al., 2002), rat (Leppard, 1998) and human (Smith et al., 1997). Two prominent pathways of apoptosis are the tumor necrosis factor (TNF) receptor pathway and the exogenous stimulus pathway (Shynlova et al., 2006). The latter route is a parallel, mitochondria-dependent pathway that can be activated by environmental stimuli and/or physiological stimuli, such as periods of hypoxia which are evident in utero during midgestation when spherical fetal growth generates tension in the uterus walls, creating ischemic conditions (Reynolds, 1950). This pathway uses cytosolic cysteine proteases (caspases) which are inactive in most cells and must be cleaved to initiate apoptosis through effector caspases that digest cellular substrate leading to cell death (Thornberry, 1996). The exogenous stimulus pathway, evident in pregnancy, is regulated by the Bcl-2 family of anti-apoptotic regulatory proteins. Shynlova et al. (2009) measured levels of anti-apoptotic factor Bcl-2 in the pregnant rat myometrium. Increased expression of Bcl-2 was apparent in the proliferative stage, aiding to attenuate apoptosis, thus further promoting the increase in cell number (Shynlova et al., 2009).

Many paracrine or endocrine contractile inhibitory molecules such as nitric oxide and relaxin, also act on the myometrium during the proliferative phase of pregnancy and throughout all of gestation to prevent spontaneous contraction. In studies examining the effect of nitric oxide on contractility in pregnant human myometrial samples, it was found that spontaneous contraction could be reduced when tissue samples were incubated in Kreb's solution containing nitric oxide (NO) (Buhimschi et al., 1995). Cyclic guanosine monophosphate (cGMP) production in these samples was also found to be increased when both a substrate (L-arginine) and nitric oxide donor (diethylamine/nitric

oxide) were present. When an NO synthase (NOS) inhibitor was added (nitro-L-arginine methyl ester), the amount of cGMP decreased (Buhimschi et al., 1995). In the pregnant rat, it has been found that diethylenetrianine-NONOate, a NO donor, and a benzylindazole derivative YC-1, a NO-independent activator of guanylate cyclase, both induced relaxation in spontaneously contracting rat myometrium strips extracted from day 21 of gestation (Demirkoprulu et al., 2005). Further radioimmunoassay experiments on these samples demonstrated that addition of these relaxation promoting molecules also coincided with an increase in cGMP. A study on ovariectomized ewes demonstrated that spontaneous myometrial contraction initiated by 17β-Estradiol injections could be inhibited by a dose of relaxin, a peptide hormone that has been associated with inhibition of myometrial contraction in laboratory animals (Kratz et al., 1950). The myometrial contraction was abruptly, but reversibly, halted in 4 out of 5 ewes (Porter et al., 1981). Bani et al. (1998) studied relaxin's mode of action in vascular smooth muscle cells from bovine artery. By measuring the expression and activity of NO synthase and the production of NO when relaxin was added, they determined that relaxin acts by activating the L-arginine-nitric oxide pathway (Bani et al., 1998).

Each contractile inhibitor has its own method of action; however, a mechanism common to many is an increase in cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) (Dayes & Lye, 1990; Grammatopoulos & Hillhouse, 1999; Negishi et al., 1995; Riemer et al., 2008), which are intracellular second messengers that influence physiological events (Yuan & Bernal, 2007). One way both nucleotides accomplish this is via activation of protein kinase A (PKA) and/or G (PKG).

Phosphorylation of myosin light chain via MLCK allows interaction of myosin with actin and actin-activated ATPase, and thus is critical for myocyte contraction (Sanborn, 2001). PKA and PKG activation promotes relaxation by maintaining actin in a globular form, thus preventing the formation of actin fibrils which are necessary for contraction (Challis et al., 2000; Gibb & Challis, 2002; Smith, 2007). Lastly, active PKA and PKG promote the uptake of extracellular calcium into intracellular storage sites, thus impeding calcium mobilization necessary for contraction (Smith, 2007).

1.4.2 The Synthetic Stage

Around day 14 of pregnancy in the rat, proliferation decreases and the expression of Bcl-2 that was evident during the proliferative phase begins to decrease (Shynlova et al., 2009). At this point in pregnancy myocyte growth switches from a proliferative phase to a phase of hypertrophic growth (Shynlova et al., 2009); this phenotype lasts until approximately day 21. Cellular hypertrophy is an increase in cell size which can lead to an organ growing in size. Smooth muscle hypertrophy can result when an obstruction impairs the outlet of a hollow organ (Gabella, 1990), such as the growing fetus that grows and imposes stress upon the uterus as pregnancy progresses.

A number of processes are critical to cellular hypertrophy. Firstly, in hypertrophic cells there is an increase in cellular protein synthesis including the thin, thick and intermediate filaments to accommodate the increase in cell size (Gabella, 1990). Secondly, synthesis of extracellular matrix proteins (ECM) such as collagen I, collagen II and elastin, as well as ECM reorganization occurs to ensure the growing cells are properly anchored for later stages of pregnancy (Steward et al., 1995). This anchoring reorganization is likely

regulated by focal adhesion kinase (FAK) – a kinase found to be highly expressed in the rat myometrium during late pregnancy in the rat (MacPhee & Lye, 2000). When FAK is activated, it forms complex structures on the cytoplasmic surface of the plasma membrane called focal adhesions, also known as dense plaques in smooth muscle (Richardson & Parsons, 1995; Juliano, 1996; Hanks & Poulte, 1997). At focal adhesion sites extracellular ligands are coupled to cytoplasmic F-actin, the main constituent of muscle fibre thin filaments, via their integrin receptors. This establishes critical cell-cell interactions that allow focal adhesion complexes to sense mechanical forces as well as provide structural linkage required for force transmission through contractile proteins to the ECM and the subsequent muscle bundles (MacPhee & Lye, 2000; Williams et al., 2005; Shynlova et al., 2009). Thirdly, an increase in mass of cell organelles, including mitochrondria, smooth and rough endoplasmic reticulum, is apparent in hypertrophic cells as a means of increasing the synthetic and secretory functions of the growing cells (Gabella, 1990). Lastly, hypertrophic cells are characterized by a transition in contractile protein content with more intermediate filaments and gap junction proteins, and a higher density of sarcoplasmic recticulum giving rise to a different structural integrity (Gabella, 1990).

The exact reason for the switch in phenotype from proliferative to hypertrophic in the myometrium is unknown, but the change coincides with the activation of an apoptotic cascade as seen by the upregulation of certain caspases such as initiator caspase-9 and effector caspases 3 and 6, which are fundamental to apoptosis (Shynlova et al., 2009). Despite activation of an apoptotic pathway, no large-scale evidence of apoptosis was seen

in the rat myometrium through terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), which labels terminal ends of nucleic acids of fragmented deoxynucleic acid (DNA) (Shynlova et al., 2006; Shynlova et al., 2009). The reason for activation of the pathway may be due to a period of hypoxia around mid-pregnancy in the rat caused by the growing fetus reaching a maximal spherical radius and thus, impeding maternal blood flow and oxygen supply (Reynolds, 1949). The shape of fetal growth then shifts to elipsoid, releasing tension and restoring maternal blood flow throughout the uterus. In the pregnant rat, this switch is evident at day 14 of pregnancy, the same timepoint when hypoxic markers such as hypoxia-induced factor 1α (HIF1α) and the hydroxyprobe pimonidazole hydrochloride are upregulated (Shynlova et al., 2010).

The hormonal environment throughout pregnancy is dominated by progesterone, a hormone found in virtually all species for maintenance of pregnancy. Progesterone seems to be a key regulator of the synthetic phase with its circulation increasing around day 10 and peaking between day 17-19 of pregnancy. When a competitive receptor antagonist to progesterone, mifepristone (RU486), was administered during this period, attenuation of hypertrophy occurred, thus demonstrating that progesterone circulation was necessary for cell growth (Lye et al., 2001). The importance of progesterone is also illustrated in terms of ECM protein synthesis, such as collagen I, collagen II and elastin. A fall in the secretion of these proteins was observed with the fall in progesterone at term, and with the administration of RU486. If progesterone was administered at late pregnancy, the synthetic phase was prolonged, preventing myocyte transition to prepare for labour and thus blocking the onset of labour (Lye et al., 2001).

Mechanical regulation is also vital to ensure that cells undergo hypertrophic in preparation for labour. In unilateral studies in pregnant rats, only the gravid horn underwent hypertrophy with evidence of fibrillar matrix protein expression (Shynlova et al., 2004). Collectively, the evidence suggests that the synthetic phase of pregnancy requires both the hormonal influence of progesterone and the mechanical tension of fetal growth to promote cell hypertrophy.

1.4.3 The Contractile Stage

At day 21 of gestation, the rat myometrium undergoes another transition to the contractile phase, which lasts until labour at day 23. At this time, myometrial hypertrophy stabilizes and a change is observed in the types of proteins secreted by the myocytes. Myocytes, which have already undergone focal adhesion remodeling to anchor themselves in the synthetic phase, are now preparing to anchor to the basement membrane (Shynlova at al., 2009), a critical event to ensure transmission of force through cohesive labour contractions (Williams et al., 2005). In the contractile phase, specialized basement membrane (BM) proteins are the main proteins expressed, surrounding each individual smooth muscle cell. Basement membrane proteins are directly apposed to the cell membrane, with a middle layer composed of collagen IV (COL4) and laminin (LAM), and an outer layer in mature BM of fibronectin (FN) (Robinson et al., 2004; Shynlova et al., 2009). This phenotypic change is evident in the remodeling of the basement membrane protein conformation from sporadic immunofluorescent detection in the synthetic phase to organized, continuous and regular structures (Shynlova et al., 2009).

Regulation of the contractile phase is again two fold – endocrine and mechanical. In unilateral pregnancy studies, expression of basement membrane proteins such as elastin, laminin fibronectin and collagen I, III, and IV was restricted to the gravid horn only in later stages of pregnancy when distension was maximal and cells were in the contractile phase (Shynlova et al., 2004). In contrast to the synthetic phase, the contractile phenotype is associated with a decrease in progesterone levels towards the end of pregnancy. This was further supported when administration of RU486 caused a premature shift in BM gene expression from interstitial to the BM proteins collagen IV, fibronectin and laminin as seen by increased mRNA levels (Shynlova et al., 2004). When progesterone circulation was prolonged with steroid injection, the transition to the contractile phenotype was prevented, as evidenced by continued interstitial matrix protein expression (Shynlova et al., 2004).

The uterus undergoes many phenotypic changes in preparation for labour including proliferation and hypertrophy as previously discussed (Section 1.4). Throughout these transformations the uterus remains fairly quiescent; however, it is not completely inactive. Low frequency, low amplitude, long duration contractions called 'contractures' occur in the human uterus, but they are poorly coordinated and do not result in labour-like waves of contraction or cervical softening associated with birth (Lye & Freitag, 1988). In the pregnant rat, at d19 of pregnancy an intrauterine catheter measuring pressure can detect low amplitude, infrequent transient rise in pressure. At d20-21, electrical bursts were detected using electrodes on the abdominal surface; however, these bursts did not coordinate with pressure readings, suggesting low

amplitude activity in local areas, quite similar to contractures seen in humans (Buhimschi et al., 1998).

1.4.4 The Labour Phase

Just prior to day 23, the rat myometrium becomes activated and is prepared to create the robust contractions of labour. During labour the myometrium is receptive to a cassette of upregulated genes associated with the formation of sodium channels, oxytocin receptors, prostaglandins receptors and gap junction proteins – which increase excitability within the myometrium (Lye et al., 2001). Substances that modulate myometrial tone and contractility are known as Contraction Associated Proteins or CAPs. During this phase, the myometrium is preparing to enter labour and there is increased electrical coupling between myometrial cells (Wray & Noble, 2008). This coupling facilitates the spread of depolarization from cell to cell, promoting phasic bursts of contractility in the uterus (Kao, 1967; Garfield et al., 1988; Miller et al., 1989; Marshall, 1962). The propagation of electrical activity combined with the frequency and duration of bursts are directly proportional to the frequency, duration and strength of uterine contraction (Marshall, 1962). Most species, such as the guinea pig, have increased gap junctions just prior to labour to form the groundwork for the synchronous contractions of labour (Garfield et al., 1977; Garfield et al., 1988). Lammers et al. (2008) determined that electrical activity in the guinea pig uterus can originate in the ovarian or cervical end of the uterus and propagate in either direction; however, it is more frequently initiated in the ovarian end.

Gap junctions are hemichannels made from connexin proteins to form interconnecting pores between smooth muscle cells. Gap junction formation is upregulated in the rat myometrium prior to labour, thus enabling the muscle to create the high amplitude, coordinated contractions (Petrocelli & Lye, 1993). This is accomplished by the low resistance pathways provided by gap junctions. During actual labour FAK kinase activity is decreased, suggesting that the myocytes have completed transformation and are now anchoring to the basement membrane, as FAK activity is associated with dense plaque turnover (MacPhee & Lye, 2000). Anchorage reinforces the ligand-integrin interaction, maintaining cell-cell communication and enabling smooth muscle cohesion required upon delivery (Shynlova et al., 2009). The focal adhesions now are main points of force transduction, ensuring that the myocytes work as a mechanical syncytium to shorten the uterine horns via contraction allowing fetal explusion (Williams et al., 2005).

Uterine distension is an important regulator of the contractile phase. In late pregnancy, the fetus keeps growing while uterine growth has ceased, thus increasing uterine tension (Lye et al., 2001). The increased expression of the transcription factor c-fos (Mitchell & Lye, 2002), gap junction protein connexin 43 (Cx43) (Ou et al., 1997) and oxytocin receptor (OXTR) (Ou et al., 1998) was only observed in the gravid horn of unilateral pregnant rats, suggesting that distension is necessary for CAP activation. Of greater importance, when a tube was used to mechanically increase pressure and stretch in the non-gravid horn of the same animals, the expression of CAPs was restored (Ou et al., 1997; Ou et al., 1998).

In addition to distension, progesterone levels must be low to allow CAP activation and decrease FAK activity to permit labour (Ou et al., 1998; MacPhee & Lye, 2000).When a polyvinyl tube was inserted into a non-gravid horn on day 17 of pregnancy in a unilaterally pregnant rat, CAP activation was not observed when circulating progesterone levels were high (Ou et al., 1998). Therefore, both endocrine and mechanical influences are necessary to increase myometrial receptivity to labour-associated agonists and activate the myometrium to initiate labour.

1.4 Hypothalamic-Pituitary-Adrenal (HPA) Axis

Although the general phases of myometrial differentiation have been described, the underlying molecular mechanisms controlling the shift from a quiescent state to one of rapid contraction is still poorly understood. However, it is known that signals residing in the fetal genome ultimately lead to myometrial activation via both mechanical and endocrine pathways (Williams et al., 2005). The mechanical pathway has been described in Section 1.4. From an endocrine standpoint, activation of the hypothalamic-pituitary-adrenal axis (HPA) during late pregnancy results in a switch of steroid production from progesterone to estrogen in most animals (Challis, 2001; Gibb & Challis, 2002), activating the expression of CAPs and promoting labour. Studies examining the fetal pituitary gland, fetal adrenal gland and pituitary stalk in fetal sheep demonstrated that ablation of any of these organs prolongs gestation (McDonald & Nathanielsz, 1991; Gluckman et al., 1991; McDonald et al., 1992). This demonstrates the importance of fetal HPA axis activation and that the failure of this activation results in the lack of hormonal input required for initiation of labour.

The maturation of the fetal HPA axis in most mammals, including primates, occurs during the later stages of pregnancy and is described in detail elsewhere (Challis et al., 2002). Briefly, work in sheep suggests that the HPA axis functions to promote a sequence of events during pregnancy that leads to parturition. During late pregnancy, increased fetal glucocorticoids are being produced (Liggins et al., 1973) and a signaling cascade results that ultimately lead to increased production of estrogens by the placenta (Rainey et al., 1991; Conley & Bird, 1997). It has also been demonstrated that estradiol synthetic capacity within the human chorion is increased with labour and is associated with the activation of the myometrium (Madsen et al., 2004), thus aiding to disrupt the relaxed uterine state and facilitating delivery (Brown et al., 2004). Cortisol production has also been found to increase the levels of prostaglandins (PG) and with PGE_2, in particular, within the fetal circulation (Lye et al., 1998). The increased prostaglandin production in intrauterine tissues plays a central role in initiation of labour by causing increased myometrial contractility (Bennett et al 1987).

One of the major factors leading to labour in most animals is the decline of placenta/ovarian progesterone output (Csapo, 1956). This decrease is coupled with an increase in circulating levels of estrogens (Liggins et al., 1973). Various pathways are thought to decrease progesterone output, including an increase in progesterone metabolizing enzymes. 20α-hydroxysteroid dehydrogenase is one enzyme which metabolizes progesterone into 20α-hydroxyprogesterone (Diaz-Zagoya et al., 1979), a biologically inactive metabolite, aiding in loss of uterine sensitivity to progesterone at term (Condon et al., 2004). Another mechanism aiding the decrease in progesterone is the

direct interaction of progesterone receptors (PR) with pro-inflammatory transcription factors such as NF-κB. A mutual antagonism between PR and the p65 subunit of NF-κB has been found in cell lines such as COS-1 and HeLa. When NF-κB was activated by TNF-α (a cytokine involved in inflammation), PR transcriptional activity was inhibited (Kalkhoven et al., 1996). In humans, the increased expression of inhibitory PR isoforms, such as PR-A and PR-C, decreases circulating levels of progesterone (Giangrande et al., 2000). Lastly, the altered expression of PR coactivators and corepressors also decreases progesterone levels (Condon et al., 2003; Mendelson, 2009). In pregnant mice at term there was a marked decrease in steroid receptor coactivator (SRC) and cAMP response element-binding protein (CREB)-binding protein (CBP) (Condon et al., 2003). These changes in coregulators may compromise PR transcriptional activity and increase sensitivity of the uterus to contractile stimuli.

Together the above pathways work together to create a decline in progesterone and a simultaneous rise in estrogens (Gibb et al., 2006). Estradiol opposes progesterone and augments myometrial contractility and excitability during labour. Estrogens function through estrogen receptors (ER) on target cells, with distinct domains of the protein responsible for ligand-binding, DNA-binding and transcription activation (Katzenellenborgen, 1997; Katzenellenborgen, 2000). There are two types of ER: ESR1 and ESR2. The receptors have different C-terminal regions, which promote different binding affinities for estrogens (Sun et al., 1999). Once the estrogen receptor is occupied by a ligand, it homodimerizes or heterodimerizes and modulates gene expression (Kumar & Chambon, 1988; Ryffel et al., 1988; Green & Chambon, 1991; Truss & Beato, 1993;

Tsai & O'Malley, 1994). There are multiple signaling pathways involved in cell regulation and cell proliferation by ER which directly affect functioning in many reproductive tissues such as the uterus, vagina, ovary, oviduct and mammary glands (Katzenellenborgen, 2000).

In terms of pregnancy, increased circulatory estradiol levels leads to the expression of CAP genes, which results in myometrial activation and subsequent labour (Gibb & Challis, 2006). ER_{α} and ER_{β} expression is low in non-pregnant women and is increased during labour (Mesiano et al., 2002), providing evidence that ERs are upregulated towards the end of pregnancy. The administration of large amounts of 17β-Estradiol at term to non-labouring pregnant women increased contractions and oxytocin responsiveness within 4-6 hours (Pinto et al., 1967).

In the rat, the increase in circulatory estradiol generally coincides with a decrease in circulating progesterone. However, in humans during pregnancy, there is no decrease in circulating progesterone and labour proceeds in the presence of high levels of progesterone. This suggests that there must be some adjustment or change in the response of one or more of the three isoforms of the progesterone receptor: (PR-A, PR-B or PR-C.) Human PR exists primarily as hPR-A and hPR-B, hPR-B activating progesterone responsive genes and hPR-A acting as a strong transdominant repressor of hPR-B while remaining transcriptionally inactive (Giangrande et al., 2000). PR-C is also an inhibitor of PR-B. It binds to progesterone, thus impeding PR function by sequestering available hormone away from PR-B (Wei et al., 1996). Towards the end of pregnancy the ratio of PR-A to PR-B mRNA is increased approximately 10-fold in the lower uterine segments

of the myometrium in labouring woman, when compared to women not in labour (Mesiano et al., 2002). Such a change in isoform could be responsible for the functional withdrawal effect in human myometrium.

1.6 Mechanical Distension

Although of great importance, endocrine influence alone is not enough to activate the myometrium for labour. Stretch, or distension, of the growing fetus(es) also initiates mechanical signals necessary for myometrial activation. Increased mechanical distension from multiple fetuses is a contributor of preterm labour (Gardner et al., 1995; Lye et al., 2001; Gibb et al., 2002) with > 50% of multiple birth cases being delivered before 37 weeks (Stock & Norman, 2010). Macrosomia, where the fetus is large for its gestational age, and polyhydramnios, where excess amniotic fluid is present in the amniotic sac, produce similar preterm effects due to uterine stretch produced by an increase in fetal contents (Mazor et al., 1996). Uterine distention leads to the upregulation of CAPs, enhance acto-myosin interactions to promote contraction, increase excitability of individual myometrial cells and promotes connectivity between cells allowing the development of synchronous contractions during labour (Smith, 2007).

The uterus also exhibits stretch dependent myogenic contraction. In vitro studies of the rat uterus showed that stretch caused intracellular calcium influx into the smooth muscle cells which promoted contraction (Kasai et al., 1995) Calcium is a second messenger that controls processes such as muscle contraction, secretion, differentiation and signal transmission through gap junction proteins (Himpens & Vereecke, 2000).

Gap junctions are structurally differentiated areas of the plasma membrane of a cell that contains transmembrane channels. These channels link cytoplasmic compartments of adjacent cells to promote cell-cell communication, transfer of small molecules and propagation of electrical impulse as seen during labour in the myometrium (Orsino et al., 1996). Such coordination allows synchronous muscle contraction. mRNA and protein levels of Cx43, in particular, are increased markedly in the myometrium during the last 48 hours of labour in the human, with the highest level seen during delivery itself, where communication is critical among cells to ensure successful delivery (Winterhager et al., 1991; Chow et al., 1994). Cx43 expression is also positively impacted by stretch. In studies on the rat uterus, when an inert tube was inserted in the lumen of the uterus to induce stretch, there was an increase in Cx43 mRNA expression as well as other CAPs such as oxytocin (Ou et al., 1997). Ca^{2+} /PKC (protein kinase C) pathways have been shown to phosphorylate Cx43 in the presence of epidermal growth factor (Park et al., 2008). Calcium influx during stretch may regulate the phosphorylation of serine and tyrosine residues in the cytoplasmic domain of Cx43 (Beyer, 1993).

Stretch is also known to stimulate the production of prostacyclin (PGI_2), a major prostaglandin secreted during pregnancy that promotes relaxation (Korita et al., 2002). PGI_2 accomplishes this through IP receptor species which increase adenylate cyclase activity and intracelluluar cAMP levels (Negishi et al., 1995). A study examining the effect of cyclic mechanical distension on human myometrial cells found that distension upregulated prostacyclin synthase (PGIS) expression, a synthase regulating biosynthesis of PGI_2, via activation of the TF activator-protein-1 (AP-1). PGI_2 elevation was also

apparent (Korita et al., 2002). As pregnancy progresses levels of PGI_2 also rise, and remain elevated even during labour, perhaps due to cyclic tension generation of the myometrium during labour (Korita et al., 2002) although the exact reason(s) remains unknown. The effects of distension in terms of growth are aided by progesterone, as this hormone is needed in circulation for stretch induced hypertrophy and uterine growth to occur (Section 1.4).

1.7 Heat Shock Proteins (Hsps)

The small heat shock proteins (Hsps) are a family of stress proteins ranging in size from 12-43 kDa in mass that are highly conserved throughout all kingdoms with the exception of some pathogenic bacteria (Haslbeck et al., 2005). The mammalian family of proteins is currently comprised of ten Hsp members (Table 1.1) (Golenhofen et al., 2004). Although the proteins belonging to this family are diverse in sequence and size, they all share the same distinguishing features such as a conserved α-crystallin domain of approximately 90 amino acid residues which spans two putative actin-binding domains (Gusev et al., 1995) Hsps have the ability to form large oligmers with other heat shock proteins, as evidenced by the formation of complexes between HspB1-HspB5, HspB6-HspB5 and HspB6-HspB1 (Sugiyama et al., 2000; Sun et al., 2004; Fontaine et al., 2005) resulting in a dynamic quarternary structure. Hsps are induced by stress conditions such as heat shock, oxidation and exposure to toxins such as anti-cancer drugs (Gusev et al., 2002). Under such conditions they perform chaperone activity by binding to denatured proteins and preventing their irreversible aggregation (Horwitz, 1992; Jakob et al., 1993). Thus Hsps aid in the assembly, disassembly, stabilization and the internal transport of

Table 1.1 List of the small heat shock proteins including the alternative nomenclature and the protein accession number to identify the protein in the NCBI protein database.

Protein	Alternate Name	Protein Accession Number
HSPB1	HSP27	P04792
HSPB2	myotonic dystrophy protein kinase binding protein (MKBP)	Q16082
HSPB3	--	Q12988
HSPB4	αA-crystallin	P02489
HSPB5	αB-crystallin	P02511
HSPB6	HSP20	O14558
HSPB7	cvHSP	Q9UBY9
HSPB8	HSP22	Q9UKS3
HSPB9	--	Q9BQS6
HSPB10	sperm outer dense fiber protein (ODFP)	Q14990

intracellular proteins (Dreiza et al., 2010). These interactions form stable complexes between the proteins and it appears that Hsps, similar to other chaperones, act in a promiscuous fashion when attaching to a substrate. They are promiscuous in that a diversity of proteins are associated with sHsps in times of heat stress such as proteins from plants, yeast and mammals (Jinn et al., 1995; Ehrnsperger et al., 1997; Haslbeck et al., 1999). It is proposed that the binding sites on the Hsps themselves are hydrophobic in nature (Lindner et al., 2000; Giese et al., 2002; Friedrich et al., 2004; Haslbeck et al., 2004; Stromer et al., 2004; Basha et al., 2004).

In addition to a conserved α-crystallin domain, Hsps all have a highly variable N-terminal region preceding the crystallin domain, followed by a C-terminal region that is involved in the stabilization of the protein via ionic interactions (Kim et al., 1998; van Montfort et al., 2001). The secondary structure of the Hsps is a compact β-sheet sandwich composed of two layers of three and five antiparallel strands that are connected by a short interdomain loop (Kim et al., 1998; van Montfort et al., 2001). The majority of Hsps also undergo post-translational modification to influence their activity, such as phosphorylation via various protein kinases. This post-translational modification affects protein functioning within the cell and oligomer formation with other Hsps (Suzuki et al., 1998; MacRae, 2000; Sugiyama et al., 2000), making it one of the most prominent modifications that occurs in Hsps, and thus very important in functional regulation (Gaestel, 2002). In smooth muscle, in particular, Hsps are involved in contraction and relaxation via their phosphorylation events. HspB1 is one Hsp whose phosphorylation at site Ser-15 is proposed to promote the association of actin and myosin through

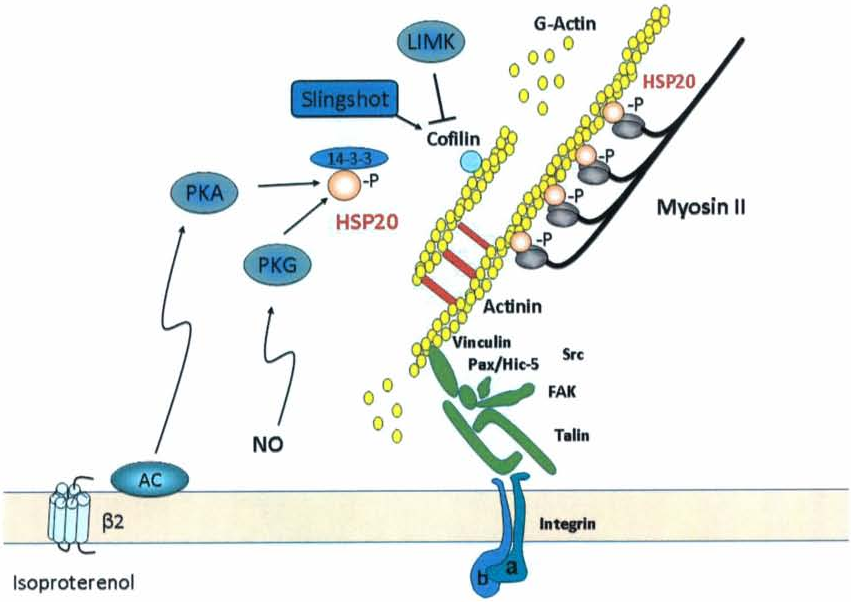
tropomyosin (Bitar, 2002), thus making phosphorylation of HspB1 a potential prerequisite for smooth muscle contraction. Relaxation of smooth muscle has been studied in relation to HspB6 and its phosphorylation at Ser-16 (Rembold et al., 2000). This will be discussed in more detail in Section 1.8.

1.8 Heat Shock Protein 20 (Hsp20/HspB6)

Hsp20, or HspB6, was initially copurified with Hsp27 and αB crystallin in human skeletal muscle and was named Hsp20 based of its molecular mass of 20 kDa following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Kato et al., 1994). As a result of this copurification, and the fact that the protein was highly similar to αB-crystallin in sequence, it was suggested that this 20 kDa protein was indeed a member of the small heat shock protein family. Today, HspB6 is recognized as one of the 10 members of the family of small heat shock proteins expressed in practically all organs and tissues, with especially high content of 1.3% of total proteins in slow skeletal muscle, diaphragm, heart and smooth muscles (Gusev et al., 2002). HspB6 forms both low and high molecular mass complexes, with the predominant aggregation being a dimer of ~ 40 kDa (Bukach et al., 2004). As Hsps are associated with chaperone activity, the chaperone capability of HspB6 was also examined. It was originally found that this level of activity was reduced compared to other Hsps (Van de Klundert et al., 1998). Later investigations by Bukach et al. (2004) performed on untagged recombinant HspB6 demonstrated that the chaperone activity of this protein is comparable or even better than HspB5. HspB6 is also capable of being phosphorylated at site Ser-16 (Rembold et al., 2000), Ser-120 (Matsuno et al., 2003) and Ser-157 (Wang et al., 1999). Phosphorylation at these

sites affects protein interactions and the functional roles of HspB6, including the prevention of thrombin-induced platelet aggregation when phosphorylated at Ser-120 (Matsuno et al., 2003), a potential role in glucose transport when indirectly phosphorylated by insulin at site Ser-157 (Wang et al., 1999), and also an anti-apoptotic effect in cardiomyocytes when phosphorylated at Ser-16 (Fan et al., 2004). A particularly intriguing role of HspB6 is in cyclic nucleotide-dependent smooth muscle relaxation when phosphorylated at Ser-16 (Rembold et al., 2000). This type of activation associated with relaxation is initiated by agonists such as isoproterenol, prostacyclin and forskolin which stimulate the adenylate cyclase/cAMP pathway, activating the cAMP dependent protein kinase (PKA) (Murray, 1990). Similarly, the cGMP dependent protein kinase (PKG) is also activated by agonists such as nitric oxide, atrial natriuretic peptide and nitroglycerin via the guanylate cyclase/cGMP pathway (Lincoln, 1989). PKA/PKG induces phosphorylation of HspB6 at Ser-16 (Rembold et al., 2000), which then is proposed to induce smooth muscle relaxation via one of two mechanisms (Figure 1.5), the first being the direct inhibition of acto-myosin cross-bridging via a troponin I like effect (Rembold et al., 2000; Yoshino et al., 2005). Troponin I is an actin-binding protein within the troponin complex that is important in striated muscle for initiating muscle contraction (Perry, 1999). In the muscle's relaxed state, troponin I binds to actin, holding the actin-tropomyosin complex in place, thus preventing actin and myosin from interacting and inihibiting muscle contraction (Perry, 1999). In smooth muscle there is no troponin I; calcium-calmodulin complex is responsible for activating the myosin light chain kinase (MCLK) to phosphorylate myosin and form an actomyosin complex (Walsh,

Figure 1.5 Phosphorylation of HspB6 begins with the activation of adenyl cyclase (AC), subsequently activating protein kinases A and G (PKA) and (PKG). These kinases phosphorylate HspB6 at site Ser16. Phosphorylated HspB6 is hypothesized to then promote relaxation either by: 1) interacting with actin via a tropinin-1 like domain, preventing actomyosin interaction or 2) outcompeting slingshot and binding to 14-3-3, leaving unbound slingshot active to promote actin depolymerization via cofilin activity. From Salinthone et al., (2008). Vinculin, Pax/Hic-5, Src, FAK and Talin comprise focal adhesion proteins which serve as anchorage for actinin. These proteins are connected to the cell membrane via integrin.


G-Actin
LIMK
Slingshot
Cofilin
HSP20
-P
-P
-P
-P
Myosin II
14-3-3
-P
PKA
HSP20
PKG
Actinin
Vinculin
Pax/Hic-5
Src
FAK
Talin
NO
AC
β2
Integrin
b
a
Isoproterenol

1991; Rembold, 1992; Jiang & Stephens, 1994; Hertelendy & Zakar, 2004; Tang et al., 2005). However, HspB6 contains a region of partial homology, at amino acid residues 110-121, to the actin binding region of cardiac and skeletal tropinin I (Rembold et al., 2000). Therefore, HspB6 might directly interact with actin, in the same manner as troponin I in striated muscle, preventing acto-myosin cross-bridging from occurring and promoting smooth muscle relaxation (Rembold et al., 2000). Yoshino et al. (2003) examined the relaxant effects of both a synthetic peptide of cardiac troponin I residues 136-147, and a highly analgous peptide corresponding to an actin-tropomyosin binding region of human HspB6 (HspB6p), residues 110-121. Both peptides were separately applied to a strip of Ca^{2+} induced contracting taenia cacei smooth muscle. Both appeared to accelerate the transfer from fast-detaching cross bridges to the latch bridge conformation indicative of a relaxed state. They proposed that HspB6p peptide could achieve relaxation directly by the interaction of the troponin-like region with thin filament actin (F-actin) (Yoshino et al., 2003). An earlier study by Brophy et al. (1999) reported that phosphorylated HspB6 associates with G-actin, whereas non-phosphorylated Hsp20 interacts with F-actin. These results were obtained by co-sedimentation of F-actin with highly aggregated His-tagged HspB6, with HspB6 being predominantly found in the pellet as it was barely soluble. However, with ultracentrifugation one would expect the largest fraction of F-actin to be present in the pellet as well, but it instead remained in the supernatant, raising doubts that HspB6 really associates with actin. To compound this, Bukach et al. (2005) studied a mixture of HspB6 or an S16D mutant peptide which mimics phosphorylated HspB6, and isolated F-actin or

F-actin containing tropomyosin, calponin or α-actinin. When this mixture was ultracentrifuged there was co-sedimentation of a miniscule amount of HspB6 monomer per mol of actin. Myofibrils of skeletal, cardiac or smooth muscle also bound a very small amount of HspB6 monomer per mol of actin, independent of phosphorylation or mutation of HspB6 (Bukach et al., 2005). This suggests that neither HspB6, nor its mutant, affects actin polymerization directly as it does not seem to be forming tight stoichiometric complexes with F-actin.

The alternate mechanism of P-Ser16 HspB6 induced smooth muscle relaxation is thought to occur via actin depolymerization. Polymerization of actin is necessary to develop tension in smooth muscle cells and contraction of smooth muscle tissues causes an increase in the pool of F-actin, which activates myosin ATPase activity and the cross-bridging cycle, and a decrease in the pool of monomeric globular (G) actin (Barany et al., 2001). Numerous studies have examined the effect of inhibiting actin polymerization on tension generation. Studies in uterine smooth muscle, in particular, have demonstrated that short-term exposure of tissues to inhibitors of actin polymerization cause a profound suppression of tension development (Shaw et al., 2003). Upon stimulation of adenyl cyclase and activation of PKA/PKG, P-Ser16 HspB6 is thought to disrupt smooth muscle contraction by promoting depolymerization of F-actin to monomeric G-actin. This may be accomplished by P-Ser HspB6 displacing phosphorylated cofilin from the adaptor protein 14-3-3, a protein involved in many cellular functions and one which has many ligands (Rubio et al., 2004). 14-3-3 protein binds to cofilin via a phosphoserine binding motif, which protects cofilin from dephosphorylation by slingshot phosphatase (Gohla &

Bokoch, 2002; Birkenfeld et al., 2003). Dephosphorylation activates cofilin and promotes F-actin depolymerization, increasing the pool of G-actin (Dreiza et al, 2005). The reaction between phoshorylated HspB6 and the scaffold protein 14-3-3 is thought to be sequence and phosphorylation state specific. The domain surrounding serine 16 phosphorylation site on HspB6 contains a sequence (RRApSAP) that is similar to a putative 14-3-3 binding motif (RSXpSXP) (Yaffee et al., 1997). In a study by Dreiza et al. (2005) they carried out pull down assays of immobilized HspB6 which demonstrated that 14-3-3 specifically associated with pHspB6 peptides. Furthermore, the immobilized pHspB6 peptides showed a band at ~ 31 kDa, suggesting binding with 14-3-3 protein (Dreiza et al., 2005). Together this suggests that both phosphorylated cofilin and phosphorylated HspB6 must share a binding site on the 14-3-3 protein (Dreiza et al., 2005). The study by Bukach et al. (2005) previously mentioned also suggests that P-Ser16 HspB6 indirectly affects F-actin polymerization as the association of HspB6, or its phosphorylated mutant, with F-actin was quite low, raising the question of how P-Ser16 HspB6 could directly affect polymerization if the stoichiometry of the complex formed by HspB6 and actin is so small.

It is clear that the field is quite divided on how P-Ser16 HspB6 is promoting relaxation. But, regardless of the mechanism, it is undeniable that expression of phosphorylated HspB6 potentially bears significant importance in many fields of healthcare. In cardiac muscle, HspB6 is thought to regulate contractility by inducing myocyte shortening via increased calcium uptake (Pipkin et al., 2003). For patients suffering from bronchospasm and asthma, activation of the PKA/PKG pathway that

results in phosphorylation of HspB6 has been shown to induce airway smooth muscle relaxation (Komalavilas et al., 2008). Lastly, and of great importance to this thesis, HspB6 has been shown to associated with relaxation in the myometrium. HspB6 phosphorylation has been shown to occur during cycle nucleotide-mediated myometrial relaxation in samples from pregnant human uterus (Tyson et al., 2008). In the MacPhee laboratory both the mRNA and protein expression of HspB6 have been demonstrated to be dramatically decreased during late pregnancy in the pregnant rat myometrium (Cross et al., 2007); however the detection of phosphorylated HspB6 was not conducted.

1.9 Hypothesis & Study Objectives

Given its potential as a smooth muscle relaxant, the hypothesis for this study was that serine 16 phosphorylated HspB6 expression in rat myometrium would be elevated during pregnancy to potentially promote myometrial relaxation during this period and participate in the phasic contractions of labour. This might occur when the uterus undergoes such dramatic mechanical stress during mid-late pregnancy and just prior to labour in the rat when marked increases in circulating levels of 17β-Estradiol are evident.

There were two main goals for this thesis: First, to characterize the expression of P-Ser16 HspB6 in pregnant rat myometrium during pregnancy, compatible with a functional significance for this protein in myometrial relaxation. Secondly, to determine the underlying regulatory mechanism of P-Ser16 HspB6 expression. To accomplish these goals, 3 major objectives were generated:

Objective 1: To determine the spatial and temporal expression of P-Ser16 HspB6 in the rat myometrium throughout pregnancy and labour.

Objective 2: To determine whether mechanical distension affects P-Ser16 HspB6 expression in rat myometrium.

Objective 3: To determine if hormonal influence (17β-Estradiol) affects P-Ser16 HspB6 expression in rat myometrium.

Experiments utilized an existing bank of uterine tissue lysates and tissue sections collected by previous students Bryan White, Brandon Cross and Joy Williams. Additional samples were collected for this thesis and added to the bank, but all experimental work described in this thesis were performed by myself.

Chapter Two

Materials and Methods

2.1 Animals

Sprague Dawley rats were acquired from the Mount Scio Vivarium (Memorial University of Newfoundland, St. John's, NL, Canada). Animals were kept at the Animal Care Unit at the Health Sciences Centre, Memorial University of Newfoundland under standard environment conditions of 12 hour light and 12 hour darkness. Rats were fed LabDiet Prolab RMH 3000 (PMI Nutrition International, Brentwood, MO, USA), while water was available *ad libitum*. Virgin female rats of approximately 220-250 g were mated to induce pregnancy. Day 1 of pregnancy was designated upon observation of a vaginal plug the morning following mating. Under these standard conditions labour occurred at day 23 of gestation. All experiments were approved by the institutional animal care committee under protocols 08-02-DM to 10-02-DM.

2.2 Experimental Design

2.2.1 *Unilateral Pregnancy Model*

Virgin female rats underwent general anaesthesia using a 1:1 ratio of ketamine hydrochloride (ketaset) (Wyeth Animal Health, Guelph, Ontario) and xylazine (rompun) (Bayer HealthCare, Toronto, Ontario) per 100 grams weight. Unilateral tubal ligation was performed through a flank incision, permitting pregnancy in only one horn (Shynlova et al., 2007). Following a 7 day recovery period, the rats were mated. Pregnant rat myometrial samples were collected from both non-gravid (empty) and gravid uterine

horns on days 15 (n = 4), 19 (n = 4) and 23 (labour; n = 8) of gestation. Labour samples were taken during active labour after the rat had delivered 2-3 pups.

2.2.2 *Exogenous 17β-Estradiol Administration*

Virgin female rats were anesthetized with 1:1 ratio of ketamine and xylazine per 100 grams weight. Ovariectomy was performed through a flank incision, removing the steroidal influence of the ovaries. Following a 7 day recovery period, rats were given either an injection of 17β-Estradiol (0.5 mg s.c. in 0.2 ml corn oil) or vehicle alone (n=4) (Mitchell & Lye, 2002). Myometrial samples were taken at 1, 3, 6, 12 and 24 hours post injection.

2.3 Tissue Collection

Individual animals were each placed in a euthanasia chamber and exposed to an increasing concentration of carbon dioxide gas resulting in death by asphyxiation within 5 minutes. For immunofluorescence, a cross sectional portion of the rat mymometrium was fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) (pH 7.4) with shaking overnight at room temperature. Tissues were processed, paraffin embedded, sectioned and mounted on microscope slides by the Histology Unit of Memorial University of Newfoundland School of Medicine. Sections contained both the longitudinal and circular muscle layer of the myometrium. For immunoblot analysis the uterine horns were first excised and then opened longitudinally, exposing the fetuses and placentae which were then discarded. Uterine tissue was placed in ice cold PBS (pH = 7.4) and the endometrial layer was gently removed by scraping with a scalpel blade

(White et al., 2005). All myometrial samples were then flash-frozen in liquid nitrogen and stored at -80 °C .

2.4 Immunoblot Analysis

Immunoblot analysis was performed on samples obtained from normal pregnancy, unilateral pregnancy experiments, and 17β-Estradiol administration experiments. Four independent sets of protein samples (n = 4 rats per gestational time point) were used for all studies with the exception of the day 23 timepoint of the unilateral pregnancy study where eight independent protein samples from 8 rats were used (MacPhee & Lye, 2000). Frozen rat myometrial samples were pulverized under liquid nitrogen and homogenized in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCL (pH 7.5), 150 mM NaCl, 1% (vol/vol) Triton X-100, 1% (wt/vol) sodium deoxycholate, and 0.1% (wt/vol) SDS) containing phosphatase inhibitor cocktail and complete and mini ethylenediaminetetraacetic acid (EDTA)-free protease inhibitors (Roche Molecular Biochemicals, Laval, Quebec, Canada). All samples were centrifuged for 15 min at 15, 000 x g at 4 °C and supernatants collected. Protein concentration was determined using the Bradford Assay (Bradford, 1976) using Bio-rad protein assay dye reagent (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Samples (100 μg/lane) were then separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 15% resolving gels according to Laemmli (1970). Proteins were transferred to 0.2 μm nitrocellulose membranes (Thermo Scientific, Rockford, Illinois, USA).

After transfer, membranes were washed with Tris-buffered saline-Tween-20 (TBST; 20 mM Tris base, 137 mM NaCl, and 0.1% Tween-20; pH 7.6) followed by a

1 h block in 5% milk powder/TBST. Membranes were all probed with two different primary antibodies for P-Ser16 HspB6. One was a rabbit polyclonal antisera raised against a synthetic phosphopeptide SWLLRRA-S-PO_3-APLPG specific for the rat, site P-Ser16 HspB6 (Nicolaou et al., 2008; a kind gift from Dr. GC Fan, University of Cincinnati College of Medicine, Ohio, USA) at 1:10,000 dilution overnight at 4 ^{o}C. The second was a new, commercially available rabbit polyclonal anti-Ser16 HspB6 antisera raised against the sequence R-A-S^{P}-A-P (Cat. No. ab58522; Abcam Inc., Cambridge, MA, USA) at a dilution of 1: 2000. After probing, only a 40 kDa band was observed with each antibody, suggesting that HspB6 formed a dimer when phosphorylated. To determine if the band was indeed a dimer, some tissues were extracted with a urea lysis buffer (30 mM Tris-CL (p.H 8.0-9.0), 7 M urea, 2M thiourea, and 4 % 3-[(3-cholamidopropyl)dimethylammino]-1-propanesulfonate (CHAPS) (wt/vol)) containing Phosphatase Inhibitor Cocktail and Complete and Mini EDTA-free protease inhibitors (Roche Molecular Biochemicals, Laval, Quebec, Canada). Samples extracted with urea buffer were incubated at room temperature for 30 min before centrifugation. Urea extraction was carried out to increase the denaturation of proteins resulting in an increase in detection of a band at 20 kDa when separated by SDS PAGE. Membranes were also probed with rabbit polyclonal antisera raised against total levels of HspB6 (Cat. No. 07-490; Upstate Biotechnology, Lake Placid, NY, USA), mouse monoclonal antisera raised against smooth muscle calponin, rabbit polyclonal antisera raised against Erk-2 (estradiol study) (Cat. No. C2687; Sigma-Aldrich, St. Louis, MO, USA), or rabbit polyclonal antisera raised against Cx43 (estradiol study) (Cat. No. ab11370; Abcam, Cambridge,

MA, USA) for 1 h at room temperature at dilutions of 1:2000, 1:100,000, 1:2000 and 1:2000 respectively. Following primary antibody incubation, blots were probed with either horseradish peroxidase (HRP) –conjugated goat anti-rabbit IgG (H + L) or HRP-conjugated goat anti-mouse IgG (H + L) (Cat. No. 31460 and 31430 respectively; Pierce, Rockford, IL, USA) at a dilution of 1:10,000. All antisera incubations were conducted with constant agitation on a shaker. Protein detection was accomplished using the Pierce SuperSignal West Pico chemiluminescent substrate detection system (MJS Biolynx, Inc., Brockville, Ontario, Canada). Multiple exposures on Amersham electrochemiluminescence (ECL) film (GE Healthcare Limited, Little Chalfont, BKM, UK) were taken to ensure the film response was in the linear range.

Calponin protein expression was analyzed as a loading control following probing with both P-Ser-16 HspB6 and total HspB6 antisera since this protein is constitutively expressed in both pregnant and non-pregnant rat myometrial samples following RIPA lysis buffer protein extraction (White *et al.* 2005; Williams *et al.* 2005). Erk-2 protein expression was analyzed as a loading control for the 17β-Estradiol study as calponin is responsive to estradiol.

2.5 Immunofluorescence

Immunofluorescence was performed on samples obtained from normal pregnancy, unilateral pregnancy experiments and 17β-Estradiol administration experiments. Four independent sets of slides (n=4) were used for all studies. Slides were de-waxed and rehydrated in a graded series of xylene (3 X 100% for 5 min each) and ethanol (1X 100%, 95%, 90%, 80%, 70% and 50% for 3 min each) washes followed by a

final wash with PBS (3 X for 7 min each). Following the washes, slides were washed once in PBS and then epitope retrieval was carried out with a 15 minincubation of sections in 1mg/mL trypsin (4 mM $CaCl_2$, 200 mM Tris, pH7.7) at room temperature. Another method of retrieval was tested, a saline sodium citrate buffer (SSC) protocol where slides were boiled 4X in SSC buffer, with 5 min between boiling periods. Slides were then rinsed with PBS. Tissue sections were blocked with 5% normal goat serum/1% horse serum in PBS for 30 min and then incubated overnight with commercial rabbit anti-P-Ser16 HspB6 (Cat. No. ab58522; Abcam Inc., Cambridge, MA, USA) or with rabbit anti-P-Ser16 HspB6 (Obtained from Dr. GC Fan) with shaking overnight at 4 °C. The antibody was diluted 1:250 in blocking solution for experiments. Rabbit IgG (Cat. No. 011-000-003; Jackson ImmunoResearch Labs, Inc., West Grove, PA, USA) was utilized at the same effective concentration to serve as a negative control. Following incubation, sections were washed in PBS and then incubated with fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit IgG (Cat. No. F7512; Sigma-Aldrich, St. Louis, MO, USA) at a 1:250 dilution for 30 min with shaking at room temperature. Sections were then washed with cold PBS containing 0.02% Tween-20 and then mounted with Vectashield (Vector Laboratories Inc., Burlington, Ontario, Canada), containing 4', 6-diamidino-2-phenylindole (DAPI) to stain the nuclear compartment. Slides were observed and images collected using a Leica DMIRE2 fluorescence microscope (Leica Microsystem (Canada) Inc., Richmond Hill, Ontario, Canada) equipped with a QImaging Retiga EXi Camera (QImaging, Surrey, British Columbia, Canada). Images were viewed using Improvision Openlab Version #5 software (PerkinElmer, Waltham, MA, USA).

2.6 Cell culture

The human myometrial cell line M-11 were a generous gift from Dr. John Copland (Mayo Clinic College of Medicine, Jacksonville, Florida). M-11 was derived from human myometrial tissue obtained from elective caesarian section. These cells were derived from dispersed primary human myometrial cells by repeated passage without the use of any immortalizing or transformating agent (Devost & Zingg, 2007). M-11 cells exhibit an elongated shape, a central nucleus and express two major smooth muscle cell markers: smooth muscle α-actin and hCaldesmon as detected by immunoblot analysis (Devost & Zingg, 2007). Scatchard plot analysis of OXTR binding revealed that this cell line also expresses large amounts of high affinity OXTR, a landmark of myometrial cells (Devost & Zingg, 2007). Lastly, the functionality of the OXTR demonstrated evidence for coupling to the MAP kinase pathway using a phospho-ERK-specific antibody. Immunoblot analysis demonstrated that the oxytocin application led to a dose-dependent increase in ERK1/2 phosphorylation. This suggested that the M-11 cell line retains the phenotypic characteristics of primary myometrial smooth muscle cells (Devost & Zingg, 2007). The cells were cultured in 75 cm^2 flasks at 37˚C with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. At 80-90% confluence, cells were seeded on coverslips at ~ 40,000 cells per coverslip and allowed to grow overnight at 37˚C, after which the cells were fixed in 4% PFA/PBS for immunflurorescence (n=3).

2.7 Data Analysis

Densitometry was performed on immunoblots using Scion Image Analysis software (Scion Image Corporation, Frederick, MD, USA). Densitometric values for P-Ser16 HspB6 and total HspB6 were normalized to the calponin loading control or Erk-2 loading control in the Estradiol study. Statistical analysis was carried out using GraphPad Instat version 3.0 (GraphPad Software, San Diego, CA, USA, www.graphpad.com) and graphs were prepared using GraphPad Prism version 4.0 (GraphPad Software). Data from the gestational profile and 17β-estradiol administration experiments data were analyzed using a One-way analysis of variance (ANOVA) and Two-way ANOVA, respectively while data from unilateral pregnancy experiments were analyzed using a student t-test. A p-value < 0.05 was considered significant in each study.

Chapter Three

Results

3.1 Normal Pregnancy and Labour

3.1.1 *Expression of P-Ser16 HspB6 protein*

To assess the commercial and donated P-Ser16 HspB6 anti-sera, immunoblot analysis was conducted on tissue homogenates extracted with the standard RIPA lysis buffer. The immunoblot analysis demonstrated that myometrial and liver homogenates extracted with RIPA lysis buffer resulted in the detection of a band of ~ 40kDa after use of both P-Ser16 HspB6 specific antibodies (Figure 3.1.1), demonstrating that this dimeric form of HspB6 was present in tissues other than the myometrium. All immunoblots in the remaining experiments were probed with both P-Ser16 HspB6 specific antibodies. Before testing the P-Ser16 HspB6 anti-sera on heart and liver tissue sections, the protocol and personal competence was validated by performing immunofluorescence on uterine sections using α-smooth muscle actin, a differentiation marker for smooth muscle cells. The results demonstrated high levels of cytoplasmic α-smooth muscle actin staining in the myometrium (Figure 3.1.2). This agrees with the results of Shynlova et al. (2005), where they found high levels of α-smooth muscle actin in longitudinal and circular muscle layers in pregnant rat myometrium. Immunofluorescence of heart and liver tissue was then performed using both P-Ser16 HspB6 antisera. The results demonstrated general cytoplasmic staining in both tissues, with brighter fluorescence in the liver (Figure 3.1.3). These observations differed from other studies where total HspB6 in non-tumor liver

Figure 3.1.1 P-Ser-16 HspB6 protein is expressed in liver and myometrial homogenates. Immunoblot analysis was performed on liver and myometrial samples using an anti-rabbit P-Ser-16 HspB6 specific antisera (commercially available antibody). Detection of HspB6 at ~40 kDa in each tissue. 100 μg of total protein was loaded per lane. Order of lanes: liver, myometrial samples: d19, d21, d22. Ladder on right indicates 25 kDa, 37 kDa and 75 kDa.

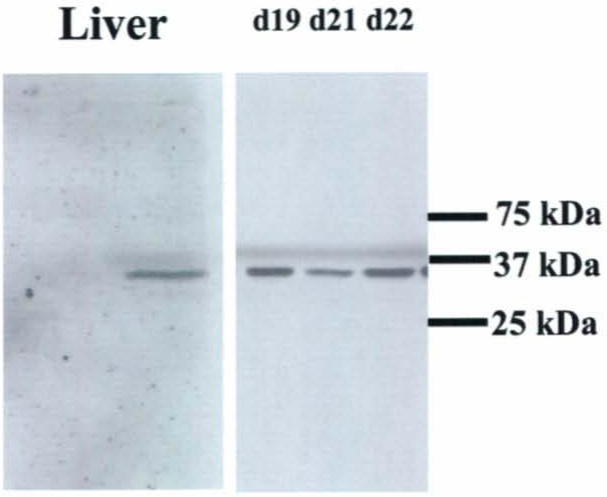
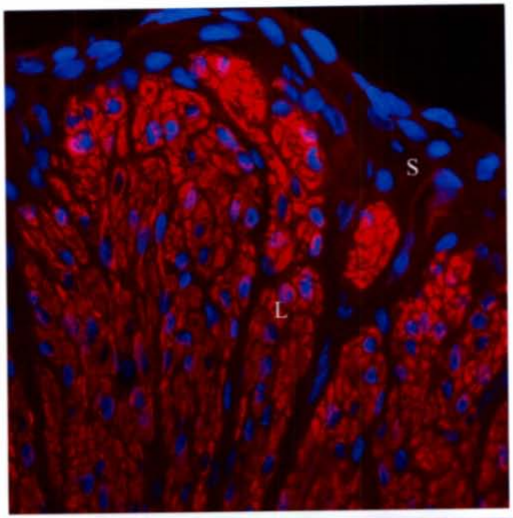
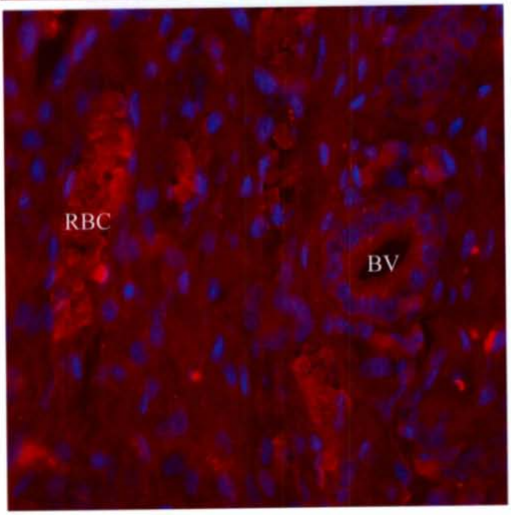

Liver
d19 d21 d22
75 kDa
37 kDa
25 kDa

Figure 3.1.2 Immunocytochemical analysis of α-smooth muscle actin detection in longitudinal muscle layer of the rat myometrium of d19 non-gravid sample using mouse anti- α-smooth muscle actin antisera. The images demonstrate high level of expression of the protein with cytoplasmic localization within myometrial cells (red). Then nuclei are counterstained with DAPI (blue). RBC = red blood cells, BV = blood vessel, S = serosa, L = longitudinal muscle layer. Control = mouse IgG. Observed magnification = 200X.

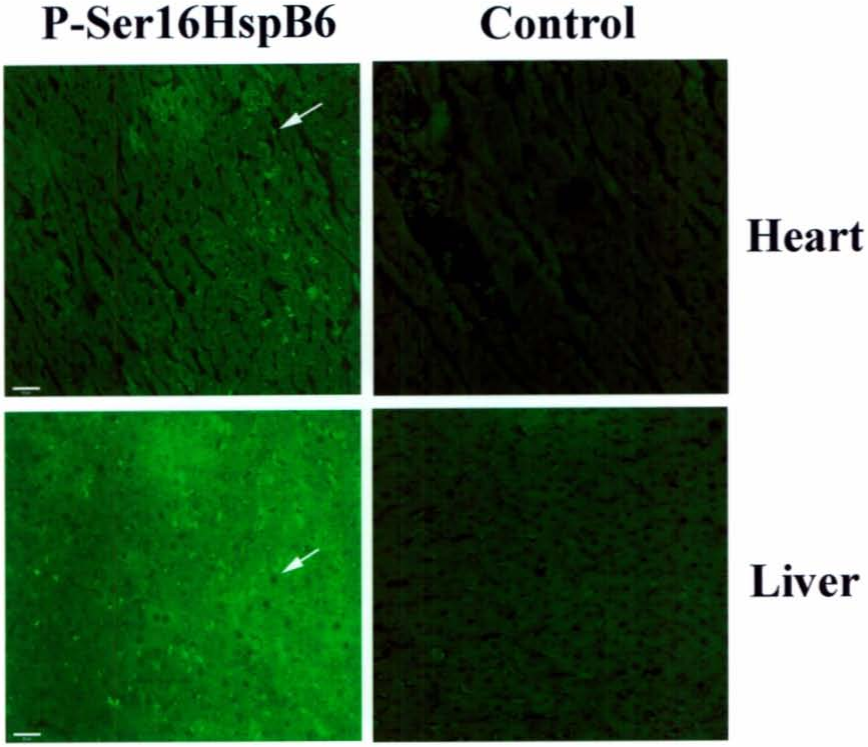


α-smooth muscle actin



Control

Figure 3.1.3 Immunofluorescent analysis of P-Ser-16 HspB6 protein detection in heart and liver sections using rabbit anti- P-Ser-16 HspB6 antisera. The images demonstrate cytoplasmic localization of the protein (green) within heart with some possible localization at intercalated discs (arrow), and the absence of any nuclear staining in the liver (arrow). Ctrl = control, rabbit IgG. Scale = 25 μM.


P-Ser16HspB6
Control
Heart
Liver

tissue was membrane-associated (Noda et al., 2007) and HspB6 in rat myocardium was clearly observed in the intercalated discs (Golenhofen et al., 2004).

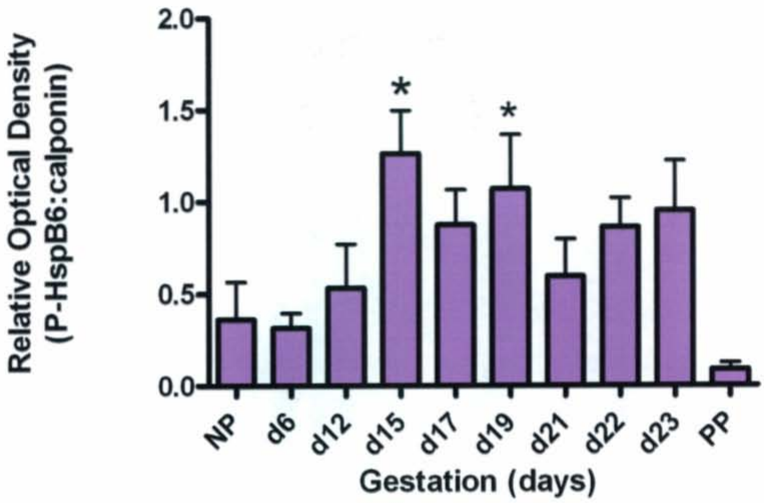
To determine the levels of P-Ser16 HspB6 protein thoughout normal pregnancy and labour in the rat, immunoblot analyses were carried out with an anti-rabbit-P-Ser16 HspB6 specific antisera at the following timepoints: NP (non-pregnant), d6, d12, d15, d17, d19, d21, d22, d23 and PP (post-partum) (Figure 3.1.4). The immunoblot analysis demonstrated that the expression of P-Ser16 HspB6 changed significantly during late pregnancy and labour (n=4; One-way ANOVA) and decreased significantly post-partum. P-Ser16 HspB6 was significantly elevated at d15 and d19 compared to expression at PP (Newman-Keuls, post-hoc test; $p< 0.05$).

3.1.2 *Immunofluorescent Detection of P-Ser16 HspB6*

To determine the spatial localization of P-Ser16 HspB6 over gestation, immunofluorescent was used. Detection of P-Ser16 HspB6 in the muscle layers demonstrated that overall the protein appeared to be primarily localized in the cytoplasm thoughout gestation (Figure 3.1.5 & 3.1.6). As both the commercial and donated P-Ser16 HspB6 anti-sera at times did not produce reproducible immunofluorescent results, different epitope retrievals were carried out including utilization of a boiling SSC protocol. Another approach was to utilize myometrial tissue preserved in zinc-based fixative (ZBF) slides, as opposed to paraformaldehyde/PBS fixed paraffin-embedded slides. The dilution of the anti-sera was varied as well from 1:10,000 to 1:2000 and 1:1000. All the results depicted in this thesis were of the original trypsin epitope retrieval method, as this gave the cleanest images. Despite these troubleshooting efforts, no clear

Figure 3.1.4 P-Ser-16 HspB6 protein is highly expressed in the rat myometrium during gestation. (A) Representative immunoblots of P-Ser-16 HspB6 protein detection found at 40 kDa and calponin detection. Analysis was performed using a anti-rabbit P-Ser-16 HspB6 specific antisera and an anti-calponin antisera, respectively. 100 μg of protein was loaded per lane. (B) Densitometric analysis illustrating P-Ser-16 HspB6 protein expression was elevated in late pregnancy and labour (n=4; One-way ANOVA) and decreased significantly post-partum. P-Ser-16 HspB6 was expressed most significantly at day 15 and day 19 compared to expression at PP (Newman-Keuls post-hoc test and t-test; $p < 0.05$) (*).Values are from four independent experiments (n=4) ± SEM. Days NP, 6, 12, 15, 17, 19, 21, 22, 23 and PP represent gestational timepoints in the pregnant rat. (C) Phases of parturition demonstrating that the significant increase in P-Ser16 HspB6 expression occurs at the transition period between the proliferative and synthetic stage.


P-Ser16 HspB6
Calponin
40 kDa
34 kDa


Relative Optical Density
(P-HspB6:calponin)
2.0
1.5
1.0
0.5
0.0
*
*
NP
d6
d12
d15
d17
d19
d21
d22
d23
PP
Gestation (days)

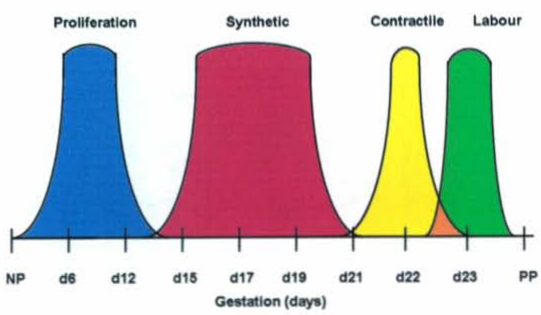

Proliferation
Synthetic
Contractile
Labour
NP
d6
d12
d15
d17
d19
d21
d22
d23
PP
Gestation (days)

Figure 3.1.5 Immunofluorescent analysis of P-Ser-16 HspB6 protein detection in longitudinal muscle layers of the rat myometrium at NP, d6, d12, d15, d17, d19, d21, d22, d23 and PP of gestation using rabbit anti-P-Ser-16 HspB6 antisera. The images demonstrate cytoplasmic localization of the protein (green) within myometrial cells and the absence of any nuclear staining (arrow). S = serosa, Ctrl = control, rabbit IgG Scale = 25 μM.

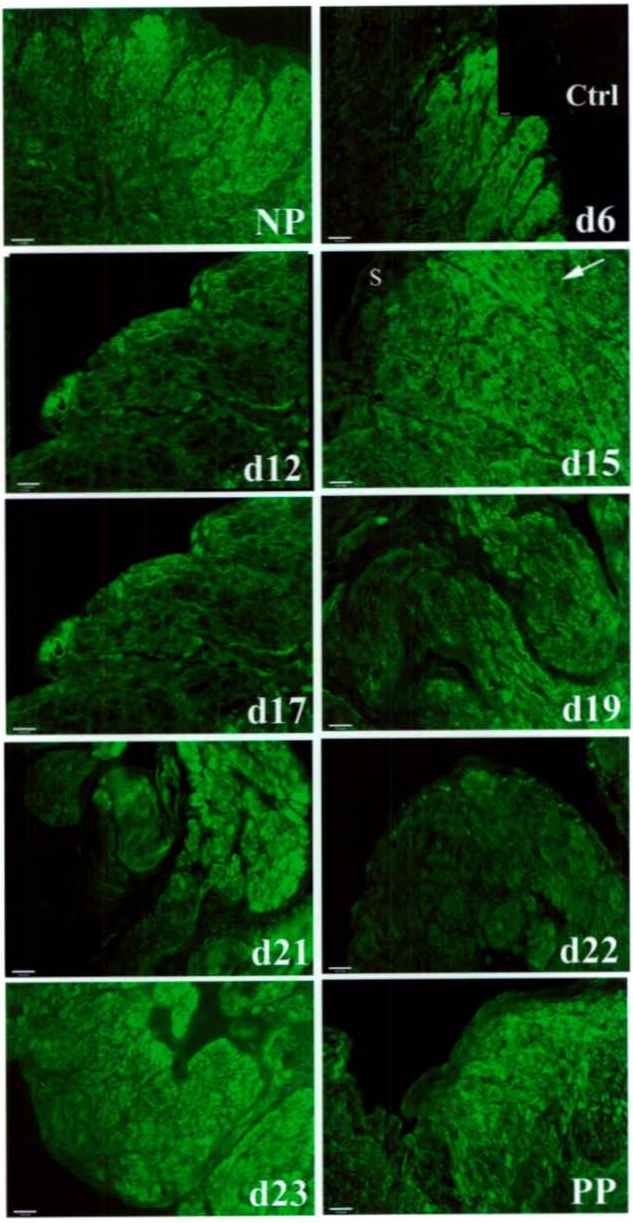
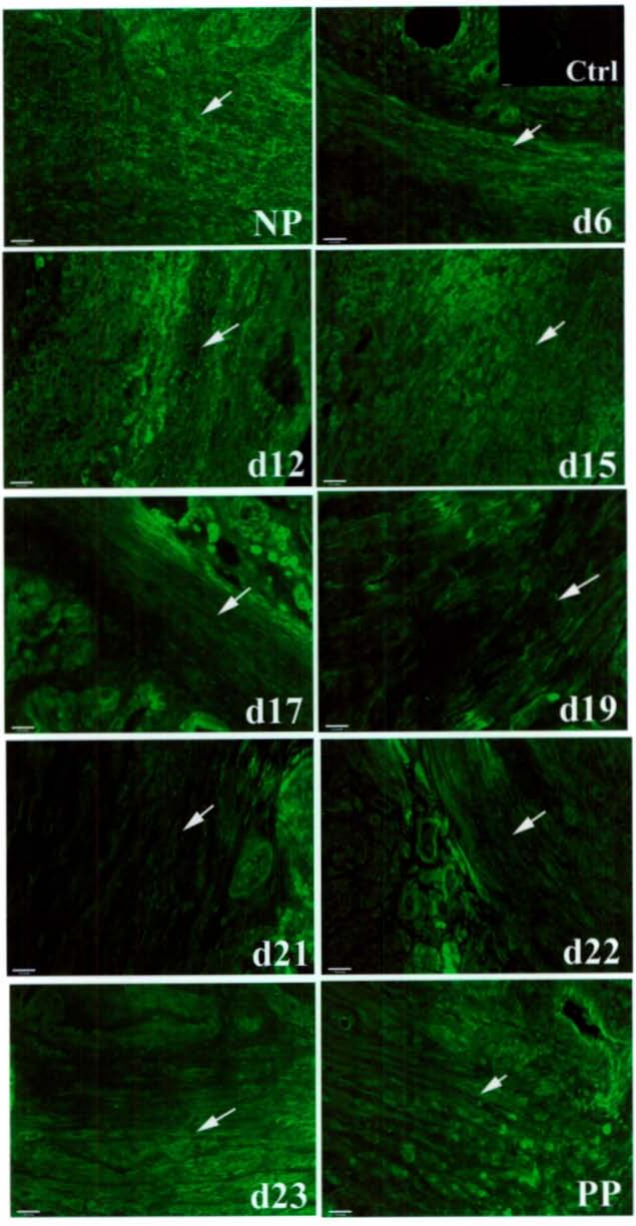

NP
Ctrl
d6
S
d12
d15
d17
d19
d21
d22
d23
PP

Figure 3.1.6 Immunofluorescent analysis of P-Ser-16 HspB6 protein detection in circular muscle layers of the rat myometrium at NP, d6, d12, d15, d17, d19, d21, d22, d23 and PP of gestation using rabbit anti-P-Ser-16 HspB6 antisera. The images demonstrate mainly cytoplasmic localization of the protein in myometrial cells (green). Arrows indicate circular muscle layer. Ctrl = control, rabbit IgG. Scale = 25 μM.


NP
Ctrl
d6
d12
d15
d17
d19
d21
d22
d23
PP

reproducible trend was observed, suggesting the antibody is not ideal for immunofluorescence.

3.2 Unilateral Pregnancy Model

3.2.1 *Expression of P-Ser16 HspB6 protein*

A unilateral pregnancy model was used (Shynlova et al., 2007) to determine the effect of stretch on P-Ser16 HspB6 protein expression at d15, d19 and d23 (labour) of gestation. Immunoblots were carried out to compare protein lysates from the gravid and non-gravid uterine horns (Figure 3.2) with an anti-rabbit-P-Ser16 HspB6 antisera. At d15, immunoblot analysis showed that detection of P-Ser16 HspB6 protein in the gravid horn was elevated but failed to reach statistical significance compared to the non-gravid horn (paired t-test; $p= 0.14$) (Figure 3.2.1). At d19 and d23, immunoblot analysis demonstrated a significant increase in detection of P-Ser16 HspB6 protein expression in the gravid horn compared to the non-gravid horn (paired t-test; $p < 0.05$; Figure 3.2.1.1 and 3.2.1.2 respectively). The increases observed between the gravid and non-gravid horn were 9.5 and 8 fold, respectively.

3.2.2 *Immunofluorescent Detection of P-Ser16 HspB6*

To determine if any changes in the spatial localization of P-Ser16 HspB6 occurred due to uterine distension, immunofluorescent analysis was conducted. Detection of P-Ser16 HspB6 in the muscle layers demonstrated that the protein was primarily localized in the cytoplasm of myometrial cell in both the non-gravid and gravid uterine samples at d19 of gestation (Figure 3.2.2).

Figure 3.2 A uterus dissected from a pregnant rat at d19 of gestation. The preparation demonstrate the difference in the size of the gravid horn that contains fetuses and the non-gravid, uterine horn.

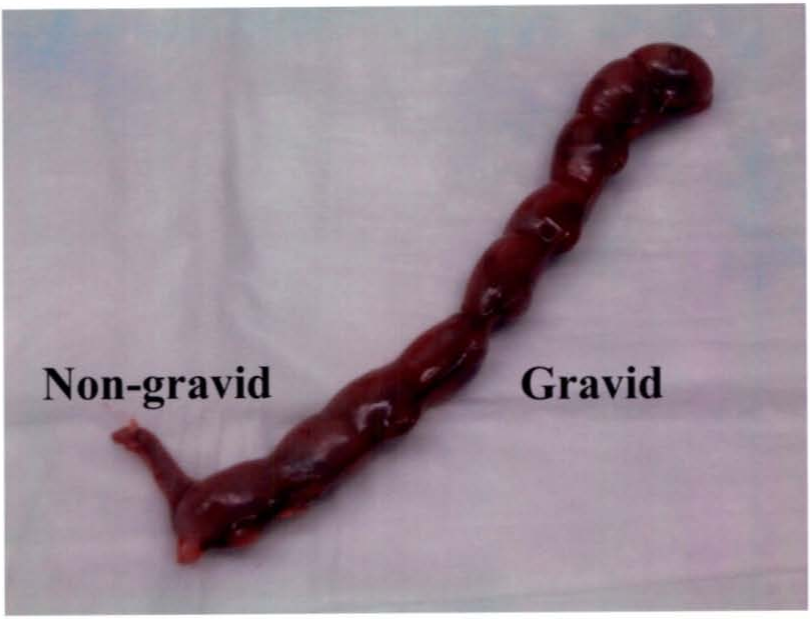

Non-gravid
Gravid

Figure 3.2.1 P-Ser-16 HspB6 protein expression is elevated in the gravid uterine horn when compared to the non-gravid horn at d15. Representative Immunoblots of P-Ser-16 HspB6 detection found at 40 kDa, total HspB6 protein detection found at 20 kDa and calponin detection. Analysis was performed using an anti-rabbit P-Ser-16 HspB6 specific antisera, an anti-rabbit total HspB6 antisera and an anti-calponin antisera respectively. 100 μg of protein was loaded per lane. Densitometric analysis illustrating the increase in P-Ser-16 HspB6 protein expression in the gravid horn compared to the non-gravid horn. Values are from 4 independent experiments ($n = 4$) ± SEM.


d15
P-Ser16 HspB6
HspB6
Calponin

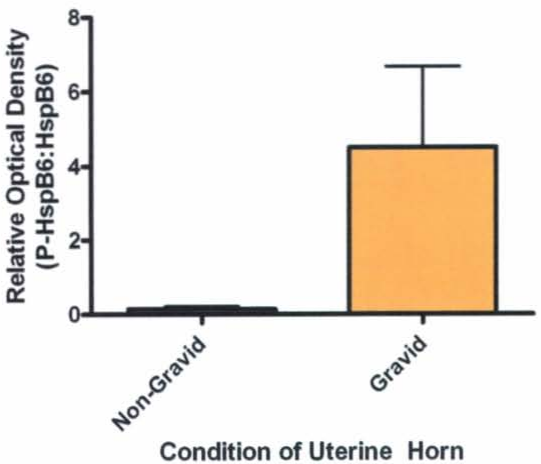
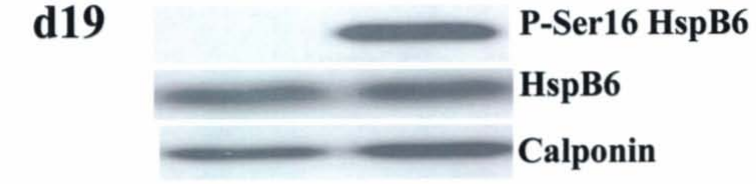

Relative Optical Density
(P-HspB6:HspB6)
8
6
4
2
0
Non-Gravid
Gravid
Condition of Uterine Horn

Figure 3.2.1.1 P-Ser-16 HspB6 protein expression is markedly elevated in the gravid uterine horn when compared to the non-gravid horn at d19. Representative Immunoblots of P-Ser-16 HspB6 detection found at 40 kDa , total HspB6 detection found at 20 kDa and calponin detection. Analysis was performed using an anti-rabbit P-Ser-16 HspB6 specific antisera, an anti-rabbit total HspB6 antisera and an anti-calponin antisera respectively. 100 μg of protein was loaded per lane. Densitometric analysis illustrating the significant increase of ~8 fold in P-Ser-16 HspB6 protein expression in the gravid horn compared to the non-gravid horn (t-test; $p < 0.05$). Values are from 4 independent experiments ($n = 4$) ± SEM.


d19
P-Ser16 HspB6
HspB6
Calponin

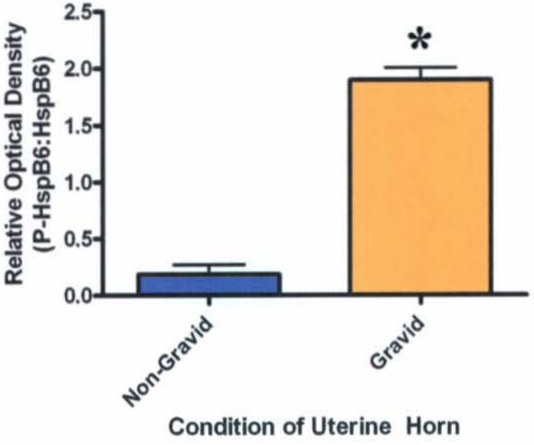

Relative Optical Density
(P-HspB6:HspB6)
2.5
2.0
1.5
1.0
0.5
0.0
*
Non-Gravid
Gravid
Condition of Uterine Horn

Figure 3.2.1.2 P-Ser-16 HspB6 protein expression is markedly elevated in the gravid uterine horn when compared to the non-gravid horn at d23 (labour). Representative Immunoblots of P-Ser-16 HspB6 detection found at 40 kDa, total HspB6 detection found at 20 kDa and calponin detection. Analysis was performed using an anti-rabbit P-Ser-16 HspB6 specific antisera, an anti-rabbit total HspB6 antisera, and an anti-calponin antisera respectively. 100 μg of protein was loaded per lane. Densitometric analysis illustrating the significant increase of ~9 fold in P-Ser-16 HspB6 protein expression in the gravid horn compared to the non-gravid horn (t-test; $p < 0.05$). Values are from 8 independent experiments ($n = 8$) ± SEM.

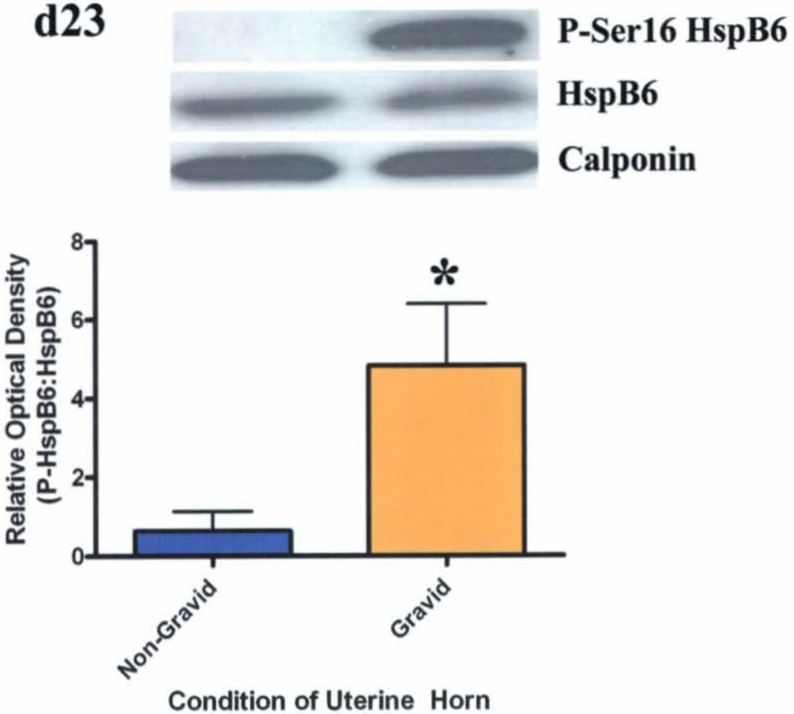
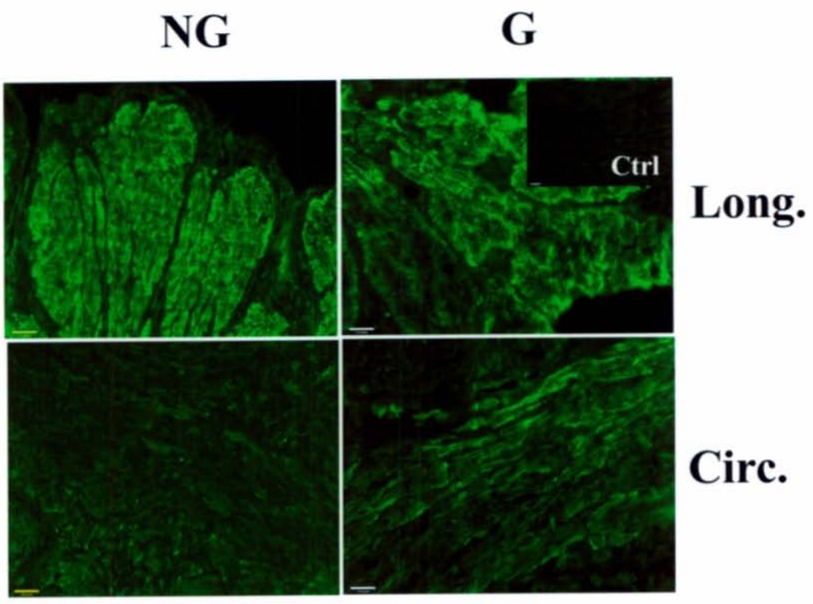

d23
P-Ser16 HspB6
HspB6
Calponin
Relative Optical Density
(P-HspB6:HspB6)
8
6
4
2
0
*
Non-Gravid
Gravid
Condition of Uterine Horn

Figure 3.2.2 Immunofluorescent analysis of P-Ser-16 HspB6 protein detection in longitudinal (Long.) and circular (Circ.) muscle layers of the rat myometrium in gravid (G) and non-gravid (NG) uterine horns. The representative timepoint shown is d19 of pregnancy. Sections were probed with an anti-rabbit P-Ser-16 HspB6 antisera. The images demonstrate cytoplasmic localization of the protein (green) in myometrial cells. Ctrl = control, rabbit IgG. Scale = 25 μM.


NG
G
Ctrl
Long.
Circ.

3.3 Exogenous 17β-Estradiol Administration

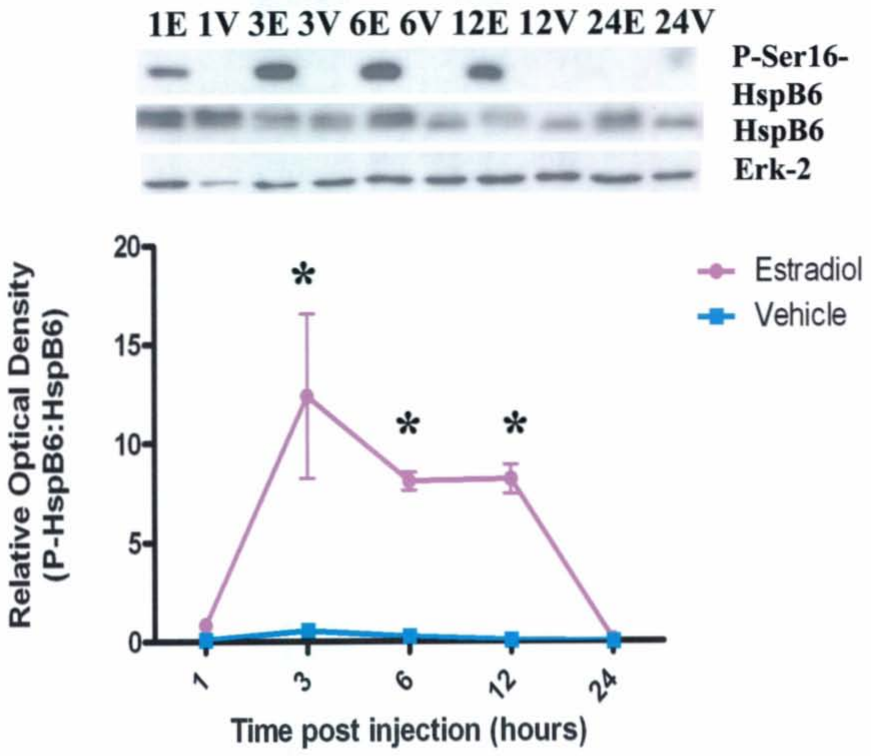
3.3.1 *Expression of P-Ser16 HspB6 protein*

To determine the effect of 17β-Estradiol on P-Ser16 HspB6 protein expression, virgin, ovariectomized rats received an injection of 17β-Estradiol or corn oil vehicle followed by collection of myometrial samples at various timepoints. Immunoblot analysis of protein lysates from 3,6,12 and 24 h post injection demonstrated a significant increase in detection of P-Ser16 HspB6 protein expression following 17β-Estradiol injection compared to rats injected with the vehicle (Two-way ANOVA; $p < 0.05$; Figure 3.3.1A). Specifically, expression was elevated at 3, 6 and 12 h representing ~ 20.6, 27 and 82 fold increases over vehicle, respectively. The effect of on total HspB6 protein expression was examined. There was no significant difference between the rats injected with 17β-Estradiol compared with those injected with the vehicle at each timepoint assayed (Two-way ANOVA; $p > 0.05$).

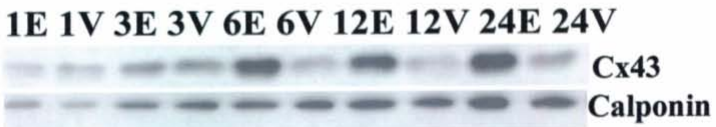
To verify the viability of the model, Cx43 responsiveness was analysed. Lye et al. 2003 compared the Cx43 densitometric values from myometrial samples collected throughout pregnancy to the ratio of estrogen: progesterone obtained by an assay of paired blood samples from the same pregnant rats throughout pregnancy. They observed a significant positive correlation between the increase in Cx43 transcripts and the increase in the ratio of estrogen: progesterone, suggesting that Cx43 expression is positively regulated by estrogen. Immunoblot analysis of the protein lysates in the 17β-Estradiol model demonstrated upregulation of Cx43 only in the lysates of rats injected with 17β-Estradiol, indicating that the model is reliable to use (Figure 3.3.1B).

Figure 3.3.1 17β-Estradiol significantly induces expression of P-Ser-16 HspB6. (A)Representative Immunoblots of P-Ser-16 HspB6 found at 40 kDa, total HspB6 protein detection found at 20 kDa and Erk-2 detection. Analysis was performed using an anti-rabbit P-Ser-16 HspB6 specific antisera, an anti-rabbit total HspB6 antisera and an anti-Erk-2 antisera respectively. 100 μg of protein was loaded per lane. E =17β-Estradiol; V = vehicle. Number corresponds to hour post-injection that sample was taken. Densitometric analysis illustrating the significant increase in P-Ser-16 HspB6 protein expression in the 17β-Estradiol injected rats compared to rats injected with the vehicle. Immunoblot analysis of protein lysates from 3,6,12 and 24 h post injection demonstrated a significant increase in detection of P-Ser-16 HspB6 protein expression following 17β-Estradiol injection compared to rats injected with the vehicle (Two-way ANOVA; $p < 0.05$; Figure 3.4.1). Specifically, expression was elevated at 3, 6 and 12 h representing ~ 20.6, 27 and 82 fold increases over vehicle, respectively. Values obtained from 4 independent experiments ($n = 4$) ± SEM. (B) Representative Immunoblots of Cx43 found at 43 kDa and calponin detection. Analysis was performed using an anti-rabbit Cx43 specific antisera and an anti-calponin antisera respectively. 100 μg of protein was loaded per lane. E =17β-Estradiol; V = vehicle. Number corresponds to hour post-injection that sample was taken.

A


1E 1V 3E 3V 6E 6V 12E 12V 24E 24V
P-Ser16-HspB6
HspB6
Erk-2
Relative Optical Density (P-HspB6:HspB6)
20
15
10
5
0
*
*
*
Estradiol
Vehicle
1
3
6
12
24
Time post injection (hours)

B


1E 1V 3E 3V 6E 6V 12E 12V 24E 24V
Cx43
Calponin

3.3.2 *Immunofluorescent Detection of Total HspB6*

Detection of total HspB6 in the muscle layers demonstrated that HspB6 was primarily localized in the cytoplasm of myometrial cells in both 17β-Estradiol injected rats and rats injected with vehicle. (Figure 3.3.2). However, some membrane-associated localization was observed at 6 h in the 17β-Estradiol exposed samples, although this was not consistent throughout.

3.3.3 *Immunofluorescent Detection of P-Ser16 HspB6*

Immunofluorescent detection of P-Ser 16 HspB6 in the muscle layers demonstrated that the phosphorylated form of the stress protein was virtually undetectable in myometrial cells in both 17β-Estradiol injected rats and rats injected with vehicle at 1h, 12h and 24 h, since detection was not greater than the IgG control (Figure 3.3.3)

3.4 Examination of P-Ser16 HspB6 dimers

When immunoblots were probed with both anti-rabbit P-Ser16 HspB6 antibodies a ~40 kDa band was observed, which is twice the predicted molecular weight of monomeric HspB6. To determine if this band represented an aggregate dimer, urea extraction of d15 normal gestation tissue samples was carried out. This extraction procedure is more efficient at denaturing and breaking apart the aggregate, and it was hypothesized that this would result in more 20 kDa bands detected instead of the 40 kDa bands observed with routine RIPA protein extraction. Urea lysis buffer extracted samples were electrophoresed with d15 samples extracted with RIPA lysis buffer for comparison. As expected, urea extraction resulted in proportionally greater detection of a band at 20

Figure 3.3.2 Immunofluorescent analysis of total HspB6 protein in longitudinal (L) and circular (C) muscle layers of the rat myometrium in 17β-Estradiol injected rats (E) compared to rats injected with vehicle (V). Timepoints are 1, 3, 6, 12 and 24 h post-injection and sections were probed with an anti-rabbit total HspB6 antisera. The images demonstrate cytoplasmic localization of the protein (green) in myometrial cells and some membrane associated localization in E 6 h. L = longitudinal muscle. Ctrl = control, rabbit IgG. Scale = 25 μM.

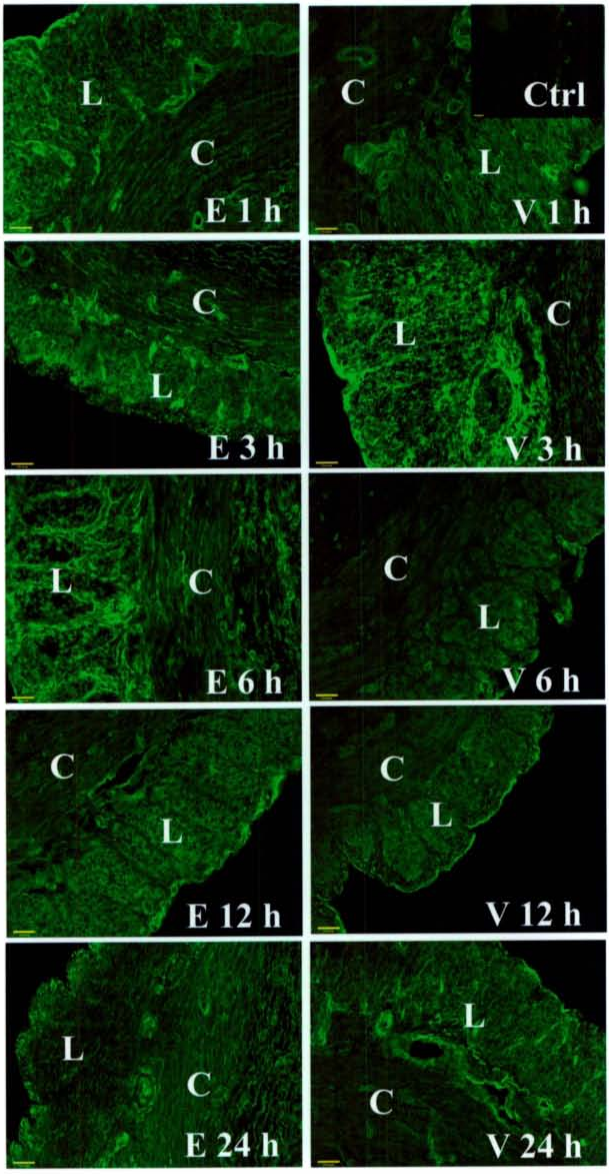
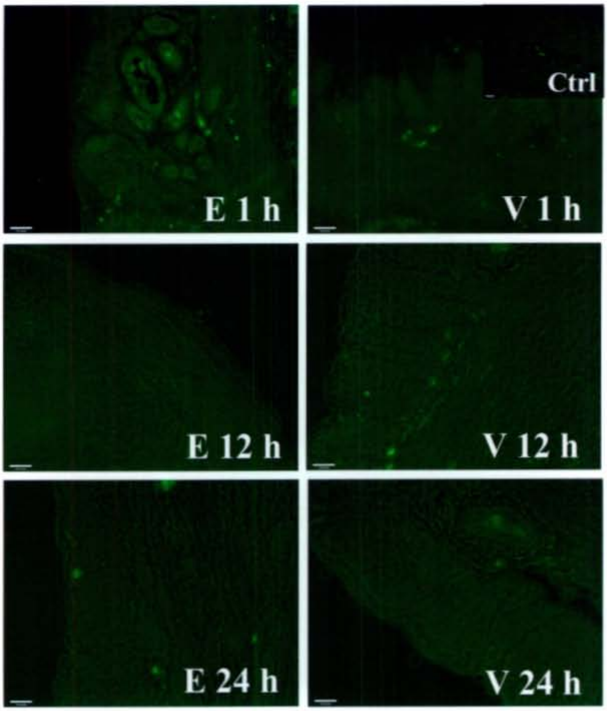

L
C
E 1 h
C
Ctrl
L
V 1 h
C
L
E 3 h
L
C
V 3 h
L
C
E 6 h
C
L
V 6 h
C
L
E 12 h
C
L
V 12 h
L
C
E 24 h
L
C
V 24 h

Figure 3.3.3 Immunofluorescent analysis of P-Ser-16 HspB6 protein detection in longitudinal and circular muscle layers of the rat myometrium in 17β-Estradiol injected rats (E) compared to rats injected with the vehicle (V). Representative timepoints shown are 1, 12 and 24 hours post-injection and sections were probed with an anti-rabbit P-Ser-16 HspB6 antisera. The images demonstrate virtually undetectable localization of the protein (green). Ctrl = control, rabbit IgG. Scale = 25 μM.


Ctrl
E 1 h
V 1 h
E 12 h
V 12 h
E 24 h
V 24 h

kDa, while RIPA extraction resulted in greater detection of the ~40 kDa band (Figure 3.4.1).

3.5 Localization of P-Ser16 HspB6 in cultured Myometrial cells

3.5.1 *Immunofluorescent Detection of HspB6*

To try and assess the quality of the P-Ser16 HspB6 antisera for immunofluorescent procedures, localization of total HspB6 and P-Ser16 HspB6 protein was examined in myometrial cells from a human myometrial cell line (M-11) (Figure 3.5.1). Immunofluorescence was performed after cells were fixed on coverslips at 80-90% confluence. Total HspB6 expression was primarily localized to the cytoplasm of cells and was also found to overlap in areas with the actin cytoskeleton. P-Ser16 HspB6 protein was instead localized mainly in the cell nucleus; with a trace amount found in the cytoplasm of cells.

Figure 3.4.1 Phosphorylated HspB6 is detected as a dimer. Immunoblot analysis was performed on samples from d15 of gestation (n=4) using an anti-rabbit P-Ser-16 HspB6 specific antisera and an anti-calponin antisera respectively. Representative set 2 shown. Lysates were prepared with a strongly denaturing urea lysis buffer or standard RIPA lysis buffer as described in Chapter 2. Urea extraction resulted in a greater detection of HspB6 at 20 kDa and lesser detecting at 40 kDa. RIPA extraction resulted in sole detection of HspB6 at ~40 kDa. 100 µg of total protein was loaded per lane. Order of lanes: d15-2 RIPA and d15-2 urea.

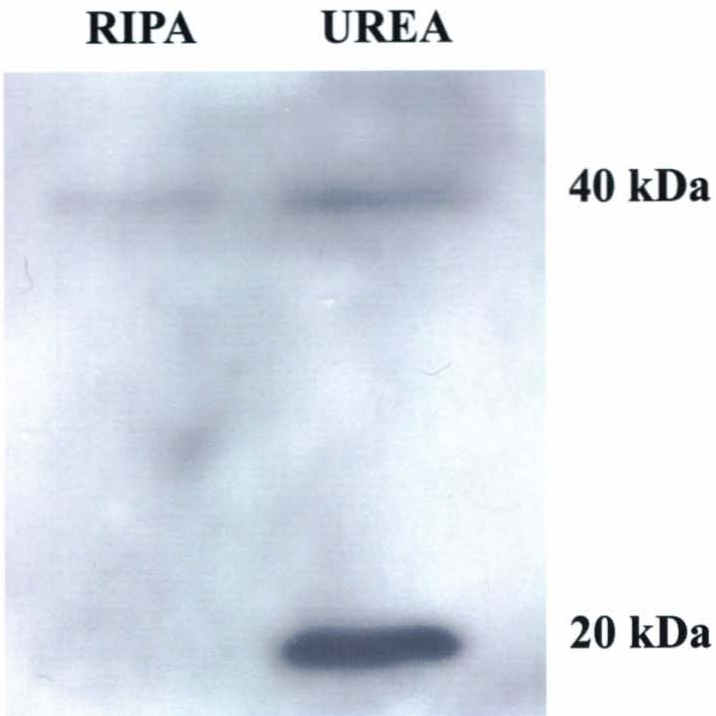
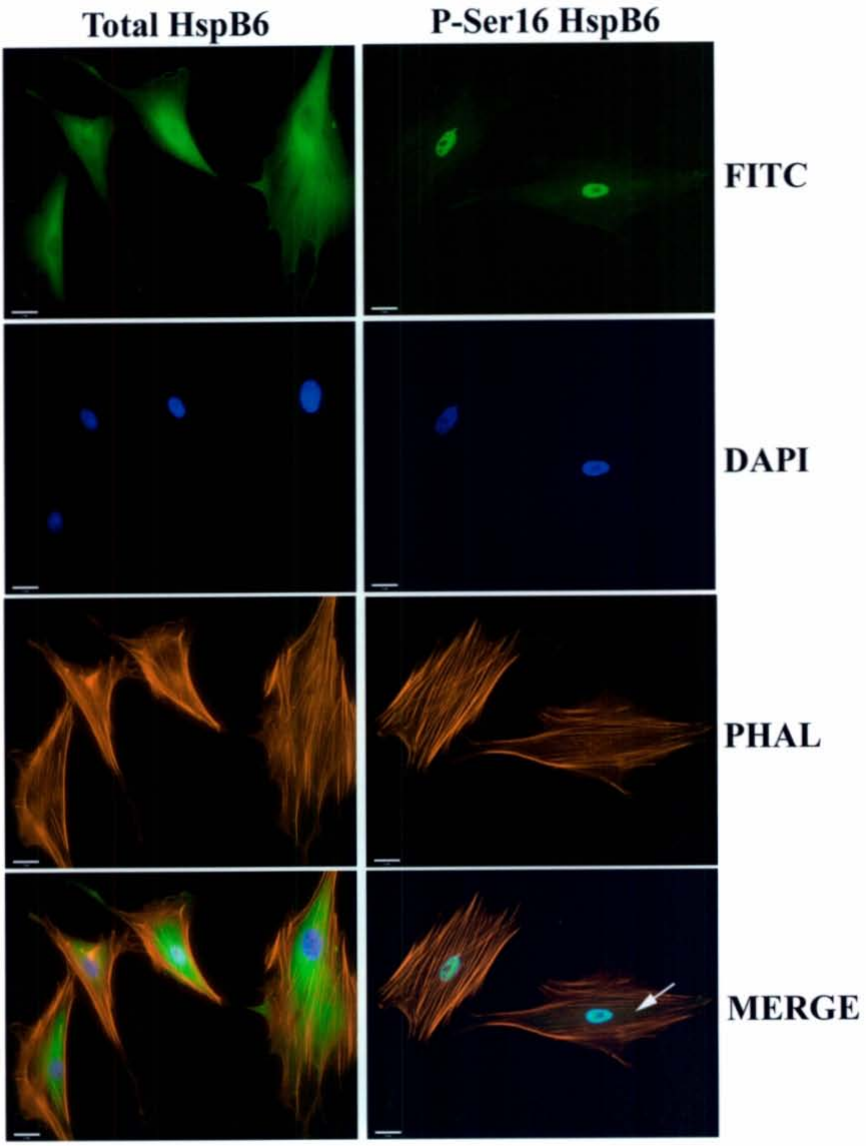

RIPA
UREA
40 kDa
20 kDa

Figure 3.5.1 Immunofluorescent analysis of total HspB6 and P-Ser-16 HspB6 protein detection in the human myometrial cell line M-11. Total HspB6 demonstrated cytoplasmic localization of the protein (FITC; green) with some co-localization with actin microfilaments (merged). P-Ser-16 HspB6 protein demonstrated primarily nuclear associated staining (FITC; green) with trace amounts found in the cytoplasm (arrow; merged). Cells were also stained with DAPI (blue) and phallodin 633 (PHAL; red). DAPI stained the nuclei blue and phallodin stained the actin cytoskeleton red. Control = rabbit IgG. Scale = 25 μM.


Total HspB6
P-Ser16 HspB6
FITC
DAPI
PHAL
MERGE

Chapter Four

Discussion

4.1 Expression of P-Ser16 HspB6 during Normal Pregnancy and Labour

It was important to first clarify how expression of P-Ser16 HspB6 changes over pregnancy to gain insight into the potential role that post-translational modification may have in the myometrium. The expression of total HspB6 during rat pregnancy has previously been examined (Cross et al., 2007). The results demonstrated that both HspB6 mRNA and protein were highly expressed in the myometrium during early and mid-pregnancy and then expression markedly decreased during late pregnancy and labour (Cross et al., 2007). This pattern was deemed consistent with a role for HspB6 to maintain myometrial quiescence during early and mid-pregnancy. However, the profile of how P-Ser16 HspB6 expression changes over pregnancy had not been examined. As the phosphorylated form is believed to be associated with smooth muscle relaxation (Rembold et al., 2000), it was imperative to determine the temporal pattern of P-Ser16 expression instead of examining total HspB6 alone. Given its potential as a smooth muscle relaxant, the ongoing hypothesis in the laboratory is that phosphorylation of HspB6 may be a mechanism to promote overall myometrial relaxation during pregnancy as well as participation in the phasic contractions of labour.

In contrast to the expression of total HspB6, P-Ser16 HspB6 protein expression increased between mid-late pregnancy and labour and then dropped dramatically post-partum. As pregnancy proceeds both the uterus and fetus grow (Smith, 2007), causing the myometrium to stretch. When a muscle fibre is stretched, contraction can be promoted

(Renfree, 2000). Therefore, during pregnancy when uterine tension is building and unwanted contraction could occur, a mechanism to maintain quiescence in the myometrium is required. P-Ser16 HspB6 has been proposed to promote smooth muscle relaxation via two mechanisms. One mechanism may be via a troponin-like effect. Troponin I is an actin-binding protein important in striated muscle for initiating muscle contraction (Perry, 1999). HspB6 contains a region of partial homology, at amino acid residues 110-121, to the actin binding region of cardiac and skeletal troponin I (Rembold et al., 2000). Therefore HspB6 might directly interact with actin, preventing acto-myosin cross-bridging from occurring and promoting smooth muscle relaxation (Rembold et al., 2000). The second mechanism of relaxation may involve actin depolymerization. This is thought to occur by P-Ser16 HspB6-mediated displacement of p-cofilin from the adaptor protein 14-3-3. 14-3-3 protein binds to cofilin via a phosphoserine binding motif, which protects cofilin from dephosphorylation by slingshot phosphatase (Gohla & Bokoch, 2002; Birkenfeld et al., 2003). Dephosphorylation activates cofilin and promotes F-actin depolymerization (Dreiza et al, 2005), preventing the anchoring of F-actin to integrins, and in doing so preventing force transmission from actin/myosin interaction to the cytoskeletal network. Thus, with these mechanisms of muscle relaxation in mind, increased P-Ser16 HspB6 may facilitate the quiescent myometrial state.

Upon labour at d23, P-Ser16 HspB6 expression was elevated. The apparent discrepancy in this finding may be resolved when realizing that labour contractions are phasic (Young, 2007). Thus, there is still a need for relaxation of the uterus during the phasic

contractions of labour. It is possible that P-Ser16 HspB6 expression at this point may help achieve this feat.

P-Ser16 HspB6 expression becomes initially upregulated at d15. This is a transitional period between the hyperplasic and hypertrophic phase of myometrial growth (Shynlova et al., 2009). At this particular transition there is evidence of caspase upregulation, an enzyme activated during the apoptotic cascade (Shynlova et al., 2006). However, no large-scale evidence of apoptosis was observed biochemically or morphologically in the myometrium. Part of the enzyme activity of caspases is vital in promoting normal differentiation, such as caspase-3 which promotes lens fibre differentiation (Weil et al., 1999). This suggests that caspase-3 upregulation is involved in initiating the normal differentiation of uterine smooth muscle while halting proliferation (Shylova et al., 2006). Mechanical influence is particularly pertinent for this transition to occur as unilateral pregnancy studies in rats demonstrated that caspase activation was only measured in the gravid horn (Shynlova et al., 2010). Hormonal influence may also play a role in regulating caspase activity as at the end of pregnancy when distension is maximal, caspase activity is low (Shylova et al., 2006). Given that various regulatory mechanisms appear to affect caspase activity, P-Ser16 HspB6 also could have a contributing role to this process by promoting cell survival during the hyperplasic-hypertrophic transitional period in the rat myometrium. This would coincide with work from Fan et al. (2004) where they examined the protective role of HspB6 in the heart after stimulation by the PKA/cAMP pathway. Fan et al. (2004) overexpressed mutant forms of HspB6, constitutively phosphorylated or non-phosphorylated, (Fan et al.,

2004) in adult rat cardiomyocytes. P-Ser16 HspB6 expression inhibited the apoptotic effects of the cAMP-PKA pathway resulting in a decrease in pyknotic nuclei, TUNEL staining, and DNA laddering - which all point to inhibition of apoptotic cascade. Therefore, phosphorylated HspB6 was associated with anti-apoptotic effects, whereas an overexpression of the non-phosphorylable mutant HspB6 exhibited no cardioprotection (Fan et al., 2004). Given this knowledge, it is possible that P-Ser16 HspB6 may promote myometrial cell survival during the phase transition at d15 by contributing to the inhibition of apoptosis. This has yet to be investigated in smooth muscle.

P-Ser16 HspB6 expression was also upregulated during the hypertrophic, or synthetic, phase of pregnancy at d19 of gestation. During this time cytoskeletal dynamics are changing within myometrial cells to accommodate the increase in cellular size. Among these changes is an increase in FAK activity – a kinase that regulates extracellular matrix reorganization (MacPhee & Lye, 2000). When activated, FAK reorganizes focal adhesions (FA), where extracellular ligands are coupled via their integrin receptors to cytoskeletal components (Geiger et al., 2001). FA are therefore important in cell-ECM interactions and are thought to provide structural linkage for force transmission in the myometrium (Williams et al., 2005). Previous studies have looked at the effect of P-Ser16 HspB6 on FA formation, since any FA disruption would ultimately promote relaxation. Dreiza et al. (2004) found that with the addition of a phospho-HspB6 peptides to fibroblast cells, a punctate localization of FA adaptor proteins - α-actinin, vinculin and paxillin – was lost, indicating a disruption of FA. The decreased presence of these adaptor proteins (α-actinin and vinculin) and intracellular signaling protein

(paxillin) together indicate focal adhesion disruption (Dreiza et al., 2004). The same cells treated with phospho-HspB6 peptide also demonstrated a statistically significant decrease in cells positive for focal adhesions when examined using interference reflection microscopy. Based on the results of Dreiza et al. (2004), P-Ser16 HspB6 may be elevated during the same period that focal adhesions are being assembled and prevent an exceeding amount of adhesions from forming. This would prevent focal adhesions from enhancing force transmission at a premature stage and lessens the potential for unwanted contraction.

4.2 The Effect of Distension on HspB6 expression

To determine the effect that uterine distension had on P-Ser16 HspB6 protein expression in the myometrium, a unilateral pregnancy model was employed to isolate the influence of distension while maintaining a constant endocrinology environment. This model produces a virgin female rat that will then only become impregnated in one uterine horn with a contralateral horn empty, or non-gravid. This model is particularly useful to observe and evaluate the effect of distension on gene expression, while both the gravid and non-gravid horn are still largely exposed to the same systemic endocrinological environment with the exception that the gravid horn is exposed to paracrine factors produced during conception by the surrounding membranes and tissues. These can include hormones, growth factors and immunological molecules. Lastly, blood flow to the gravid horn is also likely greater than the non-gravid horn. Three different timepoints in pregnancy - d15, d19 and d23 (labour) - were examined and represent periods during mid-late pregnancy when distension is a predominant stimulus of myometrial

hypertrophy (d15), extracellular matrix protein deposition (d19), FA and CAP activation (d23) (Goldspink & Douglas, 1988; Challis et al., 2001; Lye et al., 2001; Shynlova et al., 2004; Shynlova et al., 2006; Shynlova et al., 2009). Given that mechanical distension causes such stress in the uterus and activates pathways that lead to labour, it was postulated that P-Ser16 HspB6 would be upregulated in the gravid uterine horn as a way to maintain quiescence during mid-late pregnancy when distension is predominant.

Immunoblot analyses at all three timepoints indicated that there was no significant change in total HspB6 protein expression between the gravid and non-gravid uterine horns. Cross and MacPhee (unpublished results) reported preliminary results that HspB6 mRNA levels in the gravid horn of unilaterally pregnant rats appeared to be decreased at labour compared to non-gravid, while unchanged between gravid and non-gravid horns at d19. However, they utilized a small sample size compared to this present study. In contrast, there was a significant upregulation in P-Ser16 HspB6 expression in the gravid horn when compared to the non-gravid horn. The results suggest that P-Ser16 HspB6 expression may be upregulated to help maintain a relaxed state and prevent premature myometrial contraction via either the troponin-like effect (Rembold et al., 1999) or via actin depolymerization (Dreiza et al., 2005).

When examining the molecular mechanism of HspB6 induced smooth muscle relaxation, it is important to realize that HspB6 does not function exclusively as a monomeric protein. When HspB6 was initially purified, it was a by-product of the purification of HspB1 (Hsp27) (Kato et al., 1994). The aggregation of HspB6 and HspB1 was the predominant complex found in muscle extracts from heart, diaphragm and soleus

muscle, suggesting a physiological role for this macromolecular aggregate (Kato et al., 1994). HspB1, similar to HspB6, is functionally impacted by phosphorylation. Agonist-induced phosphorylation at site Ser-15 promotes actin and myosin interaction via association with tropomyosin (Gerthoffer et Gunst, 2001). HspB1 has also been found to modulate actin filament dynamics in cultured cells (Lavoie et al., 1995; Guay et al., 1997; Schafer et al., 1999), and an increase in phosphorylation has been associated with contraction in vascular smooth muscle (Bitar et al., 1991; Larsen et al., 1997; Brophy et al., 1998). Work in the MacPhee laboratory has previously investigated the levels of P-Ser15 HspB1 protein in the pregnant rat myometrium and it was found that protein expression significantly increased between days 19 and day 23 (White et al., 2005). It was thus proposed that HspB1 was a candidate CAP.

Considering that HspB6 and HspB1 have antagonistic roles and that they can form hetero-oligomers, their functions may be attenuated, in part, by their co-oligermization, particularly when phosphorylated (Sun et al., 2006). Bukach et al. (2009) examined the complexes formed by HspB6 and HspB1 and found that heteroligmeric complex formation was temperature-dependent. Furthermore, once the complexes were formed, the wild type HspB1 inhibited the rate of phosphorylation of HspB6 by cAMP-dependent protein kinase. Likewise, HspB6 inhibited phosphorylation of HspB1 via MAPKAP2 kinase (Bukach et al., 2009).

Since P-Ser16 HspB6 is upregulated in the gravid uterine horn on d15, d19 and d23, P-Ser16 HspB6 may act to inhibit phosphorylation of HspB1 to promote relaxation either during gestation or between the phasic contractions of labour. In a unilateral

pregnancy model, the same time points described above were recently examined for P-Ser15 HspB1 expression in the myometrium. It was found that P-Ser15 HspB1 was also markedly increased in the gravid horn compared to the non-gravid horn (White & MacPhee, in prep). It is thus possible that a dynamic relationship occurs between the two small heat shock proteins with functional outcome on the myometrium.

4.3 The Effect of 17β-Estradiol on HspB6 expression

Aside from mechanical distension, hormonal influence is also vital in regulating gene expression throughout pregnancy. Therefore, it was important to explore whether steroids present during pregnancy could influence the expression of HspB6. 17β-Estradiol is a hormone known to antagonize the actions of progesterone and is thus associated with labour (Madsen et al., 2004). Given that the circulating levels of this steroid are correlated with labour in the rat, the effect, if any, of 17β-Estradiol on P-Ser16 HspB6 expression was examined. Since we postulated that P-Ser16 HspB6 expression could induce myometrial relaxation, a hormone associated with labour such as 17β-Estradiol would be expected to inhibit the expression of P-Ser16 HspB6. Immunoblot analysis indicated that total expression of HspB6 was not significantly affected by 17β-Estradiol injection; however, P-Ser16 HspB6 expression was significantly upregulated in 17β-Estradiol injected rats compared to rats injected with the vehicle control. Although HspB6 mRNA levels were not investigated, this suggests that estradiol is inducing post-translational modification of the protein. As P-Ser16 HspB6 is a potential smooth muscle relaxant and estradiol is associated with labour, such a relationship seems counterintuitive. However, the ramifications of this pathway could be a finely tuned

mechanism of estradiol-regulated phasic uterine contraction. Phosphorylation of HspB6 is initiated by agonists that activate the adenyl cyclase system causing an increase in the intracellular second messengers cAMP or cGMP (Lincoln, 1989; Rembold et al., 2000). This increase causes activation of protein kinase A or G which phosphorylates HspB6 at Ser-16. In early studies examining ovariectomized rats and non-ovariectomized rats it was found that those rats lacking ovarian function and 17β-estradiol secretion had 7 times less total uterine cAMP than the intact group based on body weight (Szego & Davis, 1967). Furthermore, when the ovariectomized rats were given an intravenous injection of 17β-estradiol, uterine cAMP was elevated within 15 seconds to concentrations indistinguishable from those seen in the intact animals (Szego & Davis, 1967). This suggests a highly specific interaction between estradiol and cAMP. Perhaps estradiol interacts specifically with the membrane bound adenyl cyclase system resulting in activation. It is also possible that there is an intermediate step activated by estradiol that acts on adenyl cyclase such as site-specific amine liberation by estradiol (Szego & David, 1967). In rat uterine cell lines, 17β-Estradiol evoked an increase in cAMP levels as measured by a cAMP radioimmunoassay (Aronica et al., 1994) Therefore, in this study it is possible that Ser16-P HspB6 is upregulated upon estradiol adminstration through its activation of adenyl cyclase resulting in upregulation of cAMP/cGMP and subsequent phosphorylation of HspB6 by PKA/PKG. While making these hypotheses it is important to realize that this is a non-pregnant, ovarectomized model. Estradiol may interact with factors or receptors that are present in the pregnant state, but are not represented in the

non-pregnant state, possibly meaning our interpretations are not completely relevant to the pregnant state.

4.4 P-Ser16 HspB6 dimer

Throughout the immunoblot analysis, blots probed with both P-Ser 16 HspB6 specific antisera available always resulted in detection of a single band at ~ 40 kDa in size in contrast to detection of total HspB6 at 20 kDa. A band at this molecular weight suggested that the phosphorylated form of HspB6 aggregated into, or was detectable as a dimer (2X 20 kDa). Bukach et al. (2004) previously performed cross-linking experiments on human HspB6. The authors first produced recombinant HspB6 and performed size exclusion chromatography at neutral pH followed by analysis of the protein on SDS-PAGE. Samples of recombinant HspB6 prepared without β-mercaptoethanol, an agent commonly used to reduce sulphide bonds, resulted in the appearance of a band observed at ~40 kDa, in addition to a fainter band at 20 kDa. The 40 kDa band disappeared if the sample was treated with an excess of β-mercaptoethanol prior to electrophoresis and a darker band was observed at 20 kDa (Bukach et al., 2004). This suggested that the 40 kDa band corresponded to a HspB6 dimer and, at a neutral pH, HspB6 predominantly formed dimers of 40 kDa molecular mass. In an even earlier study examining bovine carotid muscle, Brophy et al. (1999) also found a specific band of ~40 kDa after coimmunoprecipitation. Tyson et al. (2008), using human myometrial samples, reported that HspB6 was detectable at ~40 kDa when tissue lysates were prepared with less stringent lysis buffers such as NP-40 lysis buffer and ~20 kDa when lysates were prepared in denaturing urea lysis buffer. Indeed, in our laboratory the production of tissue

lysates with a standard lysis buffer such as RIPA resulted in the detection of a 40 kDa P-Ser16 HspB6 band, regardless of β-mercaptoethanol concentration in SDS-PAGE gel loading buffer. However, upon protein extraction with urea lysis buffer, a more powerful protein denaturant, fainter detection of a band at 40 kDa and the appearance of a darker band at 20 kDa was observed on immunoblots using d15 gestation samples. Thus, it is clear that under RIPA lysis buffer extraction conditions, P-Ser16 HspB6 is detectable as a dimer at ~ 40 kDa.

Another question that arises concerning the P-Ser16 HspB6 specific antibody is why the total antibody does not pick up on the dimer; it consistently demonstrated a band at ~20 kDa only. Total antibodies should detect both the phosphorylated and unphosphorylated form of the protein. One explanation for this could be based on the fact that HspB6 forms 1.3% of total proteins in slow skeletal muscle, diaphragm, heart and smooth muscles (Gusev et al., 2002) – quite a substantial amount. Therefore, it may be that the antibody binds mainly to total protein, until it reaches saturation and has no more binding sites unoccupied. This means that the antibody may only be binding a small amount of P-Ser16 HspB6 at a level that is virtually undetectable or that the HspB6 espitope is not antibody accessible in the phosphorylated dimer form.

4.5 Detection of P-Ser16 HspB6 in Myometrium In situ

When observing both the longitudinal and circular muscle tissue sections over gestation profile, it was clear that levels of detection and spatial characterization of P-Ser16 HspB6 expression was relatively similar from NP to PP. The immunofluorescent images of d19 myometrium from the unilateral pregnancy study also displayed a parallel

trend with no significant change in P-Ser16 HspB6 detection between the non-gravid and gravid samples. The immunofluorescent data obtained from 17β-Estradiol study demonstrated virtually no significant detection of P-Ser16 HspB6. Even P-Ser16 HspB6 detection in the control heart and liver tissues did not indicate staining similar to other studies. The results for all of these experiments bare a stark contrast with the data obtained from the immunoblot analysis. The description in Section 4.4, the literature, and the lysis buffer extraction experiments, provide confidence that both antisera were appropriate for immunoblotting and that the detection of P-Ser16 HspB6 in these assays was valid. Thus, the conflicting immunofluorescent data indicate that perhaps the antisera were not suitable for immunofluorescent.

The specificity of antisera is partly dependent on the type of immunogens used to generate the antibody: synthetic peptides or purified protein (Bordeaux et al., 2010). Synthetic peptides can range from amino acids to ~25 or 30 in length and this exact sequence is used to create an antibody that will target that particular epitope of the target molecule (Saper, 2009). Although the fact that an exact sequence is being targeted seems sound in theory, this is complicated by the fact that these peptides do not necessarily recapitulate the 3-dimensional structure or post-translational modifications of the native protein (Ramos-Vara, 2005). Therefore, antisera generated against a synthetic peptide may not function optimally when the target molecule is a protein in its native state with intact 3D structure (Bordeaux et al., 2010), which is the case in immunofluorescence. In contrast, in experiments such as immunoblotting where the protein is separated under denaturing conditions after running SDS-PAGE, the sequence is more readily accessible

and the antisera can bind, resulting in most antisera generated from a synthetic peptide being ideal for immunoblotting procedures but insufficient when it comes to immunocytochemistry (Couchman, 2009). This may explain why both the donated and commercial antisera utilized in this thesis, derived from the synthetic peptide sequences SWLLRRA-S-PO_3-APLPG and R-A-S^P-A-P respectively, might have been only suitable for immunoblot analysis.

The process of fixation can further complicate the binding of antisera to either a native or denatured protein conformation. In this thesis, tissues were fixed post-collection in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) (pH 7.4) with shaking overnight at room temperature. Tissues were then processed, paraffin embedded, sectioned and mounted on microscope slides (Chapter 2, Section 2.3). The fixation process may distort the target antigen (Saper, 2009). More specifically, epitopes that are not exposed in the native proteins may be exposed in fixed tissue and vice versa, even though they may not be truly denatured. Thus an antisera that recognizes the target epitope in fresh tissue, may recognize another epitope in fixed tissue (Willingham, 1999; Saper & Sawchenko, 2003). For example, BCL-2 (41-54 amino acids) has an epitope that is exposed when BCL-2 is in the cytoplasm but is inaccessible in the nuclear compartment, perhaps due to protein interaction (Pezzella et al., 1990). However, upon phosphorylation of BCL-2 at sites near the target epitope, the epitope becomes available, as shown when the protein is extracted from cells or denatured by SDS-PAGE (Willingham, 1999). Another important point is that fixation can also alter the penetration of antibodies into the tissue itself, which is known as epitope masking (Fritschy, 2008).

Considering these points, perhaps the P-Ser16 HspB6 antisera cannot access the specific epitope due to the processing, fixation and embedding process, but the epitope is readily accessible in fresh tissue lysates used in immunoblot analysis. Support for this supposition may be demonstrated in the fact that the P-Ser16 HspB6 antisera detect the protein in the nucleus of the M-11 cell line, which were not paraffin-embedded (Section 4.6; Figure 3.5.1).

As previously mentioned, both antisera were raised against synthetic peptides, which carries its own faults. Under ideal conditions, a researcher could simply order a new antiserum that is derived from a purified protein and then use this for immunofluorescence. Unfortunately, at the time of completion of this thesis, these two P-Ser16 HspB6 specific antisera were the only such reagents available for research purposes. Even the antibody batches themselves displayed different affinities for P-Ser16 HspB6 compared to previous lots, as seen in Figure 3.3.3 and Figure 3.4.1 Therefore, the laboratory is quite limited in the resources available at this time to further validate the immunoflurescent experiments.

4.6 Cellular Studies

The human myometrial cell line (M-11) was used to study the spatial expression of P-Ser16 HspB6 at the cellular level to help determine what role this protein plays in mediating quiescence. Immunofluorescent studies demonstrated a difference in cellular localization between the total and phosphorylated form of the protein, and both indicated higher detection than the control. Total HspB6 was predominantly found in the cytoplasm of M-11 cells while P-Ser16 HspB6 was almost entirely nuclear with only trace amounts

still remaining in the cytoplasm. HspB6 localization under normal (non-heat stress) conditions in rat neonatal cardiac myocytes was predominantly cytoplasmic and upon heat stress the majority of the protein migrated into the nucleus, with small amounts left in the cytoplasm (van de Klundert et de Jong, 1999). Although the human myometrial cells used in this thesis were cultured under normal conditions, it still bears importance to show that HspB6 can translocate to the nucleus. This translocation of P-Ser16 HspB6 to the nucleus could indicate a role as a chaperone in the nuclei, preventing protein degradation/aggregation. This chaperone function could possibly be a way of P-Ser16 HspB6 indirectly regulating gene expression by chaperoning transcription factors. HspB1 is one Hsp that binds to a motif in ERα (Razandi et al., 2010), this binding promotes palmitolylation, a process required for localization of sex steroids to the plasma membrane where they can rapidly induce signal transduction and modulate gene expression (Hammes & Levin, 2007). Another study on cellular localization of HspB6 in swine carotid arteries actually added agonists such as histamine, forskolin and nitroglycerin that are known to increase cAMP and cGMP, which result in phosphorylation of HspB6 (Rembold & Zhang, 2001). They found that HspB6 was mainly found in the cytoplasm and upon addition of these agonists known to promote HspB6 phosphorylation, HspB6 remained in the cytoplasm. Although our localization for P-Ser16 HspB6 did not remain in the cytoplasm, it is possible that the localization pattern of P-Ser16 HspB6 observed may be cell or tissue specific.

Chapter 5
Summary & Future Directions

The biochemical mechanism underlying preterm birth is not well understood. Although we know generally that both mechanical and endocrine influences are involved, how they precisely regulate pathways is beyond our knowledge at this time. To be able to effectively deal with preterm labour and develop adequate treatments, we first have to understand the biochemical mechanisms underlying normal labour.

The first goal was to obtain a gestational profile of P-Ser16 HspB6 expression changes during normal pregnancy and labour in the rat as a precursor to assessing the role of HspB6 in the future. P-Ser16 HspB6 expression increased towards late pregnancy and labour and based on the available literature, P-Ser16 HspB6 may facilitate myometrial quiescence during late pregnancy when distension is a prominent influence.

Next, the contribution of mechanical distension to the regulation of P-Ser16 HspB6 expression was studied using a unilateral pregnancy model. Immunoblot analysis indicated that at d15, d19 and d23 of gestation P-Ser16 HspB6 expression was upregulated in the gravid horn when compared to the non-gravid horn. This suggests that P-Ser16 HspB6 is upregulated in the gravid horn as a way to maintain quiescence as the myometrium undergoes more and more tension due to the growing fetus. It would be important in future studies to carry out experiments with a unilateral model that involved inserting cervical tents (laminaria) into the non-gravid horn for 24 hours during pregnancy. Laminaria contains a water-absorbing substance so it would expand

overnight, producing a dynamic stretch in the absence of a fetus that would determine if the effects of stretch could be recapitulated in the non-gravid horn. As HspB6 is found to be associated with HspB1, a potential CAP, it is also possible that a dynamic relationship exists between the two where association of these two sHsps may regulate myometrial state. It would be interesting in future studies to carry out pull-down studies by immunoprecipitating HspB6-HspB1 complexes over gestation to see if the two remain associated throughout pregnancy or if they dissociate at any time. Such knowledge would give an idea as to how the two proteins are interacting and affecting one another.

From an endocrine standpoint, exogenous 17β-Estradiol injection model was used to determine if estradiol had any effect on P-Ser16 HspB6 expression. Estradiol injected rats displayed upregulation of P-Ser16 HspB6 expression when compared to the vehicle injected rats. This suggests that 17β-Estradiol is a significant inducer of P-Ser16 HspB6 expression. Previous studies have also demonstrated upregulation of heat shock proteins upon exposure to estradiol. cAMP, the second messenger and activator of protein kinase A/G which phosphorylates HspB6, has also been shown to be upregulated in the presence of 17β-Estradiol suggesting a potential signalling leading to phosphorylation of HspB6. Future studies could also investigate the effects of progesterone on P-Ser16 HspB6 expression, as this hormone can antagonize 17β-Estradiol signalling.

Additional work that is needed to solidify these findings is a reliable P-Ser16 HspB6 antibody that is suitable for immunofluorescence. Immunofluorescence was carried out in all experiments, but none proved to show any consistent or relevant results after multiple attempts. It is vital that once a new commercial P-Ser16 HspB6 antibody is

available the immunofluorescent experiments must be repeated to hopefully gain insight into what is happening to P-Ser16 HspB6 within the muscle layers of the myometrium. It would first be beneficial if the newly obtained antibody was raised in a different animal than the rabbit, which both anti-sera now are from. If one has multiple antibodies raised from different animals that all give the same results, this indicates that the antibody is specific for the target of interest. In the future it would be advantageous to either produce the antibody ourselves in house, or continue to probe the literature for new antibodies specific to P-Ser16 HspB6. Once these new antibodies are obtained the first step could be to probe a myometrial immunoblot to ensure that there are not multiple bands or bands not at the proper molecule weight present, which could indicate the same target at breakdown products, splice variants or at different post-translational modification status. In the case of P-Ser16 HspB6, the presence of 40 kDa or 20 kDa would be suffice as phosphorylation seems to induce a dimer aggregation (Chapter 4, Section 4.4). The next step could be to target P-Ser16 HspB6 through cultured cell models. One can obtain cells that are biologically proven not to expression a specific protein, therefore when one probes for this protein and no detection is seen it is evidence that antibody is targeting the protein of interest. As HspB6 is a ubiquitous protein, P-Ser16 HspB6 knock out constructs could be obtained that could be transfected into M-11 cells. Fan et al. (2004) constructed an adenovirus that prevents HspB6 from being phosphorylated, which could serve this purpose. Upon probing with P-Ser16 HspB6 antibody, if no detection is observed one can confidently say the anti-sera is specific for P-Ser16 HspB6. In

addition, our laboratory has a vector that overexpresses P-Ser16 HspB6. This could serve as a positive control for the experiment where one would expect high levels of detection.

Once the antibodies prove optimal the best immunofluorescence protocol for this particular protein in this particular tissue has to be optimized. Troubleshooting could include different methods of epitope retrieval such as trypsin and SSC, which were used in this project, but also using a pressure cooker to induce high temperatures or perhaps even proceeding without epitope retrieval and observing the effects. Once epitope retrieval is satisfactory, another method could be employed ensure the antibody is specific in situ. By applying a phosphatase to the slides, one is removing the phospho-groups, therefore an antibody specific for P-Ser16 HspB6 should not give any detection. The processing of tissue itself should be changed from paraffin embedding, which is known to have deleterious effects on antigen preservation for immunodetection, to formalin staining of fresh-frozen tissue which would avoid these issues.

Lastly, the early stages of cellular studies demonstrated that HspB6 localization changes upon phosphorylation from cytoplasm to mainly nuclear. This does coincide with heat stress studies on neonatal rat cardiac myocytes indication that HspB6 can translocate to the nucleus. However, the localization pattern of P-Ser16 HspB6 may be cell or tissue specific. Future cellular work could be based on the fact that P-Ser16 HspB6 expression is upregulated during the synthetic phase of pregnancy when hypertrophy is prominent. One could observe how P-Ser16 HspB6 expression changes spatially when the cells are exposed to a period of hypertrophy by additional of a

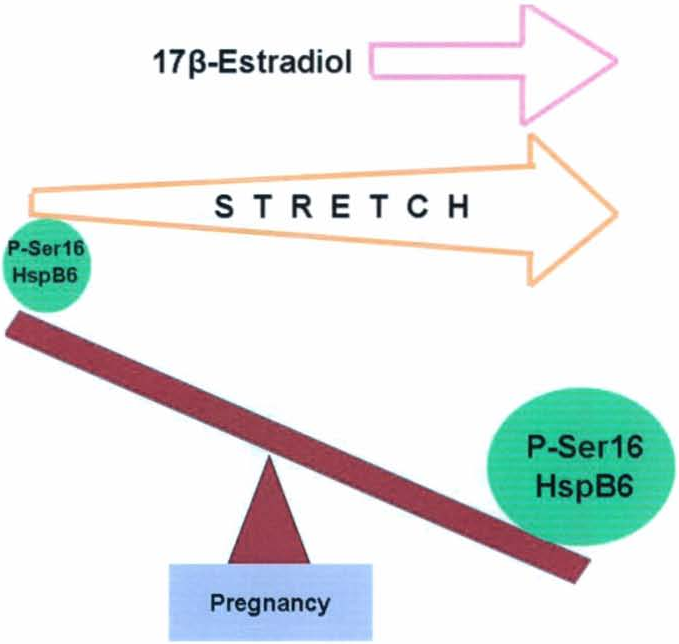
hypertrophic agent, such as fluprostenol. This would give us an idea of what is happening at the cellular level to P-Ser16 HspB6 during a period when its upregulation is maximal.

If resources were available in the future a really interesting study would be to functionally prove the relevance of P-Ser16 HspB6 in myometrial relaxation by extracting myometrial muscle strips from pregnant and non-pregnant rats and performing isometric studies in the presence/absence of a P-Ser16 HspB6 peptide with a PKA inhibitor. Flynn et al. (2003) developed a peptide containing enhanced protein transduction sequences and phosphopeptide analogs of HspB6. Protein transduction domains have been shown to cross biological membranes efficiently without transporters or receptors and promotes the delivery of peptides into the cell (Frankel & Pabo, 1988; Fawell et al., 1994; Schwarze et al., 1999). This peptide has been shown to promote relaxation in vascular smooth muscle (Flynn et al., 2003). If the stretched myometrial muscle can be relaxed by the addition of the peptide with a PKA inhibitor, functionally we would have a stronger foundation to say P-Ser16 HspB6 is indeed a smooth muscle relaxant and that PKA is not inducing relaxation via one of its many pathways. To take this a step further *in vivo* studies could be performed using the same peptide but this time injecting it exogenously into a pregnant rat on d23 of pregnancy to see if labour could be delayed. Such experiments certainly are difficult to execute as so many factors must be taken into consideration, but if it was carried out properly the results would be substantial.

In summary my results demonstrate that P-Ser16 HspB6 expression is increased towards late pregnancy and labour, that distension, or strech, is a significant inducer of P-

Ser16 HspB6 expression during mid-late pregnancy and that 17β-Estradiol is also a significant inducer of P-Ser16 HspB6 expression (Figure 5.1).

Figure 5.1 Increasing stretch and circulating levels of 17β-Estradiol are the tipping point during pregnancy, inducing increased expression of P-Ser16 HspB6 in the myometrium.


17β-Estradiol
S T R E T C H
P-Ser16
HspB6
P-Ser16
HspB6
Pregnancy

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Appendix A

From: Chun Seow
To: mpeach@mun.ca
Subject: Re: Figure from paper
Date: Tuesday, August 10, 2010 10:38:47 PM

Dear Mandy,

You have my permission to use the figure. Good luck with your thesis.

Chun Y. Seow, Ph.D.
Professor, Department of Pathology and Laboratory Medicine,
University of British Columbia,
James Hogg Research Laboratories,
Providence Heart + Lung Institute,
St. Paul's Hospital,
Office: Room 370A, Burrard Building,
Mail/Courier: Room 166, Burrard Building
1081 Burrard Street, Vancouver, BC V6Z 1Y6
P: 604-806-9268
F: 604-806-9274

Lab Phone: (604) 682-2344 Ext. 63562
E-mail: chun.seow@hli.ubc.ca

>>> <mpeach@mun.ca> 8/10/2010 2:51 pm >>>
Dr.Seow:

My name is Mandy Peach and I am a graduate student in a reproductive physiology lab in Memorial University. I am currently writing up my thesis and I was wondering if I could use Figure 9 from your paper " Contractile filament architecture and force transmission in swine airway smooth muscle". I will be using it to explain the mechanical syncytium formed in the myometrium upon labour.

Thank you,

Mandy

Appendix B

From:	William Gerthoffer
To:	mpeach@mun.ca
Subject:	Re: sHsps in smooth muscle article
Date:	Thursday, July 29, 2010 6:51:22 PM
Attachments:	Ba et al HSP20 Airway Smooth Muscle Relaxation.pdf Presentation1.pptx

Dear Mandy,

Of course you may use the figure. I am attaching the original powerpoint file if that will be useful.

I am also sending a reprint of a paper we published last year in an online journal that is not indexed in PubMed. The paper describes some recent mechanistic data on HSP20 and relaxation of airway smooth muscle. We showed it disrupts actin filaments, but also inhibits actin binding to myosin (at least in vitro). Let me know if you have found similar (or different) results in uterus. The HSP20 protein is pretty interesting. I think it is underappreciated in smooth muscle cell biology.

Good luck with your thesis.

William T. Gerthoffer, Ph.D.
Professor and Chair
Department of Biochemistry & Molecular Biology
University of South Alabama, College of Medicine
307 University Boulevard
Mobile, AL 36688-0002
Phone: 251-460-6856
FAX: 251-460-6850
wgerthoffer@usouthal.edu

>>> <mpeach@mun.ca> 7/29/2010 1:52 PM >>>

Dr. Gerthoffer:

My name is Mandy Peach and I am a graduate student in a reproductive physiology lab with Memorial University of Newfoundland. I am currently writing my thesis on P-Hsp20 in rat myometrium during pregnancy. Figure 4 in your 2008 paper "Small heat shock proteins in smooth muscle" is fantastic. I was wondering if I may have permission to use this in my thesis to demonstrate the hypothesized mechanisms of relaxation by Hsp20?

Thanks so much,

Mandy