Expression of small heat shock-related protein 20 (HSP20) in rat myometrium is markedly decreased during late pregnancy and labour

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Abstract

The underlying mechanisms regulating uterine contractions during labour are still poorly understood. Heat shock protein 20 (HSP20) is known to be present at high levels in smooth muscle and implicated in muscle relaxation, but HSP20 expression in the myometrium is completely undetermined. Since HSP20 has been implicated in smooth muscle relaxation, we hypothesized that HSP20 would be highly expressed in the rat myometrium during early and mid-pregnancy when the myometrium is relatively quiescent. Northern blot analysis particularly demonstrated that HSP20 mRNA detection was significantly decreased from day (d) 22 of pregnancy to 1-day *post-partum* (PP) compared with d6 ($P < 0.05$). HSP20 mRNA detection was also significantly decreased from d22 to d23 of gestation compared with non-pregnant (NP) samples. Immunoblot analysis showed that detection of HSP20 was significantly decreased at d23 compared with d12 and d15 ($P < 0.05$). HSP20 detection also significantly decreased at PP compared with d15 ($P < 0.05$). Immunofluorescence analysis demonstrated that after d15, plasma membrane-associated localization of HSP20 decreased markedly in both circular and longitudinal muscle layers. In addition, HSP20 was detectable near cell membranes at much higher levels in the longitudinal muscle layer of progesterone-treated pregnant rats (delayed labour) at all gestational time points examined, compared with controls. Our results demonstrate that HSP20 mRNA and protein are highly expressed during early and mid-pregnancy and then the expression markedly decreases during late pregnancy and labour. The observed patterns of HSP20 expression are consistent with a potential role for HSP20 in facilitating myometrium quiescence during early and mid-pregnancy.

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Introduction

The underlying mechanisms regulating uterine contractions during pregnancy and labour are still poorly understood. However, it is clear that the initiation of parturition is controlled by the integration of endocrine and mechanical (uterine stretch) signals which originate within the foetal genome (Lye *et al.* 2001). The combination of these signalling pathways leads to phenotypic changes in the myometrium, resulting in myometrial activation. Endocrine signalling requires the activation of a cascade which involves the foetal hypothalamic–pituitary–adrenal axis. This endocrine cascade yields, in most species, an increase in circulating oestrogen levels and a decrease in circulating progesterone levels in the maternal plasma (Challis *et al.* 2002). Although important, complete activation would not occur with the influence of the endocrine pathway alone. Mechanical signals imparted by uterine stretch

(as a result of foetal growth during pregnancy) are also required for myometrial activation (Ou *et al.* 1997).

The result of activation is a switch from a muscle, which is quiescent during the majority of pregnancy, to one which is spontaneously active, excitable and highly responsive to uterotonic stimuli (Challis *et al.* 2002). This activation leads to an increase in the expression of a cassette of genes known collectively as contraction-associated proteins, which include ion channels, gap junction proteins and the oxytocin receptor (Challis *et al.* 2002).

The small heat shock proteins (sHSPs) are a family of proteins which range in mass from 12 to 43 kDa and exhibit chaperone activity, participating in the folding of proteins in both normal and extreme conditions such as heat shock, oxidation and exposure to toxins such as anti-cancer drugs (Gusev *et al.* 2002, 2005). These proteins also possess other common properties, including the presence of an

80-residue conserved α -crystallin domain, which spans two putative actin-binding domains (Gusev *et al.* 2005).

HSP20 is expressed practically in all tissues, but reaches a maximal level of 1.3% of total proteins in skeletal and smooth muscle (Bukach *et al.* 2003). This 20 kDa protein can be phosphorylated by both cAMP- and cGMP-dependent protein kinases (PKA/PKG) and increases in phosphorylation are associated with smooth muscle relaxation (Beall *et al.* 1999). Beall *et al.* (1999) have shown that the introduction of phosphopeptide analogues of HSP20 into bovine carotid artery smooth muscle inhibited agonist-induced muscle contractions. Upon phosphorylation, HSP20 has been shown to associate with actin (Rembold *et al.* 2000) and the actin-binding protein α -actinin (Tessier *et al.* 2003), which is located at focal adhesions in smooth muscle cells. Amino acid residues 110–121 of HSP20 bear sequence homology with the actin-binding region of both cardiac and skeletal troponin I and this region is necessary for binding of HSP20 to actin filaments (Rembold *et al.* 2000). This binding is believed to inhibit cross-bridge cycling (myosin–actin interactions), thus leading to relaxation of smooth muscle (Rembold *et al.* 2001). The HSP20 has been shown in both skeletal and smooth muscle to exist in macromolecular aggregates with HSP27, another sHSP that we have identified as a candidate contraction-associated protein in the myometrium (White *et al.* 2005). This interaction is thought to be a functional one, with HSP20 regulating the function of HSP27 (Brophy *et al.* 1999).

The apparent importance of HSP20 in smooth muscle contraction, coupled with its interaction with the potential contraction-associated protein HSP27, led us to the hypothesis that HSP20 would be expressed in the rat myometrium during early and mid-pregnancy, but that its expression would be down-regulated during the late stages of pregnancy, as the muscle becomes activated and the onset of labour approaches.

Materials and Methods

Animals

Sprague–Dawley rats were obtained from the Mount Scio Vivarium (Memorial University of Newfoundland, St John's, NL, Canada). Animals were held and cared for under standard environmental conditions (12 h light:12 h darkness) in the Animal Care Unit at the Health Sciences Centre, Memorial University of Newfoundland. Rats were fed LabDiet Prolab RMH 3000 (PMI Nutrition International, Brentwood, MO, USA) and water was available *ad libitum*. All experiments were approved by the institutional animal care committee under animal care protocols 02-02-DM to 06-02-DM. Virgin female rats (~220 g each) were mated with stud males, and observation of vaginal plugs in the following morning was designated day 1 of pregnancy. The time of delivery under these standard conditions was day 23 of gestation.

Experimental design

Normal pregnancy and term labour

Individual animals were each placed in an euthanasia chamber and exposed to an increasing concentration of carbon dioxide gas resulting in death within 5–10 min. Tissues were collected at the following time points: non-pregnant (NP), gestation days (d) 6, 12, 15, 17, 19, 21, 22, 23 (labour) and 1-day *post-partum* (PP). Labour samples were taken during active labour and only after the rat had delivered two to three pups.

Progesterone-delayed labour

The onset of labour is coupled with a withdrawal of the inhibitory effects of progesterone on the myometrium following a decline in plasma levels of this steroid. To determine whether progesterone withdrawal might modulate HSP20 expression, pregnant rats were given either a daily injection of progesterone (4 mg s.c. in 0.2 ml of corn oil) to maintain elevated plasma levels of this steroid, or vehicle alone (0.2 ml corn oil, s.c.) beginning on d20 of gestation. Individual animals were each placed in an euthanasia chamber and exposed to an increasing concentration of carbon dioxide gas resulting in death within 5–10 min. Samples were collected from vehicle control animals on d21, d22 and d23 (during delivery), while samples were collected from progesterone-treated rats on d21, d22, d23 and d24; however, on d23 and d24, the rats were not in labour.

Tissue collection

For northern and immunoblot analyses, uterine horns were removed, opened longitudinally and fetuses and placentae discarded. Uterine tissue was placed in ice-cold PBS (pH 7.4) and endometrial tissue was removed by gentle scraping with a scalpel blade. All myometrium samples were flash-frozen in liquid nitrogen and stored at -80°C . For immunocytochemistry, rat uterine tissue samples were fixed in 4% paraformaldehyde in PBS (pH 7.4) overnight at room temperature with shaking. The tissues were paraffin-embedded, sectioned and mounted on microscope slides by the Histology Unit of Memorial University of Newfoundland School of Medicine. Sections of rat uterine tissue contained both longitudinal and circular smooth muscle layers and sections were processed under identical conditions and at the same time in each experiment.

Northern blot analysis

RNA isolation

Northern blot analysis for both normal pregnancy and delayed labour regimes was performed on four separate, independent sets of RNA samples ($n=4$; i.e. four rats used per gestational time point). RNA was isolated from

tissues using TRIzol Reagent (Invitrogen Corporation) exactly according to the manufacturer's instructions. RNA quality and quantity ($A_{260/280}$) were determined using a Shimadzu Bio-Mini Spectrophotometer (Mandel Scientific, Guelph, ON, Canada) and samples were stored at -70°C .

Electrophoretic separation and capillary transfer of RNA

RNA samples (10 μg per lane) were loaded on a 1% agarose gel containing 0.66 M formaldehyde and $1\times\text{MOPS}$ buffer (0.02 M MOPS pH=7.0, 2 mM sodium acetate, 10 mM EDTA pH=8.0) and electrophoretically separated at 80 V in $1\times\text{MOPS}/0.22$ M formaldehyde running buffer. RNA was transferred overnight to a nylon membrane (Hybond-XL; Amersham Pharmacia Biotech) by upward capillary action using $2\times\text{SSC}$ (0.3 M sodium chloride, 0.03 M sodium citrate) in DEPC-treated ddH_2O . RNA was cross-linked to nylon membrane with a UVC-508 u.v. cross-linker (Ultra-Lum Inc., Paramount, CA, USA) and all blots were stored at -20°C until required.

Northern blot hybridization

Northern blot hybridization procedures have been described in detail elsewhere (White *et al.* 2005, Williams *et al.* 2005). Briefly, membranes were pre-hybridized in hybridization buffer, then hybridized overnight at 42°C in hybridization buffer containing a ^{32}P -labelled HSP20 cDNA probe. The 729 bp Bam HI/Xba I fragment of the rat HSP20 cDNA (Genbank accession no. NM_138887) was kindly provided, within the vector pRTL2, by Dr Colleen Brophy and Dr Charles Robb Flynn (Arizona State University, Tempe, AZ, USA). Radiolabelled cDNA probes were prepared with a Megaprime DNA Labelling Kit (Cat. No. RPN 1607; Amersham Biosciences) according to manufacturer's specifications. Following hybridization, blots were washed in $2\times\text{SSC}/0.1\%$ SDS and exposed to X-ray film (Hyperfilm MP; Amersham Pharmacia Biotech), with the exception of the delayed labour experiments where blots were exposed to multisensitive phosphor imaging screens (cyclone storage phosphor screen; Perkin Elmer, Boston, MA, USA). Multiple exposures were produced for each northern blot to ensure that the results were within the linear range of the film or screens.

Following the analysis of HSP20 gene expression, northern blots were stripped by incubating the membranes in a solution of 1 M Tris-Cl, 1 mM EDTA, $0.1\times\text{Denhardt's}$ solution in ddH_2O . Membranes were washed at room temperature with $0.1\times\text{SSPE}$ and then subsequently analysed for expression of 18S rRNA using the same procedures described previously. A 750 bp EcoRI fragment of the rabbit 18S ribosomal cDNA (Genbank Accession: X06778) was generously provided, within the vector pBluescript, by Dr I Skerjanc (University of Western Ontario, London, Ontario,

Canada; Petropoulos & Skerjanc 2002, Rogerson *et al.* 2002). 18S rRNA is constitutively expressed in rat myometrial cells and has been utilized as a loading control for analysis of myometrial gene expression (Oldenhof *et al.* 2002, Shynlova *et al.* 2004, White *et al.* 2005, Williams *et al.* 2005).

Immunoblot analysis

Immunoblot analysis for both normal pregnancy and delayed labour experiments was performed on four separate, independent sets of protein samples ($n=4$; i.e. four rats used per gestational time point) according to the method described by MacPhee & Lye (2000). Briefly, frozen rat myometrial samples were pulverized under liquid nitrogen and homogenized in RIPA lysis 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 100 μM Na_2VO_3 and Complete, Mini EDTA-free protease inhibitors (Roche Molecular Biochemicals). Samples were centrifuged at 15 000 g at 4°C for 15 min, and the supernatants were collected. Protein concentrations were determined by the Bradford assay (Bradford 1976) using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories) and protein samples (100 $\mu\text{g}/\text{lane}$) were separated by PAGE in 12% resolving gels according to the method described by Laemmli (1970). The gels were electroblotted to BioTrace NT nitrocellulose membranes (Pall Life Sciences, Pensacola, FL, USA).

Membranes were rinsed in Tris-buffered saline-Tween-20 (TBST; 20 mM Tris base, 137 mM NaCl and 0.1% Tween-20; pH 7.6) and blocked in 5% milk powder/TBST for 45 min. Unless otherwise stated, all incubations were performed at room temperature and with constant agitation. Rabbit polyclonal antisera raised against HSP20 (Cat. No. 07-490; Upstate Biotechnology, Lake Placid, NY, USA) or mouse monoclonal antisera raised against smooth muscle calponin (Cat. No. C2687; clone hCP; Sigma-Aldrich) used at dilutions of 1:2000 and 1:50 000 respectively were incubated with the blots for 1 h and then they were rinsed with TBST. 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) were used as secondary antisera at dilutions of 1:10 000 and 1:150 000 respectively. Proteins were detected using the Pierce SuperSignal West Pico chemiluminescent substrate detection system (MJS Biolynx, Inc., Brockville, Ontario, Canada) and multiple exposures were produced to ensure the linearity of the film exposures.

Following the detection of HSP20, all blots were stripped with Restore western blot stripping solution (Pierce) according to the manufacturer's instructions. Analysis of calponin protein expression was subsequently performed. We have determined that calponin

protein is constitutively expressed in non-pregnant and pregnant rat myometrial tissue under our protein extraction conditions (i.e. RIPA buffer extraction protocol) and, as a result, serves as a sufficient loading control for our analyses (White *et al.* 2005, Williams *et al.* 2005).

Immunocytochemistry

Two separate, independently collected sets of rat tissues ($n=2$; i.e. 2 rats used per gestational time point) were utilized for immunocytochemistry experiments and the experiments were repeated thrice. Following de-waxing and rehydration, tissue sections were permeabilized with 0.125% trypsin in PBS for 10 min at room temperature and then rinsed with PBS. Unless otherwise noted, all incubations were performed at room temperature and with constant agitation. Tissue sections were blocked with 5% normal goat serum/1% horse serum in PBS for 30 min. The sections were then incubated overnight with constant agitation at 4 °C in rabbit anti-HSP20 (Cat. No. H1830-48D; US Biological, Swampscott, MA, USA) at a dilution of 1:200 in blocking solution or rabbit IgG (Cat. No. 011-000-003; Jackson ImmunoResearch Labs, Inc., West Grove, PA, USA) at the same final concentration to serve as a negative control. Tissue sections were washed in PBS and then incubated in fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG (Cat. No. F7512; Sigma-Aldrich) at a dilution of 1:200 in blocking solution for 30 min. For nuclear staining, TO-PRO-3 iodide (Cat. No. T3605; Molecular Probes, Eugene, OR, USA) was added at a dilution of 1:250 to the secondary antibody solution. The sections were washed with cold PBS containing 0.02% Tween-20, followed by mounting in Vectashield (Vector Laboratories Inc., Burlington, Ontario, Canada). Prepared slides were observed and images were collected with an Olympus Fluoview laser scanning confocal microscope (Olympus Optical Company Ltd, Melville, NY, USA). For each immunocytochemistry experiment (i.e. normal pregnancy and term labour, progesterone-delayed labour) image collection parameters (PMT (photomultiplier tube), gain and black level settings) were the same within each muscle layer.

Data analysis

Densitometric analysis of northern and immunoblots was performed with the aid of Scion Image software (Scion Image Corporation, Frederick, MD, USA). Densitometric measurements of HSP20 mRNA were normalized to those of 18S rRNA while measurements of HSP20 protein on immunoblots were normalized to those of calponin. Statistical analysis was performed with GraphPad InStat version 3.0 (GraphPad Software, San Diego, CA, USA, www.graphpad.com) and data graphed using GraphPad Prism version 4.0 (GraphPad Software). Data from northern and immunoblot analyses of HSP20 expression

during normal gestation were subjected to a one-way ANOVA and a Tukey–Kramer multiple comparisons test. Data from analysis of HSP20 expression during progesterone-delayed labour were subjected to a two-way ANOVA and a Bonferroni post-test. Values were considered significantly different if $P<0.05$.

Results

Normal pregnancy and labour

Expression of HSP20 mRNA and protein

To characterize the levels of HSP20 mRNA within myometrial samples, northern blots of myometrial total RNA from NP, d6, d12, d15, d17, d19, d21, d22, d23 and PP were analysed with radiolabelled probes generated from a rat HSP20-specific cDNA (Fig. 1A). Our experiments demonstrated that HSP20 mRNA detection significantly decreased during late gestation

Figure 1 Northern blot analysis of HSP20 mRNA detection in rat myometrium during pregnancy, labour and 1-day *post-partum*. A: Representative northern blots of HSP20 mRNA detection and 18S rRNA detection. Analysis was performed using an HSP20-specific rat cDNA and an 18S-specific rabbit cDNA as templates for radiolabelled probe production. Ten micrograms of total RNA were utilized per lane. B: Densitometric analysis illustrating the decrease in HSP20 mRNA detection during pregnancy and labour. Detection levels on d22, d23 were significantly decreased ($*P<0.05$) when compared with NP and values on d22 to PP were significantly decreased ($^{\dagger}P<0.05$) when compared with d6. Values are from four independent experiments ($n=4$) $\pm$ S.E.M. Days 6, 12, 15, 17, 19, 21, 22 and 23 represent gestational time points. NP, nonpregnant; PP, 1-day *post-partum*.

(one-way ANOVA, $P < 0.05$; $n = 4$). Specifically, HSP20 was highly detectable in NP and d6 samples, then detection appeared to decrease by d12. HSP20 mRNA detection was significantly decreased at d22 and d23 when compared with NP samples and between d22 and PP, inclusive, when compared with d6 samples (Tukey–Kramer post-test, $P < 0.05$; Fig. 1A and B).

Immunoblot analysis using HSP20-specific antisera demonstrated that HSP20 detection decreased significantly during late gestation (one-way ANOVA, $P < 0.05$; $n = 4$). Protein detection levels remained high until d15 of gestation and then began to decrease thereafter. Detection levels at d23 were significantly lower than those at d12 and d15, while PP levels were significantly decreased compared with d15 samples (Tukey–Kramer post-test, $P < 0.05$; Fig. 2A and B).

Immunocytochemical detection of HSP20

Detection of HSP20 in the longitudinal muscle layer demonstrated that HSP20 was primarily localized at or near cell membranes from NP and throughout gestation (Figs 3 and 4). The level of detection gradually decreased

Figure 2 Immunoblot analysis of HSP20 protein detection in rat myometrium during pregnancy, labour and 1-day post-partum. A: Representative immunoblots of HSP20 protein and calponin detection. B: Densitometric analysis illustrating the decrease in HSP20 protein detection during pregnancy and labour. Values on d23 were significantly decreased ($*P < 0.05$) when compared with d12 and values on d23, PP were significantly decreased ($†P < 0.05$) when compared with d15. Values are from four independent experiments ($n = 4$) $\pm$ s.e.m. Days 6, 12, 15, 17, 19, 21, 22 and 23 represent gestational time points. NP, nonpregnant; PP, 1-day post-partum.

Figure 3 Immunocytochemical analysis of HSP20 protein detection in the longitudinal smooth muscle layer of rat myometrium between NP and d12 of pregnancy using polyclonal rabbit anti-HSP20 antisera. These images demonstrate the observed localization of HSP20 (green) at or near the cell membrane. TO-PRO-3 iodide was used for nuclear staining (red). Cont, control, rabbit IgG. Scale bar = 50 μ m.

throughout gestation, reaching its lowest detection level in the PP samples; however, detection was lower on d12 than at the adjacent d6 and d15 time points. A similar pattern of down-regulation of HSP20 detection was seen in the circular muscle layer (Figs 5 and 6). Although HSP20 appeared to be localized at or near cell membranes, there was also evidence of HSP20 detection in the cytoplasm.

Progesterone-induced delayed labour

Expression of HSP20 mRNA and protein

Throughout the majority of pregnancy, circulating levels of progesterone in the rat are high, reaching peak levels between d15 and d19 then declining thereafter including at labour (Lye *et al.* 1993). The finding that HSP20 expression also decreased significantly during late gestation and labour suggested that the decline in progesterone levels and its subsequent effects on the myometrium might be responsible, at least in part, for our results. Animals treated with progesterone beginning on d20 of gestation did not enter labour on d23. Our experiments demonstrated that, at each time point, there were no significant differences in HSP20 mRNA detection levels between the progesterone- and vehicle-treated animals (two-way ANOVA, $P > 0.05$; $n = 3$; Fig. 7). Immunoblot analysis also demonstrated that there were no significant differences in HSP20 protein detection levels (two-way ANOVA, $P > 0.05$; $n = 4$; Fig. 8).

Figure 4 Immunocytochemical analysis of HSP20 protein detection in the longitudinal smooth muscle layer of rat myometrium between d15 of pregnancy and PP using polyclonal rabbit anti-HSP20 antisera (green). TO-PRO-3 iodide was used for nuclear staining (red). Scale bar = 50 μ m.

Immunocytochemical detection of HSP20

There was a striking difference in detection levels between progesterone-treated and vehicle control tissue samples in the longitudinal muscle layer (Fig. 9). HSP20 detection in the progesterone-treated samples was markedly higher than that of the vehicle controls at all examined gestational time points. In both cases, HSP20 was also localized at or near cell membranes. In the circular muscle layer, no marked differences in HSP20 detection levels or spatial

Figure 5 Immunocytochemical analysis of HSP20 protein detection in the circular smooth muscle layer of rat myometrium between NP and d12 of pregnancy using polyclonal rabbit anti-HSP20 antisera (green). TO-PRO-3 iodide was used for nuclear staining (red). Scale bar = 50 μ m.

localization were observed between the progesterone-treated and vehicle control samples (Fig. 10).

Discussion

We recently reported that HSP27 was highly expressed in the rat myometrium during pregnancy and postulated that it could be a contraction-associated protein (White *et al.* 2005). Until now the expression of no other sHSP had been reported in the rat myometrium during pregnancy. HSP20, highly expressed in smooth muscle (Bukach *et al.* 2003), may have a role in smooth muscle relaxation (Beall *et al.* 1999), and can exist in macromolecular aggregates with HSP27 (Brophy *et al.* 1999). Therefore, we hypothesized that HSP20 would be expressed in the rat myometrium during early and mid-pregnancy, but that its expression would be down-regulated during the late stages of pregnancy, as the muscle became activated and the onset of labour approached.

Normal pregnancy and labour

Our northern blot analysis showed that the detection of HSP20 mRNA decreased significantly from d22 to PP, while detection of HSP20 protein on immunoblots showed a significant decrease by labour. Thus, the observed decrease in HSP20 protein expression could be the result of decreased HSP20 mRNA available for subsequent translation. Together with our results from immunofluorescence experiments demonstrating a marked reduction in HSP20 immunostaining after d15,

Figure 6 Immunocytochemical analysis of HSP20 protein detection in the circular smooth muscle layer of rat myometrium between d15 of pregnancy and PP using polyclonal rabbit anti-HSP20 antisera (green). TO-PRO-3 iodide was used for nuclear staining (red). Scale bar = 50 μ m.

the observed patterns of HSP20 expression during pregnancy and labour potentially support a role for HSP20 in regulating myometrial quiescence during early and mid-pregnancy. Previous research in smooth muscle has demonstrated an association between HSP20 and actin, which has been suggested to lead to actin depolymerization and inhibition of contraction (Beall *et al.* 1999, Tessier *et al.* 2004, Dreiza *et al.* 2005). A decrease in HSP20 expression late in gestation could also lead to a decrease in the association of HSP20 with actin thereby relieving, at least in part, contractile

Figure 7 Northern blot analysis of HSP20 mRNA detection in a delayed-labour model following administration of progesterone or corn oil (vehicle control) to pregnant rats. A: Representative northern blots of HSP20 mRNA detection and 18S rRNA detection. B: Densitometric analysis illustrating no significant change in the level of detection between treatment groups. Values are from three independent experiments ($n=3$) $\pm$ S.E.M. P4, progesterone. Designations 21-Oil, 22-Oil, 23-Oil, 21-P4, 22-P4, 23-P4, 24-P4 represent gestational time points in the two treatment groups.

inhibition of the myometrium. Significant rat uterine contractile activity only becomes evident within the last 24 h prior to labour (Buhimschi & Garfield 1996, Buhimschi *et al.* 1998).

Immunocytochemistry experiments revealed that HSP20 was primarily localized at or near cell membranes. HSP20 has been shown to be associated with the actin-binding protein α -actinin, which is a known component of focal adhesions (or dense plaques) in smooth muscle cells (Tessier *et al.* 2003). Furthermore, the spatial immunolocalization of HSP20 correlates, at least in part, with Serine 15 (Ser15) phosphorylated HSP27 expression, as Ser15- phosphorylated HSP27 was also localized to cell membranes prior to labour (White *et al.* 2005). The significance of this finding is not yet clear, but Fuchs *et al.* (2000) has demonstrated that phosphorylated HSP27 can inhibit phosphorylation of HSP20 leading to reduction of vascular smooth muscle relaxation. In total, our findings are consistent with a report that HSP20 and HSP27 proteins may exist in aggregates in smooth muscle (Brophy *et al.*

Figure 8 Immunoblot analysis of HSP20 protein detection in a delayed-labour model following administration of progesterone or corn oil (vehicle control) to pregnant rats. A: Representative immunoblots of HSP20 and calponin detection. B: Densitometric analysis illustrating no significant change in the level of detection between treatment groups. Values are from four independent experiments ($n=4$) $\pm$ S.E.M. P4, progesterone. Designations 21-Oil, 22-Oil, 23-Oil, 21-P4, 22-P4, 23-P4, 24-P4 represent gestational time points in the two treatment groups.

1999) and our future work will examine the phosphorylation state of HSP20 in the myometrium during gestation and the interdependence of HSP20 and HSP27 in uterine smooth muscle function.

Physiological growth of the pregnant uterus occurs by two mechanisms: an increase in cell number (hyperplasia) followed by an increase in cell size (hypertrophy; Shynlova *et al.* 2006). The transition between these two phases of growth is associated with a transient activation of the caspase cascade, at $\sim$ d12–d14 of gestation, which initiates the differentiation of uterine smooth muscle but does not lead to the biochemical or morphological features of apoptosis (Shynlova *et al.* 2006). Interestingly, within relatively the same period, we observed a transient but marked decrease in immunodetection of HSP20 in both muscle layers by d12 of gestation followed by an increase in detection by d15. We believe these results may be related to the initiation of uterine smooth muscle differentiation. The subsequent increase in HSP20 detection by d15 in both muscle layers could be a reflection of a protective mechanism against cell death since HSP20 has

Figure 9 Immunocytochemical analysis of HSP20 protein (green) detection in the longitudinal smooth muscle layer of rat myometrium in a delayed-labour model following administration of progesterone (4 mg in 0.2 ml corn oil, s.c.) or oil (vehicle control; 0.2 ml corn oil, s.c.). These images illustrate the observed maintenance of HSP20 detection in the progesterone treatment samples. P, progesterone; O, oil; Cont, control, rabbit IgG. Days 21-O, 22-O, 23-O, 21-P, 22-P, 23-P, 24-P represent gestational time points. TO-PRO-3 iodide was used for nuclear staining (red). Scale bar = 50 μ m.

previously been shown to protect against apoptosis in cardiomyocytes (Fan *et al.* 2004).

Progesterone-induced delayed labour

Circulating levels of progesterone in the rat peak at d19 of gestation declining thereafter including at labour

Figure 10 Immunocytochemical analysis of HSP20 protein (green) detection in the circular smooth muscle layer of rat myometrium in a delayed-labour model following administration of progesterone (4 mg in 0.2 ml corn oil, s.c.) or oil (vehicle control; 0.2 ml corn oil, s.c.). P, progesterone; O, oil; Cont, control, rabbit IgG. Days 21-O, 22-O, 23-O, 21-P, 22-P, 23-P, 24-P represent gestational time points. TO-PRO-3 iodide was used for nuclear staining (red). Scale bar = 50 μ m.

(Lye *et al.* 1993); thus, the observed drop in HSP20 expression late in pregnancy could be related to the decline in circulating levels of this steroid. Evidence suggests that expression of sHSPs can be regulated by both progesterone and oestrogen (Ciocca *et al.* 1993, Tabibzadeh *et al.* 1996, Bany & Schultz 2001, Kato *et al.* 2002). Progesterone administration to pregnant rats did

not result in significant induction of HSP20 mRNA and protein expression compared with vehicle controls as measured by northern and immunoblot analysis respectively. However, immunocytochemical analysis demonstrated a maintenance of HSP20 expression at or near cell membranes from d21–d24 in the longitudinal muscle layer of progesterone-treated rats compared with vehicle controls suggesting a regional sensitivity of HSP20 expression to progesterone. It has been reported that the longitudinal and circular muscle layers exhibit different contractile and physiological characteristics (Osa & Katase 1975, Kawarabayashi & Osa 1976, Chow & Marshall 1981, Mlynarczyk *et al.* 2003); however, it is important to note that we demonstrated similar HSP20 immunolocalization patterns in the two muscle layers across normal gestation and particularly during early to mid-pregnancy when circulating levels of progesterone are high (Lye *et al.* 1993). Thus, the reason(s) for increased HSP20 expression from d21 to d24 in the longitudinal muscle layer during progesterone-induced delayed labour is unknown and requires future clarification. In total, our findings indicate that progesterone regulation of HSP20 expression, if any, may be quite complex. Future work will attempt to more specifically determine the role of both progesterone and oestrogen in the regulation of HSP20 gene expression.

HSP20 expression and relevance to initiation of labour

A recent study by Girotti & Zingg (2003) using DNA microarrays of rat uterus has found that as the uterus progresses toward labour, the degree of gene suppression is greater than the degree of gene activation. This finding suggests that for the initiation of labour, the suppression of genes related to the control of quiescence may be equally as important as the up-regulation of the contraction-associated proteins. Our work has identified that HSP20 expression decreases as the uterus progresses toward labour. Although it is not yet possible to determine the specific functional role(s) of HSP20 in the rat myometrium during pregnancy and labour, our results, coupled with documented evidence of a role for HSP20 in smooth muscle relaxation, implicate a role for HSP20 in the regulation of myometrial quiescence. Subsequent down-regulation of HSP20 expression prior to and during labour may also be important for the initiation of parturition.

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