Distension of the Uterus Induces HspB1 Expression in Rat Uterine Smooth Muscle.

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ABSTRACT

The uterine musculature or myometrium demonstrates tremendous plasticity during pregnancy under the influences of the endocrine environment and mechanical stresses. The expression of the small stress protein heat shock protein B1 (HspB1) has been reported to increase dramatically during late pregnancy – a period marked by myometrial hypertrophy induced by fetal growth-induced uterine distension. Thus, we examined the effect of uterine distension on myometrial HspB1 expression using unilaterally pregnant rat models and ovariectomized, non-pregnant rats with uteri containing laminaria tents to induce uterine distension. In unilaterally pregnant rats, HspB1 mRNA and pSer15-HspB1 protein expression were significantly elevated in distended gravid uterine horns at both d19 and d23 (labour) of gestation compared to non-gravid horns. Similarly, pSer15-HspB1 protein detection in situ was only readily detectable in the distended horns compared to the non-gravid horns at both d19 and d23; however, pSer15-HspB1 was primarily detectable in situ at d19 in membrane-associated regions while it had primarily a cytoplasmic localization in myometrial cells at d23. Both HspB1 mRNA and pSer15-HspB1 protein expression were also markedly increased in ovariectomized, non-pregnant rat myometrium distended for 24h with laminaria tents compared to empty horns. Therefore, uterine distension plays a major role in the stimulation of myometrial HspB1 expression and increased expression of this small stress protein could be a mechanoadaptive response to the increasing uterine distension that occurs during pregnancy.
INTRODUCTION

The uterus during pregnancy undergoes considerable physical distension. In response to such mechanical stress, adaptation of the uterus occurs and is exemplified by increases in uterine size and weight. Before pregnancy, the human uterus weighs approximately 40-70g and can hold a volume of 10mL, whereas at labour the uterus weighs about 1100-1200g and can hold on average a volume of 5L (30). The size of human uterine smooth muscle cells have also been found to increase up to 10 times in length and 3 times in width during gestation (30).

During gestation the rat uterine smooth muscle, or myometrium, goes through a series of differentiation phases (43). Early in pregnancy, the myometrium is characterized by a phase of hyperplasia marked by significantly increased mRNA expression of IGF-I and IGFBP1 as well as activation of the mTOR signalling pathway (11,42). Subsequently, there is a transition to a synthetic phase with a period of hypertrophic myometrial growth that requires uterine distension and is supported by circulating progesterone. This growth is marked by an increase in the protein:DNA ratio of the myometrium and the increased expression of interstitial matrix proteins such as collagen I (39,41).

Recently, Shynlova and colleagues (44) demonstrated that the significant increase in rat myometrial growth during late pregnancy was due to a threefold increase in myocyte size and confirmed that it was dependent on uterine distension. Towards the end of gestation the myometrium switches to a contractile phenotype with an increase in basement membrane matrix synthesis, marked by increased expression of fibronectin, as well as increased detection in situ of the fibronectin receptor protein subunits α5 and β1 integrins (42,50,51). The rat myometrium then undergoes the labour phase and becomes activated by the increased presence of contraction associated proteins (CAPs) such as the gap junction protein Cx43 and oxytocin receptors as well as decreased focal adhesion kinase activity thus marking the stabilization of myometrial cell-ECM contacts to facilitate the initiation of labour (28,35,47).
In this differentiation sequence, both endocrine and mechanical pathways are required for induction of CAP gene expression (25,26,33-35,43).

Heat shock protein B1 (HspB1; previously known as Hsp27) is one of 11 members of the mammalian small heat shock protein (sHSP) family (13,22). All members of this class have a molecular weight between 15 and 40 kDa and share a structural domain in their carboxyl-terminal halves named the α-crystallin domain that spans two putative actin binding domains (36). Furthermore, these proteins have demonstrated ATP-independent chaperone activity (14,22).

Phosphorylation of sHSPs is a post-translational modification that is very important for regulation of sHSP structure and function. For example, HspB1 phosphorylation results in dissociation of large oligomers of HspB1 and loss of chaperoning activity (15,17). Two phosphorylation sites have been reported for HspB1 in rodents, serine (Ser)-15 and Ser-86 (homologous to Ser-82 in humans and Ser-90 in hamsters; 8). The latter site is necessary for the dissociation of large sHSP multimers, but at the cellular level it is not always sufficient (9,16,19). Ser-15 phosphorylation of HspB1 may produce a conformational change in HspB1 that aids the direct binding of HspB1 with actin microfilaments (19). A number of past reports have indicated a role for HspB1 in actin polymerization, remodelling, stabilization and actin-myosin crossbridge cycling (1,10,19,23,24,29). In terms of a role in smooth muscle, the facilitation of actin formation and actin-myosin interaction by HspB1 has been shown to be essential for the contraction of colonic smooth muscle (2).

Many different cellular stress signals initiate HspB1 expression and phosphorylation, such as heat shock and oxidative stress (16,20), and HspB1 expression is reportedly induced by stretch of epithelial cells and heavy resistance training of skeletal muscle (27,31). Recently, Chaudhuri and Smith (6) reported that cyclic mechanical stress of airway smooth muscle cells resulted in increased HspB1
phosphorylation, yet the role of mechanical stress on HspB1 expression within the uterine musculature during pregnancy remains unknown.

We have previously reported that HspB1 gene expression is highly upregulated in the rat myometrium during late pregnancy and labour (49), which parallels the increase in mechanical stress or distension exerted on the myometrium due to growing foetuses. It was also reported that during late pregnancy Ser-15 phosphorylated HspB1 (pSer15-HspB1) had a predominantly membrane-associated localization in situ within cells of both the circular and longitudinal muscle layers. We have thus suggested that HspB1 is a potential contraction-associated protein, but the regulatory mechanism(s) underlying myometrial HspB1 expression during pregnancy are still unknown. Utilizing a unilaterally pregnant rat model, we examined the effect of uterine distension on myometrial HspB1 expression. We hypothesized that this stress would increase HspB1 expression within the uterine musculature.
MATERIALS AND METHODS

Animals and Tissue Collection

Sprague-Dawley rats were maintained in the Animal Care Unit at the Health Sciences Centre (Memorial University of Newfoundland, St. John’s, Newfoundland and Labrador, Canada) under standard environmental conditions (12-h light, 12-h dark) and the institutional animal care committee approved all experiments under protocols 06-02-DM - 08-02-DM. Animals were fed Purina Rat Chow #5012 (Purina Mills, St. Louis, Missouri, USA) and water ad libitum. For collection of pregnant rat samples, virgin female rats (~220g) were mated with stud males. Day 1 of pregnancy was determined by the observance of vaginal plugs the next morning. Under this timing scheme the rat gestational period was 23 days.

Carbon dioxide induced asphyxiation was used for euthanisation of all animals prior to sample collection. Uterine horn samples to be used for immunocytochemical analysis were fixed in 4% paraformaldehyde in 1X PBS overnight with agitation, followed by washing in 1X PBS for 24 hours. Tissue samples were then subjected to a graded ethanol series and xylene penetration prior to embedment in paraffin wax. In other cases, excised uterine tissue was placed in ice-cold PBS (pH 7.4) and opened longitudinally for collection of the myometrium as has been previously described (49). The endometrial layer was removed from all samples by gentle scraping with a scalpel blade prior to flash freezing in liquid nitrogen and storage at -80°C.

Experimental Design

Unilaterally pregnant rat model
Virgin female rats (~220g) were administered an intramuscular injection of anesthesia (100mg/kg ketamine, 20mg/kg xylazine; Ketaset®, Wyeth Animal Health, Guelph, ON, Canada; Rompun®, Bayer Inc., Toronto, ON, Canada) and then received a unilateral tubal ligation through bilateral flank incisions (dorsal surface), approximately 1cm distal of the spine, as described elsewhere (34,35). Animals were monitored post-operatively and subsequently allowed to recover for at least 5 days before matings were attempted. Samples of gravid and non-gravid horns were collected on gestational day (d) 19 and d23 (n=4).

Non-pregnant ovariectomized rat models

To investigate the role of uterine distension on HspB1 expression in the absence of any endocrinological contributions from the feto-placental units or ovarian steroids, we utilized a non-pregnant ovariectomized rat model. Prior to experiments using laminaria tents, female rats (~220g) were administered an intramuscular injection of anesthesia (100mg/kg ketamine, 20mg/kg xylazine; Ketaset®). Rats were then bilaterally ovariectomized as previously described in detail (34). Animals were allowed to recover for at least 5 days post-operatively before laminaria tent insertion.

To generate a dynamic uterine distension over 24h in the uterine horns of these rats, we used extra-small (2 x 50mm) laminaria tents (Catalogue #021002, MedGyn, Lombard, IL, USA). These tents consist of dried, sterilized seaweed stems that are hygroscopic and gradually expand over 24h. Larger versions of these tents can be used clinically as disposable devices for gentle dilation and softening of the cervix. Following administration of anesthesia to the rats (as described above), bilateral flank incisions (dorsal surface) approximately 1cm distal of the spine were made to expose the uterine horns. Laminaria tents were then surgically inserted into the lumen of one of the uterine horns of the ovariectomized rats (n= 4) through a small distal incision in the horn that was then sutured closed.
control for the presence and effect of an intra-uterine device (IUD), polyethylene tubes of the same size and diameter (~2mm OD, Cat #7446, Clay Adams) as pre-expanded laminaria tents were also inserted into some uterine horns (n=4) in place of the laminaria tents. Samples were subsequently collected from empty, polyethylene tube-containing and laminaria-containing horns 24hrs post insertion.

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**Northern Blot Analysis**

For each experimental model studied, 4 independent sets of RNA samples (n=4) were used. TRizol® Reagent (Invitrogen Corporation, Carlsbad, California, USA) was used for RNA extractions, and extractions were completed according to the manufacturer’s instructions. Spectrophotometric analysis of RNA was performed using a Shimadzu Bio-Mini Spectrophotometer (Mandel Scientific, Guelph, Ontario, Canada) to assess RNA purity and concentration before storage at -80°C.

Northern blot preparation and hybridization were performed as previously described (49). Briefly, RNA (10μg) was prepared, separated on a 1% agarose-formaldehyde-MOPS gel, transferred to a nylon membrane (Hybond-XL nylon membrane; GE Healthcare, Little Chalfont, Buckinghamshire, England), and stored at -20 C. Pre-hybridization of membranes for 1-2 hours at 42°C was followed by hybridization with radiolabelled (32P-dCTP) probes overnight at 42°C. Probes were produced utilizing a hamster HspB1 cDNA template, following instructions provided in the Megaprime DNA Labeling kit (GE Healthcare, Little Chalfont, Buckinghamshire, England). Blots were subsequently washed and multiple exposures of X-ray film were produced to confirm the linearity of the film response. Following detection of HspB1 mRNA, northern blots were stripped and re-probed for ribosomal 18S RNA for use as a loading control. The hamster HspB1 cDNA (Genbank Accession #X51747) and rabbit 18S ribosomal cDNA (Genbank Accession #X06778) were kind gifts from Dr. J. Landry (Laval University, Quebec, Canada) and Dr. I. Skerjanc (University of Ottawa, Ontario, Canada), respectively. 18S rRNA is constitutively expressed...
in rat myometrial cells and has been utilized in the past as a loading control for analysis of myometrial gene expression (33, 39, 49).

Immunoblot Analysis

Immunoblot analysis was performed on at least 4 independent sets of samples as previously described in detail (49). Flash-frozen myometrial samples were used to extract total protein in RIPA lysis buffer containing COMPLETE™ Mini EDTA-free protease inhibitors and PhosSTOP phosphatase inhibitors (Roche Molecular Biochemicals, Laval, Quebec, Canada). Protein concentrations were determined using the Bradford assay (4). Protein samples (100μg/lane) were separated under reducing conditions in 12% SDS-polyacrylamide gels (18) and transferred to nitrocellulose membranes (0.45μm, Pierce membranes, Fisher Scientific, Ottawa, ON, Canada; 0.45μm, Pall Corporation, East Hills, NY, USA).

Membranes were first probed for detection of the serine 15 phosphorylated form of HspB1 with a pSer15-HspB1-specific rabbit polyclonal antibody (1 μg/mL final concentration; Cat # PA1-016, Affinity BioReagents, Golden, CO, USA), then stripped with Restore Western blot stripping solution (Cat# PI-21059, Fisher Scientific, Ottawa, ON, Canada) and re-probed for Calponin, which served as a loading control, using a mouse monoclonal antibody provided as an ascites fluid (0.01 μg/mL final concentration, clone hCP; Cat # C2687, Sigma-Aldrich Canada, Oakville, Ontario, Canada). The appropriate HRP-conjugated anti-rabbit (Cat #W4011, Promega Corporation, Madison, WI, USA) and anti-mouse secondary antibodies (Cat #W4021, Promega Corporation, Madison, WI, USA) were utilized in 1:10,000 and 1:150,000 dilutions respectively. The pSer15-HspB1 and calponin-specific antisera recognized protein bands at the predicted 27 kDa and 34 kDa molecular weights, respectively. Blots were developed with the Pierce SuperSignal West Pico chemiluminescence substrate detection system (Cat# PI34080,
Fisher Scientific, Ottawa, ON, Canada) according to the manufacturer’s instructions. Multiple exposures were generated to ensure the linearity of the film exposures.

**Immunocytochemistry**

Immunocytochemical analysis was performed on two independent sets of uterine tissue samples for each experimental design and repeated at least 2 times as previously described (49). All paraformaldehyde fixed samples were processed, embedded, and sectioned by the Histology Unit of Memorial University of Newfoundland School of Medicine. Five micrometer thick tissue sections were adhered to silane-coated slides. Sections were de-waxed and rehydrated for immunocytochemistry as previously described in detail (49). Briefly, the following steps were conducted at room temperature unless otherwise stated. Antigen retrieval was performed by incubating tissue sections in 0.125% Trypsin in 1XPBS for 15 minutes. Sections were then incubated in blocking solution (5% horse, 1% goat, and 1% fetal bovine serum in PBS) for 30 minutes followed by incubation in pSer15 HspB1-specific rabbit polyclonal antiserum (5 ug/mL final concentration, Cat # PA1-018, Affinity BioReagents, Golden, CO, USA), or a pre-immune sera at the same effective concentrations for 1 hour as previously conducted (49). After washing with PBS, sections were incubated for 1 hour with an FITC-conjugated anti-rabbit secondary antibody (20 ug/mL final concentration; Cat # F7512, Sigma-Aldrich, St. Louis, MO, USA). Following additional washes with PBT (PBS with 0.02% Tween-20), sectioned were mounted in Vectashield (Vector Laboratories Inc., Burlington, Ontario, Canada) anti-fade mounting media and sealed around the edges with nail polish. Image collection was conducted using an Olympus Fluoview 300 laser scanning confocal microscope (Olympus Optical Company Ltd., Melville, New York, USA).
Data Analysis

Differences in expression levels between samples within each experimental group were determined through densitometric analysis of x-ray films. Following acquisition of digital scans of x-ray films with an HP Scanjet G4050 scanner, Image J (National Institutes of Health, Frederick, Maryland, USA) was used to generate raw densitometric values for each sample lane. Densitometric measurements of HspB1 mRNA were normalized to those of 18S ribosomal RNA while measurements of pSer15-HspB1 protein on immunoblots were normalized to those of calponin. Statistical significance between data sets was assessed by performing a two-tailed t-test using Graphpad Prism version 5.0 (GraphPad Software, San Diego, California, USA, www.graphpad.com). Values with a p < 0.05 were considered significantly different.
RESULTS

HspB1 Expression in Unilaterally Pregnant Rat Myometrium

HspB1 mRNA Expression

Northern blot analysis was employed to observe any change in myometrial HspB1 mRNA expression due to uterine distension. Initial investigation was performed using a unilaterally pregnant model consisting of rats at d19 and d23 (labour) of gestation with one gravid (distended) and one non-gravid (empty) horn (n=4). HspB1 mRNA expression was significantly increased in the gravid horn myometrium from unilaterally pregnant rats on both d19 and d23 compared to samples from the non-gravid horn (Fig. 1A, B; p<0.05).

pSer15 HspB1 Protein Expression and Localization in situ

Total myometrial protein extracts (n=4) were utilized for immunoblot analysis to determine any distension-induced changes in HspB1 protein expression. Investigation with pSer15 HspB1-specific antibodies in unilaterally pregnant rats showed pSer15 HspB1 expression in the myometrium was significantly induced with uterine distension at both d19 and d23 (Fig. 2A,B; p<0.05).

To investigate changes in sub-cellular localization and detection levels of pSer15 HspB1 between gravid and non-gravid uterine horns, immunofluorescence analysis was utilized. At day 19 the gravid horn exhibited a high level of detection of pSer15 HspB1 in both muscle layers and localization was primarily found to be membrane-associated; however, some peri-nuclear localization was also observed (Fig. 3). In contrast, the non-gravid horn exhibited virtually no pSer15-HspB1 immunostaining above the pre-immune control. At d23, pSer15 HspB1 was also highly detectable in both muscle layers of the gravid horn and localized at some membrane-associated regions, but was much more detectable in the
cytoplasm of myometrial cells (Fig. 4). The non-gravid horn exhibited virtually no pSer15 HspB1 immunostaining above the pre-immune control.

**HspB1 Expression in Non-Pregnant Rat Myometrium**

*HspB1 mRNA Expression*

It was possible that the increased myometrial HspB1 expression observed in the gravid horns during pregnancy could be due, at least in part, to fetal or placental paracrine influences absent in the non-gravid horns. Thus, to more precisely confirm the induction of HspB1 expression by uterine distension, in the absence of any underlying influence from the fetoplacental unit or even ovarian steroids, a non-pregnant ovariectomized (OV) rat model was also employed for experiments. In this model, laminaria tents were surgically inserted into one of the two uterine horns of ovariectomized female rats for 24 h. The result was the production of uterine distension stress in the laminaria-distended horn compared to the contralateral empty horn (Fig. 5A). In other experiments a polyethylene tube of similar length and diameter to the pre-expanded laminaria tents (i.e. intra-uterine device control) was surgically inserted in one of the horns while the contralateral horn was empty.

Northern blot analysis (n=4) revealed a significant increase in myometrial HspB1 mRNA expression in the laminaria-distended horn of ovariectomized rats compared to empty horns (Fig. 5B; p<0.05). There was no significant difference observed in myometrial HspB1 mRNA expression between uterine horns containing a polyethylene tube and empty uterine horns (Fig. 5C).

*pSer15 HspB1 Protein Expression*
Following laminaria tent insertion, immunoblot analysis (n=4) also demonstrated that the expression of pSer15 HspB1 in the myometrium of the distended horn was significantly increased compared to the empty horn (Fig. 6A; p<0.05). In contrast, there were no significant differences in myometrial pSer15-HspB1 protein expression between uterine horns containing a polyethylene tube, mimicking a pre-expanded tent, and empty uterine horns demonstrating a lack of any intra-uterine device effect on protein expression (Fig. 6B).

*pSer15 HspB1 Localization in situ*

Overall we observed a decrease in detectable pSer15 HspB1 immunolocalization as compared to detection in the pregnant state. However, there was an increase in pSer15 HspB1 detection in the myometrium of laminaria distended horns compared to detection in the myometrium of empty horns, particularly in the longitudinal muscle layer (Fig. 7A). Localization of pSer15 HspB1 in the distended horn appeared to be more cytoplasmic and peri-nuclear as compared to the membrane-associated pattern of detection observed in the unilaterally pregnant rat model at d19 of gestation. As expected, there were no observable changes in myometrial pSer15 HspB1 detection levels or localization between the uterine horns containing a polyethylene tube and empty uterine horns (Fig. 7B).
DISCUSSION

In the myometrium during pregnancy, uterine distension has been reported to increase the expression and/or post-translational modification of contraction-associated (CAP) proteins, focal adhesion proteins and signaling kinases such as connexin-43, FAK, and extracellular signal-regulated kinases (ERK), respectively (25,26,33-35). While investigation has been undertaken into the effects of stretch on the larger class of heat shock proteins in smooth muscle such as HSP70, research on sHSPs is still emerging (3). In particular, there has still been limited investigation of the response of HspB1 to mechanical stress. Chaudhuri and Smith (6) showed that cyclic strain of airway smooth muscle cells resulted in significant and rapid increases in expression of phosphorylated HspB1, although the exact phosphorylated form studied was not specified. Thus, we took the opportunity to investigate the effects of uterine distension or mechanical stress on myometrial HspB1 expression in the unilaterally pregnant rat model that at the same time might advance knowledge and stimulate research with additional types of smooth muscle.

Uterine Distension Induces Expression of HspB1 mRNA and pSer15-HspB1 Protein

We have previously reported that HspB1 mRNA expression was significantly elevated in the pregnant rat myometrium at d19 then significantly decreased by d23 (labour) while pSer15-HspB1 expression was significantly increased from d19 to d23 compared to earlier points in pregnancy (49). Our results presented here show that uterine distension induced expression of HspB1 mRNA and pSer15-HspB1 protein levels at both d19 and d23 demonstrating the important role of distension or mechanical stress on the regulation of expression regardless of the period of late pregnancy. However, the increased expression in the gravid horns was observed at times when circulating levels of progesterone and 17β-estradiol are reported to be elevated in the rat at d19 and d23, respectively (37). As a result, it was still...
possible that these steroids as well as other contributions from the fetoplacental unit could contribute to the observed increases in HspB1 expression during late pregnancy. Examination of HspB1 expression in ovariectomized, non-pregnant rats subjected to uterine distension with laminaria tents clearly showed that HspB1 mRNA and pSer15-HspB1 protein expression could be potently induced by uterine distension in vivo in the absence of fetal, placental paracrine or ovarian steroid influences. However, we cannot exclude the likelihood that a substance(s) could leach out of the laminaria tents or that intrauterine and interstitial fluid could be excessively absorbed by the tents and contribute signals, in addition to uterine distension, leading to the observed increase in HspB1 expression.

Despite our findings, hormonal influences cannot be excluded as a regulatory mechanism underlying HspB1 expression during pregnancy. We did observe an overall decrease in detectable pSer15-HspB1 immunolocalization in ovariectomized, non-pregnant rats compared to detection in the pregnant state. This could be a result of the loss of the specific hormone environment of late pregnancy. Lastly, marked differences in levels of pSer15-HspB1 immunolocalization were also observed between the two uterine muscle layers following laminaria distension in ovariectomized, non-pregnant rats compared to detection in gravid uterine myometrium. Laminaria tents cannot completely simulate the timing and extent of fetal growth-induced stretch of the uterus during pregnancy and it is conceivable that, as a result, there are different uterine muscle layer-specific responses to the distension produced by the laminaria tents compared to growing fetuses.

It was previously reported that the rat HspB1 gene promoter contains overlapping Sp-1 and AP-2 transcription factor binding sites and a heat shock element (HSE; 32). Thus, it is possible that that Sp-1, AP-2 and/or Heat Shock Factor 1 or 2 (HSF1, 2) could induce transcription of HspB1 mRNA in response to uterine distension. Importantly, HSF1 has been found to be necessary for stress induced HspB1 upregulation in mouse embryonic fibroblasts (53) and Xu and colleagues (52) have reported that HSF1
activation was induced by cyclic mechanical stress in vascular smooth muscle cells. However, the expression of HSF-1 has yet to be determined in pregnant rat myometrium.

HspB1 is a substrate for phosphorylation by the p38 MAPK pathway, utilizing members of the MAPK-activated protein kinase (MK) family, although phosphorylation of Ser-15 and Ser-86 does not appear to occur in any specific obligate order (17, 21, 46). Static stretch of primary cultures of rat myometrial smooth muscle cells \textit{in vitro} strongly induces activated p38 MAPK expression and uterine distension also led to increased p-p38 MAPK expression beginning ~d19 of pregnancy in the rat myometrium reaching significantly elevated levels by d22 (33). Thus, the increased expression of pSer15-HspB1 protein at d19 and d23 may be due, at least in part, to some contributions from the p38 MAPK signaling pathway.

\textit{HspB1 and the Actin Cytoskeleton}

The dynamic modulation of actin microfilament formation likely plays a large role in smooth muscle contraction (48). For example, Shaw et al (38) reported that agonist induced constriction of non-pregnant rat myometrium was reduced by inhibition of actin polymerization with cytochalasin D. All three actin isoforms (\(\alpha\), \(\beta\), \(\gamma\)) are expressed in rat myometrium, but only \(\gamma\)-actin appears to undergo increased expression and changes in localization as term approaches (40). Analysis of term pregnant mouse uterus demonstrated that actin microfilaments were densely packed and ran parallel to the longitudinal axis of uterine smooth muscle cells (48). Smooth muscle dense plaques, or focal adhesions, are sites on the plasma membrane where clusters of integrins, signalling molecules and adapters such as FAK and vinculin can provide a structural link between the ECM and the actin cytoskeleton. FAK activation is highly induced in rat myometrium during late pregnancy where focal adhesion signalling may be necessary to remodel cell-ECM adhesion during myometrial hypertrophy (28). Shynlova et al
(44) recently demonstrated, using a stereological approach, that there were significant increases in uterine smooth muscle cell sizes at late pregnancy (d19) that were not significantly decreased until post partum. Myometrial hypertrophy was also significantly higher in gravid uterine horns compared to non-gravid horns at d19 and at term in unilaterally pregnant rats. Therefore, in this study the increased expression of pSer15-HspB1 protein levels in distended uterine horns at d19 and labour, and the immunolocalization of the protein to membrane-associated regions indicate pSer15-HspB1 could be part of a mechano-adaptive response to regulate actin cytoskeleton dynamics at focal adhesion sites and support hypertrophy-induced focal adhesion reorganization during late pregnancy. Evidence to support this possibility can be found in recent reports. During et al (7) demonstrated that HSPB1 is a G-actin sequestering protein and that HspB1 phosphorylation enhances actin filament assembly. Jia et al (12) has also shown, with mass spectrometry analysis, that phosphorylated HspB1 co-immunoprecipitated with members of the actin regulatory complex Arp2/3.

Since our results demonstrated significantly induced pSer15-HspB1 levels at d23 (labour) upon uterine distension and immunolocalization of pSer15-HspB1 to the cell cytoplasm at this time, we cannot rule out a role for this protein in myometrial contraction. Increased myometrial stretch, as a result of multiple gestation pregnancies for instance, has been suggested as one factor that could lead to the increased incidence of premature uterine contractions (5). The facilitation of actin formation and actin-myosin interaction by HspB1 has been shown to be essential for the contraction of colonic smooth muscle (2) and it is postulated that the mechanism involves phosphorylated HspB1-mediated modulation of caldesmon association with tropomyosin, a thin filament protein critical for actin-myosin interaction (2,45). Taken with the recent findings of stretch inducing phosphorylation of caldesmon in the myometrium (25), a specific association or relationship of HspB1, caldesmon and stretch in uterine smooth muscle contraction may exist.
Perspectives and Significance

Cellular stress signals such as heat shock and oxidative stress are known to initiate HspB1 expression and phosphorylation (16,20). The results of this investigation provide novel insights into the mechanism of regulation of HspB1 expression in uterine smooth muscle during pregnancy and demonstrate that mechanical forces have a major role in regulating HspB1 expression. The data presented also add to a limited literature on the importance of mechanical stress in inducing HspB1 mRNA and phosphorylated protein expression in smooth muscle per se. The increasing identification of proteins, such as HspB1, that are highly expressed and regulated by uterine distension in the myometrium during late pregnancy also provides new potential targets for the development of effective therapeutic strategies to mitigate premature myometrial contraction. Further identification of the exact mechanism(s) of action of HspB1 on the myometrial cell actin cytoskeleton and on contraction will require a molecular approach at the cellular level.
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Figure Legends

Figure 1. HspB1 mRNA expression in rat myometrium is significantly induced by uterine distension. Representative northern blot analysis of HspB1 mRNA and 18S rRNA expression in myometrium from non-gravid and gravid uterine horns at A) d19 and B) d23 of rat pregnancy are shown. Densitometric analyses of experimental data are shown below each set of appropriate representative blots. Northern blot analysis was performed with an HspB1- specific hamster cDNA and an 18S-specific rabbit cDNA as templates for radiolabelled probe production. Values (means +/- SEM) plotted are from four independent experiments (n=4) and values were considered significantly different from one another when p < 0.05.

Figure 2. Expression of pSer15-HspB1 protein in rat myometrium is significantly induced by uterine distension. Representative immunoblot analysis of pSer15-HspB1 and calponin protein expression in myometrium from non-gravid and gravid uterine horns at A) d19 and B) d23 of rat pregnancy are shown. Densitometric analyses of experimental data are shown below each set of appropriate representative blots. Values (means +/- SEM) plotted are from four independent experiments (n=4) and values were considered significantly different from one another when p < 0.05.

Figure 3. Immunofluorescence detection of pSer15-HspB1 in rat myometrium was markedly increased by uterine distension at d19 of pregnancy. A pSer15 HspB1-specific rabbit polyclonal antiserum was used to detect this phosphorylated form of HspB1 in longitudinal and circular muscle layers of distended gravid uterine horns and non-gravid horns. The gravid horns exhibited a high level of detection of pSer15 HspB1.
in both muscle layers, compared to the non-gravid horns, and localization was primarily found to be membrane-associated (arrows). Pre = pre-immune serum control. Scale bar = 50μm.

Figure 4. Immunofluorescence detection of pSer15-HspB1 protein in rat myometrium was markedly increased by uterine distension at labour. A pSer15 HspB1-specific rabbit polyclonal antiserum was used to detect pSer15-HspB1 in longitudinal and circular muscle layers of distended gravid uterine horns and non-gravid horns. pSer15 HspB1 was highly detectable in both muscle layers of the gravid horns compared to the non-gravid horns and localized at some membrane-associated regions, but was much more detectable in the cytoplasm of myometrial cells (arrows). Pre = pre-immune serum control. Scale bar = 50μm.

Figure 5. HspB1 mRNA expression in myometrium from non-pregnant, ovariectomized (OV) rats is significantly elevated in uterine horns exposed to stretch with laminaria tents. A) Hematoxylin and Eosin-stained tissue sections of an empty rat uterine horn or horns after removal of Laminaria tents or polyethylene tubes demonstrating uterine diameter differences. B) and C) Northern blot analysis. Laminaria tents (B) or polyethylene tubes of the same size and diameter as pre-expanded laminaria tents (C) were surgically inserted into one of the uterine horns of non-pregnant, ovariectomized rats. Myometrial mRNA samples were collected from empty (E), polyethylene tube-containing (P) and laminaria-containing (Lam) horns 24hrs post insertion. Northern blot analysis was performed with an HspB1- specific hamster cDNA and an 18S-specific rabbit cDNA as templates for radiolabelled probe production. Representative northern blot analysis of HspB1 mRNA and 18S rRNA expression in myometrium are shown. Densitometric analyses of experimental data are shown below each set of appropriate representative blots. Values (means +/- SEM) plotted are from four independent
experiments (n=4) and values were considered significantly different from one another when p < 0.05. Scale Bar = 250 μm.

Figure 6. Myometrial expression of pSer15-HspB1 protein is significantly induced in uterine horns of non-pregnant, ovariectomized rats (OV) exposed to stretch with laminaria tents. Laminaria tents (A) or polyethylene tubes of the same size and diameter as pre-expanded laminaria tents (B) were surgically inserted into one of the uterine horns of non-pregnant, ovariectomized rats. Samples were subsequently collected from empty (E), polyethylene tube-containing (P) and laminaria-containing (Lam) horns 24hrs post insertion. Representative immunoblot analyses of pSer15-HspB1 (pHspB1) and calponin protein expression in myometrium are shown. Densitometric analyses of experimental data are shown below each set of appropriate representative blots. Values (means +/- SEM) plotted are from four independent experiments (n=4) and values were considered significantly different from one another when p < 0.05.

Figure 7. Immunofluorescence detection of pSer15-HspB1 in ovariectomized, non-pregnant rat myometrium was increased in laminaria-distended uterine horns. Laminaria tents (A) or polyethylene tubes of the same size and diameter as pre-expanded laminaria tents (B) were surgically inserted into one of the uterine horns of non-pregnant, ovariectomized rats. Samples were subsequently collected from empty, polyethylene tube-containing and laminaria-containing horns 24hrs post insertion. A) An increase in pSer15 HspB1 detection in the myometrium of laminaria-distended horns compared to detection in the myometrium of empty horns was observed, particularly in the longitudinal muscle layer. Localization of pSer15 HspB1 in the distended horn appeared to be more cytoplasmic and peri-nuclear as compared to the membrane-associated pattern of detection observed in the unilaterally pregnant rat model at d19 of gestation. B) pSer15 HspB1 detection was very low within circular and longitudinal muscle layers.
muscle layers and no observable changes were noted in the muscle layers from empty uterine horns compared to horns containing polyethylene tubes (plastic). Pre = pre-immune serum control, C= circular muscle layer, L= longitudinal muscle layer. Scale bar = 50μm.
Figure 1

A

\[
\begin{array}{c}
\text{d19} \\
\text{G} & \text{NG} \\
\text{HspB1} & \text{18S} \\
\end{array}
\]

B

\[
\begin{array}{c}
\text{d23} \\
\text{G} & \text{NG} \\
\text{HspB1} & \text{18S} \\
\end{array}
\]

Relative Optical Density

\(\text{HspB1:18S}\)

Gravid | Non-Gravid
---|---
\* |
Figure 2

A

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B

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<td>Calponin</td>
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Relative Optical Density (pSer15 HspB1 : Calponin)

Gravid    Non-Gravid

Gravid    Non-Gravid

*
Figure 5

A

Laminaria  Empty  Polyethylene Tube

B

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<tr>
<th></th>
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Relative Optical Density (HspB1:18S)

C

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Relative Optical Density (HspB1:18S)
Figure 6

A

pHspB1
Calponin

Relative Optical Density (pSer15 HspB1 : Calponin)

E  Lam

OV-Empty  OV-Laminaria

B

pHspB1
Calponin

Relative Optical Density (pSer15 HspB1 : Calponin)

E  P

OV-Empty  OV-Plastic