

Small heat shock protein 27 (Hsp27) expression is highly induced in rat myometrium during late pregnancy and labour

B G White, S J Williams, K Highmore and D J MacPhee

Division of Basic Medical Sciences, Health Sciences Centre, Faculty of Medicine, Memorial University of Newfoundland, St John's, Newfoundland and Labrador, Canada A1B 3V6

Correspondence should be addressed to D MacPhee; Email: dmacphee@mun.ca

Abstract

The underlying mechanisms that regulate uterine contractions during labour are still poorly understood. A candidate regulatory protein is heat shock protein 27 (Hsp27). It belongs to the small heat shock protein family and can regulate actin cytoskeleton dynamics, act as a chaperone, and may regulate contractile protein activation. As a result, we hypothesized that Hsp27 expression would be highly induced during late pregnancy and labour. Hsp27 mRNA expression was significantly elevated ($P < 0.05$) on days 17 to 22 of gestation. In addition, immunoblot analysis demonstrated that detection of total Hsp27 increased ($P < 0.05$) between day 21 and 1 day post-partum (PP) inclusive. Since phosphorylation of Hsp27 has been reported to be a prerequisite for smooth muscle contraction, we examined the temporal and spatial expression of Ser-15 phosphorylated Hsp27. Immunoblot analysis showed that the detection of Ser-15 phosphorylated Hsp27 significantly increased ($P < 0.05$) between days 19 and 23 (active labour) inclusive, in parallel with detection of total Hsp27. Immunocytochemical analysis of Ser-15 phosphorylated Hsp27 expression *in situ* demonstrated that phosphorylated Hsp27 in circular muscle became detectable in peri-nuclear and membrane regions on days 19 to 22, but was primarily restricted to the cytoplasm on days 23 to PP. In contrast, phosphorylated Hsp27 in longitudinal muscle was primarily detected in myocyte membranes on days 15 to 22, and then also became detectable in the cytoplasm of myocytes on days 23 and PP. Our results demonstrate that Hsp27 expression is highly upregulated during late pregnancy and labour and suggest that Hsp27 is a potential candidate contraction-associated protein.

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Introduction

The underlying mechanisms that induce or regulate uterine contractions during labour are still poorly understood; however, it is clear that the fetal genome contributes signals that facilitate the initiation of parturition (Challis *et al.* 2000, 2002). These signals ultimately lead to phenotypic changes in the myometrium, from a muscle which during pregnancy is quiescent to one which is spontaneously active, excitable, and responsive to uterine agonists and characteristically exhibits a high degree of cell–cell coupling. This myometrial activation results from the increased expression of a group of genes that encode contraction-associated proteins such as ion channels, agonist receptors and gap junctions (Challis *et al.* 2002).

Activation requires the triggering of two separate, but interdependent signalling pathways in the fetus (Challis *et al.* 2002). The first requires activation of an endocrine cascade involving the fetal hypothalamic–pituitary–adrenal–placental axis. In most animals, the yield of this axis at term results in a decreased influence of progesterone on

the myometrium. While important, this endocrine pathway is not sufficient for myometrial activation, and work by Ou *et al.* (1997) has demonstrated that mechanical distension (or stretch) within the myometrium (as a result of fetal growth during pregnancy) is also required. Ultimately, the endocrine and mechanical signalling pathways may potentially be integrated at focal adhesion sites (i.e. smooth muscle dense plaques) by integrin-mediated signal transduction via focal adhesion kinase (FAK) and downstream effectors (MacPhee & Lye 2000).

The family of mammalian small heat shock proteins (sHSP) comprises seven members: α A and α B-crystallin, Hsp27, Hsp20, Myotonic dystrophy protein kinase binding protein (MKBP), Hsp27L and cardiovascular (cv) Hsp with molecular masses of 15–30 kDa (Kato *et al.* 2002). All members of this class share a structural domain named the α -crystallin domain in their carboxyl terminal halves that also spans two putative actin binding domains (Quinlan 2002). Hsp27, in particular, has been demonstrated to play an important role in actin polymerization, remodelling and even cross-bridge cycling in smooth

muscle cells (Miron *et al.* 1991, Lavoie *et al.* 1993a,b, Benndorf *et al.* 1994, Ibitayo *et al.* 1999, Gerthoffer & Gunst 2001).

Phosphorylation is one of the most prominent modifications that occurs in sHSP and is considered to be very important for regulation of sHSP structure and function (Gaestel 2002). It is known to be one of the earliest events induced by a stress such as heat shock, oxidative stress, and stimuli such as cytokines and growth factors (Landry *et al.* 1992). Two phosphorylation sites are known to exist for Hsp27 in rodents, Ser-15 and Ser-86 (homologous to Ser-82 in humans and Ser-90 in hamsters). The latter site has been demonstrated to be the major phosphorylation site for this protein and is necessary for the dissociation of large sHSP multimers, although at the cellular level it is not always sufficient (Lambert *et al.* 1999, Gaestel 2002, Kato *et al.* 2002). In contrast, phosphorylation of Hsp27 on Ser-15 is proposed to produce a conformational change in Hsp27 that aids the direct binding of Hsp27 with actin filaments (Lambert *et al.* 1999, Gerthoffer & Gunst 2001, Mounier & Arrigo 2002). Recent work by Bitar (2002) has demonstrated that agonist-induced phosphorylation of Hsp27 promotes the association of actin and myosin, likely through association with tropomyosin. Therefore, phosphorylation of Hsp27 could be a prerequisite for smooth muscle contraction (Gerthoffer & Gunst 2001).

Small heat shock proteins have been detected in rat and mouse uterine tissue during implantation where they are thought to play a role in decidualization (Tabanelli *et al.* 1992, Bany & Schultz 2001). In addition, Hsp27 has been examined in human endometrial stromal cells during *in vivo* and *in vitro* decidualization (Tang *et al.* 1993, Ciocca *et al.* 1996, Shah *et al.* 1998). Yet, there is a complete lack of information regarding the expression of sHSP in the myometrium during late pregnancy and labour. Since Hsp27 appears to be important in smooth muscle contraction, we hypothesized that Hsp27 expression would be highly induced during late pregnancy and labour in the rat.

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 protocol 02-02-DM. For our studies, virgin female rats (~220 g each) were mated with stud males and the observation of vaginal plugs the following morning was designated day 1

of pregnancy. The time of delivery under these standard conditions was day 23 of gestation.

Tissue collection

All animals were killed by carbon dioxide inhalation on the desired day of sampling and pregnancy (e.g. non-pregnant (NP), day (d) 6, 12, 15, 17, 19, 21, 22, 23 (labour), 1 day post-partum (PP)). Samples on d23 were taken during active labour and only after the rat had delivered 2–3 pups. Uterine horns were removed, opened longitudinally, and fetuses and placentae discarded. Uterine tissue was placed in ice cold phosphate-buffered saline (PBS; pH 7.4) and endometrial tissue was removed by gentle scraping with a scalpel blade. Liver and heart tissues were taken from non-pregnant rats for use as controls. All samples were flash-frozen in liquid nitrogen and stored at -80°C .

Northern blot analysis

RNA isolation

Northern blot analysis was performed on four separate, independent sets of RNA samples ($n = 4$, i.e. four rats used per gestational timepoint). RNA was isolated from tissues using TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA, USA) exactly according to the manufacturer's instructions. RNA quality and quantity (A260/280) were determined using a Shimadzu Bio-Mini Spectrophotometer (Mandel Scientific, Guelph, Ontario, Canada) and samples were stored at -70°C .

Electrophoretic separation and capillary transfer of RNA

RNA samples were prepared by precipitating RNA with two volumes of 100% ethanol/2% potassium acetate and resuspending pellets in sample buffer (50% formamide, 0.066 M formaldehyde, and $1 \times$ MOPS buffer in diethyl pyrocarbonate (DEPC)-treated double distilled (dd) H_2O). Samples were incubated for 30 min at 65°C and quick cooled on ice for 10 min. RNA samples (10 μg per lane) were loaded on a 1% agarose gel containing 0.66 M formaldehyde and $1 \times$ 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 $1 \times$ MOPS/0.22 M formaldehyde running buffer. RNA was transferred overnight to a nylon membrane (Hybond-XL; Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) by upward capillary action using $2 \times$ SSC (0.3 M sodium chloride, 0.03 M sodium citrate) in DEPC-treated dd H_2O . RNA was crosslinked to nylon membrane with a UVC-508 ultraviolet crosslinker (Ultra-Lum Inc., Paramount, CA, USA) and all blots were stored at -20°C until required.

Northern blot hybridization

Membranes were pre-hybridized in hybridization buffer consisting of 50% formamide, $5 \times$ sodium chloride-sodium phosphate EDTA (SSPE; 0.75 M sodium chloride,

0.05 M sodium phosphate, 0.005 M EDTA), 1% SDS, 5 × Denhardt's solution, and 0.1 mg/ml herring sperm DNA for 1–2 h at 42 °C in a hybridization oven (Hybaid Instruments, Franklin, MA, USA). Hamster Hsp27 cDNA (Genbank Accession No. X51747) was a kind gift from Dr J Landry (Laval University, Quebec, Canada). Radiolabelled cDNA probes were prepared with a Megaprime DNA Labelling kit (Amersham Biosciences, Little Chalfont, Bucks, UK) exactly according to the manufacturer's specifications. Hybridization was performed overnight at 42 °C, then blots were washed 5 × for 5 min at 42 °C in 2 × SSC/0.1% SDS and exposed to X-ray film (Hyperfilm MP; Amersham Pharmacia Biotech). Multiple exposures were produced for each Northern blot to ensure the results were within the linear range of the film.

Following analysis of Hsp27 gene expression, Northern blots were stripped by incubating membranes for 2 h at 75 °C in a solution of 1 M Tris-Cl, 1 mM EDTA, 0.1 × Denhardt's solution in ddH₂O. Membranes were washed at room temperature with 0.1 × SSPE and then subsequently analyzed for expression of 18S rRNA using the same procedures described above and a rabbit 18S ribosomal cDNA template generously provided by Dr I Skerjanc (University of Western Ontario, London, Ontario, Canada). 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 (Mitchell & Lye 2002, Oldenhof *et al.* 2002, Shynlova *et al.* 2004).

Immunoblot analysis

Immunoblot analysis was performed on three separate, independent sets of protein samples ($n = 3$, i.e. three rats used per gestational timepoint) according to MacPhee and 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% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS) containing 100 µM Na₂VO₃ and COMPLETE, Mini EDTA-free protease inhibitors (Roche Molecular Biochemicals, Laval, Quebec, Canada). Protein concentrations were determined by the Bradford assay (Bradford 1976) using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Mississauga, Ontario, Canada), and protein samples (50 µg/lane) were separated by polyacrylamide gel electrophoresis in 9% resolving gels according to the method of Laemmli (1970). Gels were electroblotted to Pierce 0.45 µm nitrocellulose membranes (MJS BioLynx, Inc., Brockville, Ontario, Canada).

Proteins were detected using the Pierce SuperSignal West Pico chemiluminescent substrate detection system (MJS BioLynx, Inc., Brockville, Ontario, Canada) according to the manufacturer's instructions. Rabbit polyclonal antisera (S15) raised against serine-15 (Ser-15) phosphorylated Hsp27, rabbit polyclonal antisera (CT) raised against the c-terminus of Hsp27, or mouse monoclonal anti-smooth muscle calponin (clone hCP; Sigma-Aldrich

Canada, Oakville, Ontario, Canada) were used as primary antisera at dilutions of 1:2000, 1:2000 or 1:50 000 respectively. Horse-radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H + L) (Pierce, Rockford, IL, USA) or HRP-conjugated goat anti-mouse IgG (H + L) (Pierce) were used as secondary antisera at dilutions of 1:25 000 and 1:150 000 respectively. Multiple exposures were generated to ensure the linearity of the film exposures.

Following detection of Ser-15 phosphorylated Hsp27, all blots were stripped with Restore J Western blot stripping solution (Pierce) according to the manufacturer's instructions. Analysis of calponin protein expression was subsequently performed, followed by stripping and analysis of total Hsp27 protein expression. 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.

Immunocytochemistry

Two separate, independently collected sets of rat tissues ($n = 2$, i.e. two rats used per gestational timepoint) were utilized for immunocytochemistry experiments and the experiments were repeated three times. Rat uterine tissue samples were fixed in 4% paraformaldehyde in PBS (pH 7.4) overnight at room temperature with shaking. 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.

Following de-waxing and rehydration, tissue sections were permeabilized with 0.125% trypsin in PBS for 10 min at room temperature. Tissue sections were blocked with 5% normal goat serum/1% horse serum and then incubated with rabbit anti-Hsp27 primary antisera (SRQL or S15) at dilutions of 1:200. To serve as negative controls for Hsp27 detection, separate tissue sections from the same gestational timepoints were incubated with pre-immune sera at the same concentration as the Hsp27-specific antisera. Tissue sections were incubated with FITC-conjugated sheep anti-rabbit IgG (Sigma-Aldrich Canada) at dilutions of 1:200 and then washed twice with cold 0.02% Tween-20 in PBS, 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).

Hsp27 antisera

Three Hsp27-specific antisera were employed for our experiments. A novel rabbit polyclonal antisera, designated SRQL for our experiments, was obtained from

Affinity Bioreagents (Golden, CO, USA) and utilized exclusively for immunofluorescent detection of total pools of Hsp27. It was raised against a human Hsp27 sequence encompassing amino acids 73–83 (Genbank Accession No. AB020027; YSRALSRQLSS). The targeted human sequence is 82% identical with the rat sequence (Genbank Accession No. M86389; FSRALNRQLSS) corresponding to amino acids 77–87. A rabbit polyclonal phosphorylation state-specific antisera, termed S15 for our experiments, was obtained from Affinity Bioreagents (Cat. no. PA1-018) and utilized for immunofluorescent detection and immunoblot analysis of Ser-15 phosphorylated Hsp27. It was raised against a phosphorylated peptide encompassing amino acids 10–20 (Genbank Accession No. AB020027; LLRGP(pS)WDPFR) of the human sequence. The targeted human sequence is 82% identical with the corresponding rat sequence (Genbank Accession No. M86389; LLRSP(pS)WEPFR). Lastly, a rabbit polyclonal antisera, designated CT, was obtained from Upstate Biotechnology (Lake Placid, NY, USA; Cat. No. 06-517) and utilized for immunoblot analysis of total pools of Hsp27 (Lavoie *et al.* 1995, Lambert *et al.* 1999). It was raised against a C-terminal sequence of hamster Hsp27 encompassing amino acids 204–213 (Genbank Accession No. X51747; AGKSEQSGAK) and is specific for rodent Hsp27. The targeted hamster sequence is 70% identical to the corresponding rat sequence of amino acids 196–205 (Genbank Accession No. M86389; GPESEQSGAK).

Data analysis

Densitometric analysis was performed with the aid of Scion Image software (Scion Image Corporation, Frederick, MD, USA). Densitometric measurements of Hsp27 mRNA were normalized to those of 18S ribosomal RNA while measurements of Hsp27 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 were subjected to a one-way analysis of variance and a Tukey-Kramer multiple comparisons test. Values were considered significantly different if $P < 0.05$.

Results

Analysis of Hsp27 expression

To characterize Hsp27 mRNA expression within myometrial samples, Northern blots of rat myometrial total RNA from non-pregnant (NP), days (d) 6, 12, 15, 17, 19, 21, 22, 23 (labour) of pregnancy, 1 day post-partum (PP), and heart and liver controls were analyzed with radiolabelled probes generated from a hamster Hsp27-specific cDNA (Fig. 1A). In agreement with a previous report (Lu & Das 1993), Hsp27 mRNA was detected in heart tissue, but very low expression was detected in liver tissue under our assay conditions. Our experiments demonstrated that

Hsp27 mRNA expression significantly increased during late pregnancy (one-way ANOVA, $P < 0.0001$; $n = 4$). Specifically, Hsp27 mRNA expression was barely detectable from NP to d12 and then began to increase by d15 of pregnancy. Hsp27 mRNA expression then became significantly elevated (Tukey-Kramer post test, $P < 0.05$) between d17 and d22 inclusive, compared with the NP, d6, d12, d23 and PP timepoints (Fig. 1A,B).

Immunoblot analysis utilizing the Hsp27 CT antisera demonstrated that detection of total Hsp27 increased significantly during late pregnancy, labour and PP (one-way ANOVA, $P < 0.0001$; $n = 3$). Specifically, Hsp27 protein was virtually undetectable from NP to d12 and then began to accumulate by d15. Detection of Hsp27 between d21 and PP, inclusive, then significantly increased over the NP, d6 and d12 timepoints (Tukey-Kramer post test, $P < 0.05$; $n = 3$; Fig. 2A,B). In addition, detection of Hsp27 on d23 and PP was also significantly elevated over d15 ($P < 0.05$; $n = 3$). Immunoblot analysis of Ser-15 phosphorylated Hsp27, utilizing the S15 antiserum, demonstrated that significantly increased detection of phosphorylated Hsp27 during late pregnancy and labour (one-way ANOVA, $P < 0.0001$; $n = 3$) paralleled the increased detection of total Hsp27. In particular, phosphorylated Hsp27 became detectable by d15, and between d19 and d23 inclusive, detection of phosphorylated Hsp27 significantly increased (Tukey-Kramer post test, $P < 0.05$; $n = 3$) over the NP, d6, d12, and d15 timepoints (Fig. 2A,C). By PP, and in contrast to total Hsp27, detection of phosphorylated Hsp27 was markedly decreased.

Immunocytochemical detection of Hsp27

Localization in the circular muscle layer

Hsp27 was virtually undetectable on d15 and d17 of gestation by immunocytochemical analysis with the SRQL antisera. In contrast, Hsp27 was readily detectable on days 19 to 22, followed by a decrease in detection on days 23 and PP that approached the background levels observed in pre-immune controls (Fig. 3). Spatially, Hsp27 was localized to the cytoplasm in peri-nuclear regions on d19 to d22, likely representing newly synthesized Hsp27, but also became localized to myocyte membranes on d22.

Phosphorylated Hsp27 was also undetectable on d15 and d17 of gestation but became more readily detectable thereafter. On d19 to d22, phosphorylated Hsp27 was localized in peri-nuclear and membrane regions; however, in contrast to results with the SRQL antisera phosphorylated Hsp27 was also detected on d23 and PP. During these periods, phosphorylated Hsp27 was primarily restricted to the cytoplasm of myocytes (Fig. 4) although not consistently to any specific sub-cellular domain (e.g. peri-nuclear).

Localization in the longitudinal muscle layer

Detection of Hsp27 in the longitudinal muscle layer with the SRQL antisera demonstrated that Hsp27 was primarily

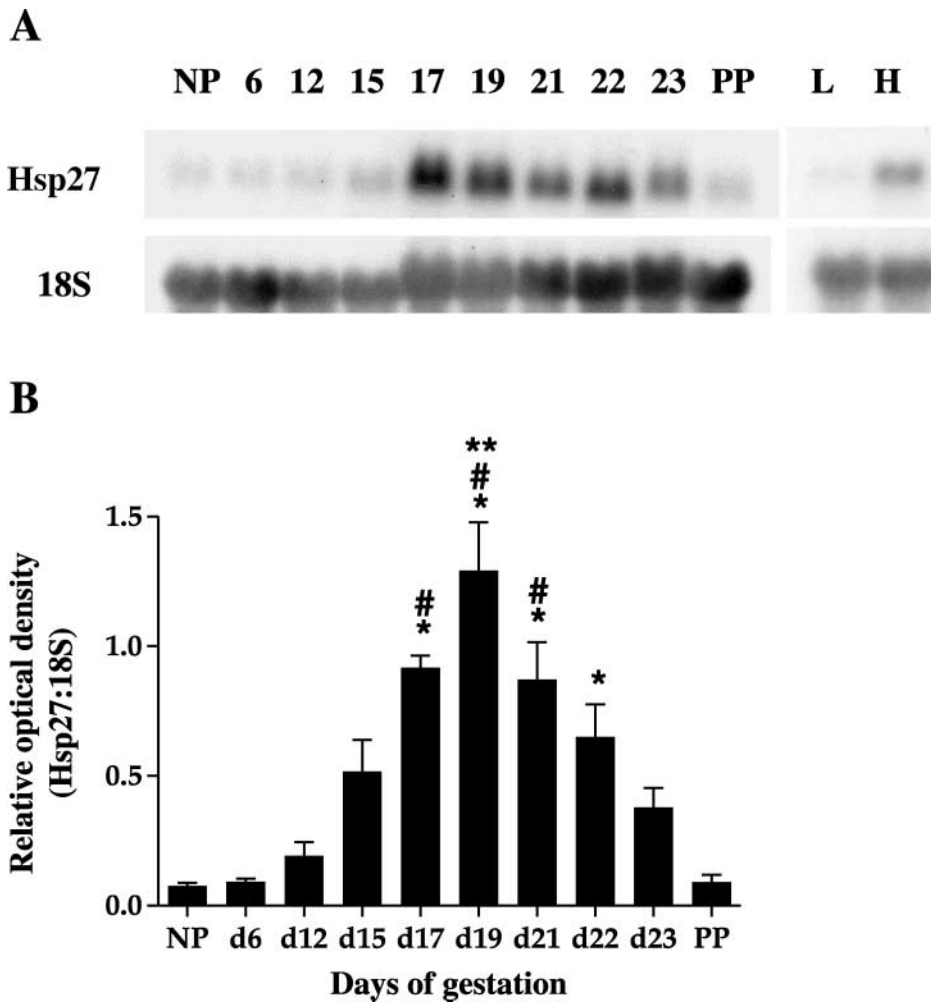


Figure 1 Northern blot analysis of Hsp27 mRNA expression in rat myometrium during pregnancy, labour and post-partum. (A) A representative Northern blot analysis of Hsp27 mRNA and 18S rRNA expression. Northern blot analysis was performed with an Hsp27-specific hamster cDNA and an 18S-specific rabbit cDNA as templates for radio-labelled probe production. Ten micrograms total RNA were utilized per lane. (B) Densitometric analysis illustrating the increase in Hsp27 mRNA expression during late pregnancy. Values on d17 to d22 were significantly elevated ($*P < 0.05$) compared with NP, d6 and PP and values on d17 to d21 were also significantly elevated ($\#P < 0.05$) compared with d12 and d23. Expression on d19 was significantly elevated compared with d15 and d22 ($**P < 0.05$). Days 6, 12, 15, 17, 19, 21, 22, and 23 represent gestational timepoints. Values are from four independent experiments ($n = 4$) \pm S.E. NP, non-pregnant; PP, 1 day post-partum; L, liver tissue control; H, heart tissue control.

localized to membrane regions from d15 and was increasingly detected by d22, followed by decreased membrane detection on d23 and undetectable Hsp27 expression at PP that was similar to pre-immune controls (Fig. 5). Utilizing the S15 antisera, phosphorylated Hsp27 was also detected on d15 but was readily detectable on d17 to d22, primarily localized to myocyte membranes but also detectable at a low level in the cytoplasm of myocytes (Fig. 6). In contrast to results with the SRQL antisera, however, phosphorylated Hsp27 was readily detected on d23 and PP. In addition, phosphorylated Hsp27 was detected in the cytoplasm of myocytes during these two latter timepoints.

In addition to the temporal and spatial localization differences observed for total and Ser-15 phosphorylated Hsp27 expression during gestation, our immunocytochemistry experiments also demonstrated that Hsp27 was differentially expressed between the two muscle layers of the myometrium during gestation. Both total Hsp27 (measured with the SRQL antisera) and Ser-15 phosphorylated Hsp27 were much more highly detectable in the longitudinal muscle layer than in the circular muscle layer.

Discussion

Hsp27 expression during pregnancy

Our Northern blot analysis demonstrated that detection of Hsp27 mRNA began to increase by d15 of pregnancy, increased significantly from d17 to d22, and then dropped significantly thereafter. This pattern of expression may be due to gestation-specific changes in Hsp27 mRNA synthesis. Putative AP-1-binding sites exist within the promoter region of sHSP genes (Iwaki *et al.* 1990, Srinivasan & Bhat 1994) and it is possible that AP-1 transcription factors, which are expressed in the myometrium during late pregnancy and labour (Piersant & Lye 1995, Mitchell & Lye 2002, Shynlova *et al.* 2002), might, at least partially, be responsible for regulating Hsp27 mRNA expression during gestation. We also cannot rule out the possibility that changes in Hsp27 mRNA stability, in addition to mRNA synthesis, could be a contributing factor to the observed pattern of expression. Lastly, the steroid hormone environment during pregnancy may also regulate Hsp27 mRNA expression. In most animal species, including the rat, the circulating levels of progesterone begin to drop significantly prior to labour and the circulating levels

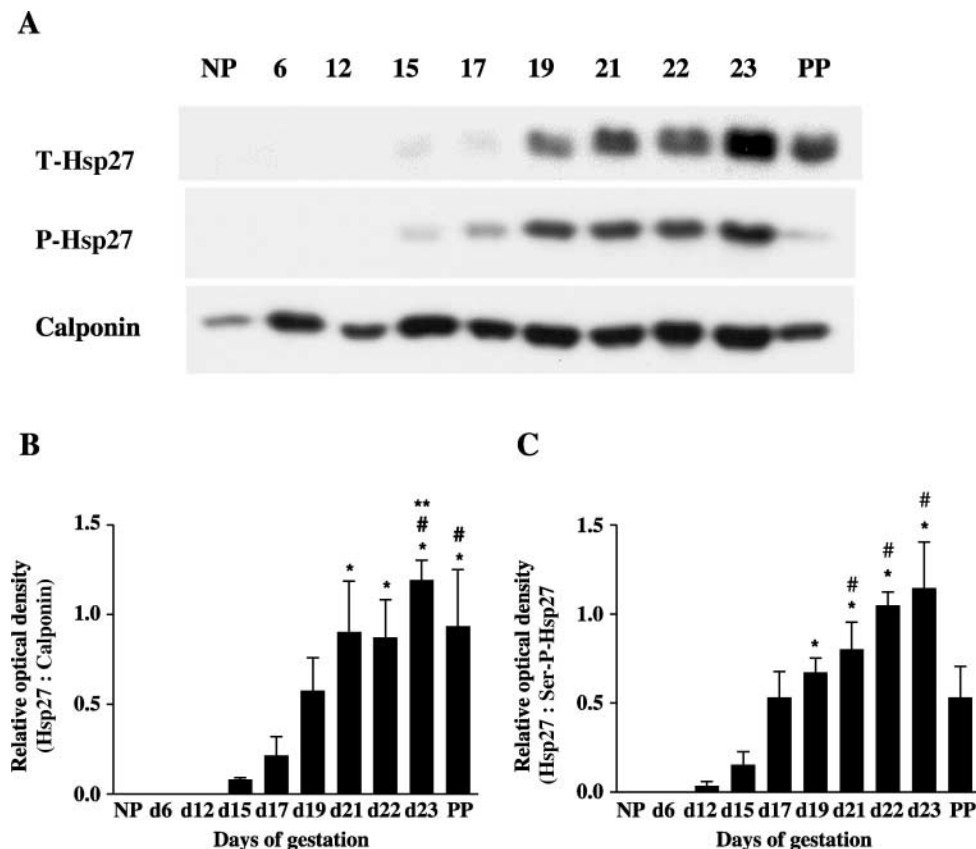


Figure 2 Immunoblot analysis of serine 15 (Ser) phosphorylated Hsp27 and total Hsp27 during pregnancy, parturition and post-partum. (A) Representative immunoblots from analysis of Hsp27 and calponin expression during gestation. (B) Densitometric analysis illustrating the dramatic increase in detection of total Hsp27 during pregnancy, labour and post-partum. Values on d21 to PP were significantly ($*P < 0.05$) increased compared with NP, d6, and d12 timepoints, values on d23 to PP were significantly ($#P < 0.05$) increased compared with d15 and expression on d23 was significantly elevated compared with d17 ($**P < 0.05$). (C) Densitometric analysis illustrating the dramatic increase in detection of Ser-15 phosphorylated Hsp27 during pregnancy and labour. Values on d19 to d23 were significantly increased ($*P < 0.05$) compared with NP, d6, and d12 timepoints while values on d21 to d23 were significantly ($#P < 0.05$) increased compared with d15. Days 6, 12, 15, 17, 19, 21, 22, and 23 represent gestational timepoints. Values are from three independent experiments ($n = 3$) \pm s.e. NP, non-pregnant; PP, 1 day post-partum; T-Hsp27, total Hsp27; P-Hsp27, phosphorylated Hsp27.

of estrogen increase sharply (Challis *et al.* 2000). However, both progesterone and estrogen are thought to induce Hsp27 expression (Tabibzadeh *et al.* 1996, Kato *et al.* 2002); therefore, the role these steroid hormones play in regulating Hsp27 expression in the rat myometrium is unclear.

Detection of total Hsp27 protein, by immunoblot analysis, also began to increase by d15 and was significantly elevated during late pregnancy, labour, and PP. Despite the fact that Hsp27 was virtually undetectable from the NP to d12 timepoints, it is still possible that low basal expression of Hsp27 exists because it may be beyond the detection level of our immunoblot analysis under the conditions utilized for our assay. Since the increased detection of phosphorylated Hsp27 towards labour paralleled the increase in detection of total Hsp27, our results suggest that at least a basal activity of an Hsp27 kinase is required for this post-translational modification. The markedly decreased detection of phosphorylated Hsp27 at PP, compared with total Hsp27, suggests that this

result was likely due to dephosphorylation of Hsp27 by a phosphatase.

Immunocytochemistry experiments revealed that Ser-15 phosphorylated Hsp27 was primarily localized to myocyte membranes during late pregnancy. Phosphorylated Hsp27 might be associated with focal adhesions during this period since Hsp27 has been reported to bind to the focal adhesion protein Hic-5, a member of the paxillin protein family (Turner 2000). Focal adhesions are necessary for transmission of tension from the extracellular matrix to the internal contractile apparatus and may be remodelled during late pregnancy to support myometrial hypertrophy (MacPhee & Lye 2000, Gerthoffer & Gunst 2001). The phosphorylation of Hsp27 on Ser-15 is proposed to produce a conformational change in Hsp27 that aids the direct binding of Hsp27 with actin filaments (Lambert *et al.* 1999, Gerthoffer & Gunst 2001, Mounier & Arrigo 2002) and Benndorf *et al.* (1994) has demonstrated that Hsp27 phosphorylation can relieve Hsp27 inhibition of actin polymerization. Therefore, in the myometrium

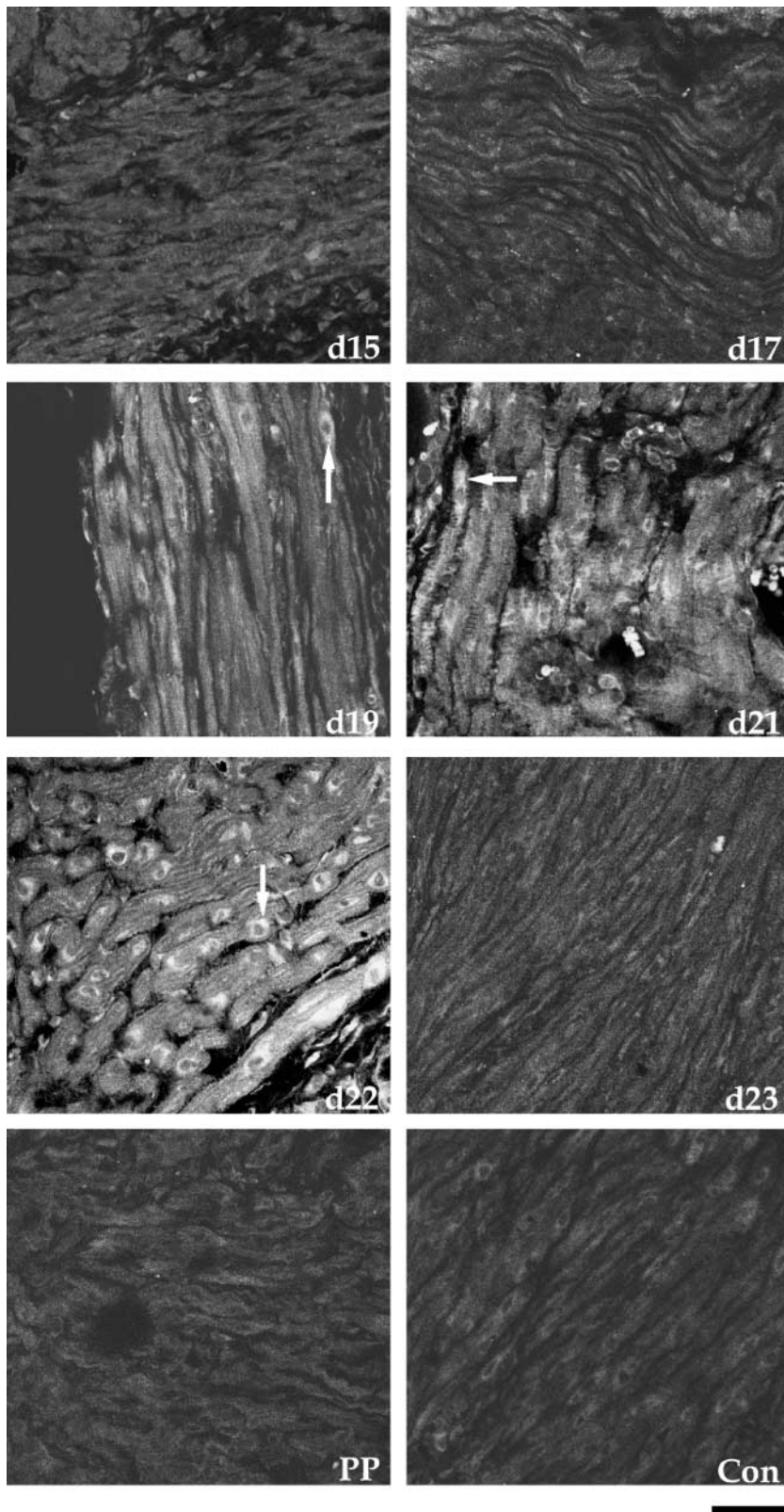


Figure 3 Immunocytochemical analysis of total Hsp27 protein expression in circular muscle layers of pregnant rat myometrium between day 15 of pregnancy (d15) and 1 day post-partum (PP) using polyclonal anti-human Hsp27 antisera (SRQL). Arrows indicate peri-nuclear localization. Con, pre-immune serum control. Scale bar = 50 μ m.

during late pregnancy, membrane localized, phosphorylated Hsp27 may regulate actin cytoskeleton dynamics at focal adhesion sites to support the hypertrophy-induced reorganization of these structures during late pregnancy.

Ser-15 phosphorylated Hsp27 was also readily detected in cytoplasmic regions of myocytes during labour. Phosphorylation of Hsp27 also appears to be an essential step for smooth muscle contraction by promoting

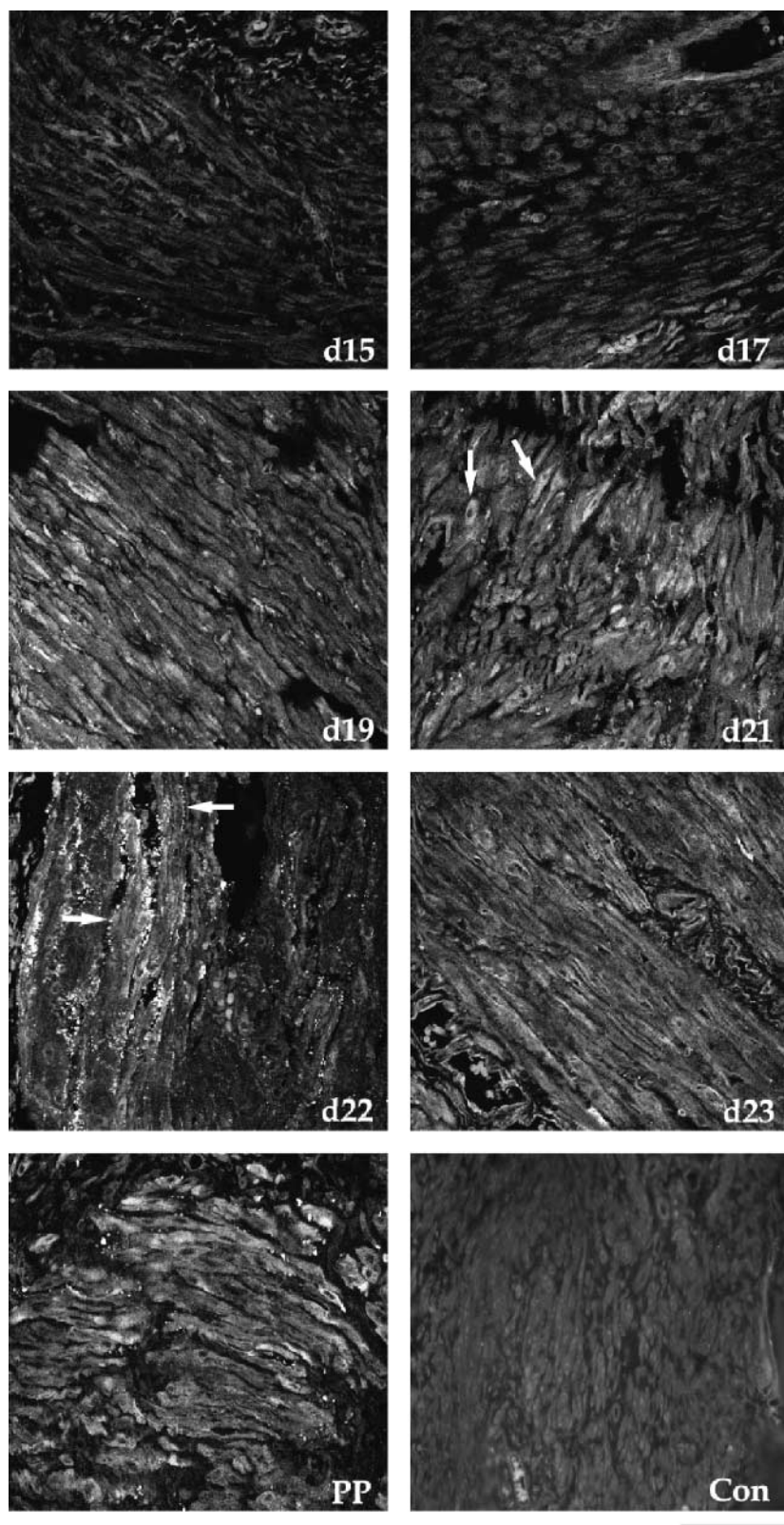


Figure 4 Immunocytochemical analysis of Ser-15 phosphorylated Hsp27 protein expression in circular muscle layers of pregnant rat myometrium between day 15 of pregnancy (d15) and 1 day post-partum (PP) using phosphorylation state-specific polyclonal anti-human Hsp27 antisera (S15). Con, pre-immune serum control. Arrows on d21 indicate peri-nuclear localization while arrows on d22 demonstrate membrane localization. Scale bar = 50 μ m.

actin–myosin interaction (Gerthoffer & Gunst 2001, Bitar 2002). In colonic smooth muscle cells, Hsp27 co-immunoprecipitates with actin, tropomyosin, and caldesmon (Ibityayo *et al.* 1999, Bitar 2002). Colonic smooth muscle cells

that were transfected with constitutively phosphorylated Hsp27 mutants, and stimulated with contractile agonists, also exhibited an increased binding of actin with myosin (Bitar 2002). Therefore, during labour phosphorylated

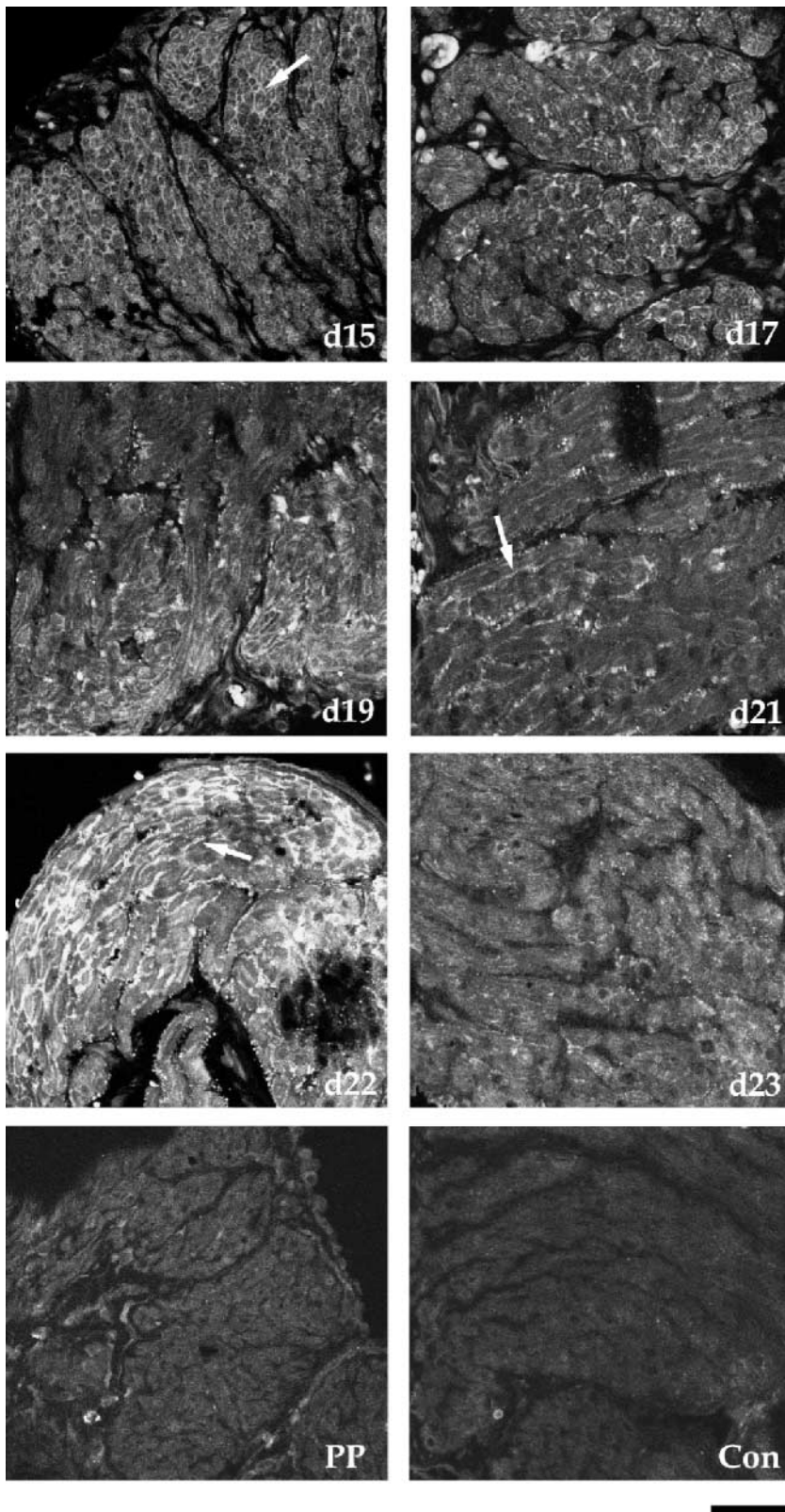


Figure 5 Immunocytochemical analysis of total Hsp27 protein expression in longitudinal muscle layers of pregnant rat myometrium between day 15 of pregnancy (d15) and 1 day post-partum (PP) using polyclonal anti-human Hsp27 antisera (SRQL). Arrows indicate membrane localization. Con, pre-immune serum control. Scale bar = 50 μ m.

Hsp27 in cytoplasmic regions might be associated with the contractile machinery mediating contraction.

Despite published evidence of a role for Hsp27 in both actin polymerization and smooth muscle contraction, we

cannot rule out a role for phosphorylated Hsp27 as a chaperone. Phosphorylated Hsp27 was detected in the cytoplasm of myometrial cells during late pregnancy, labour, and PP. Actin cytoskeleton dynamics and

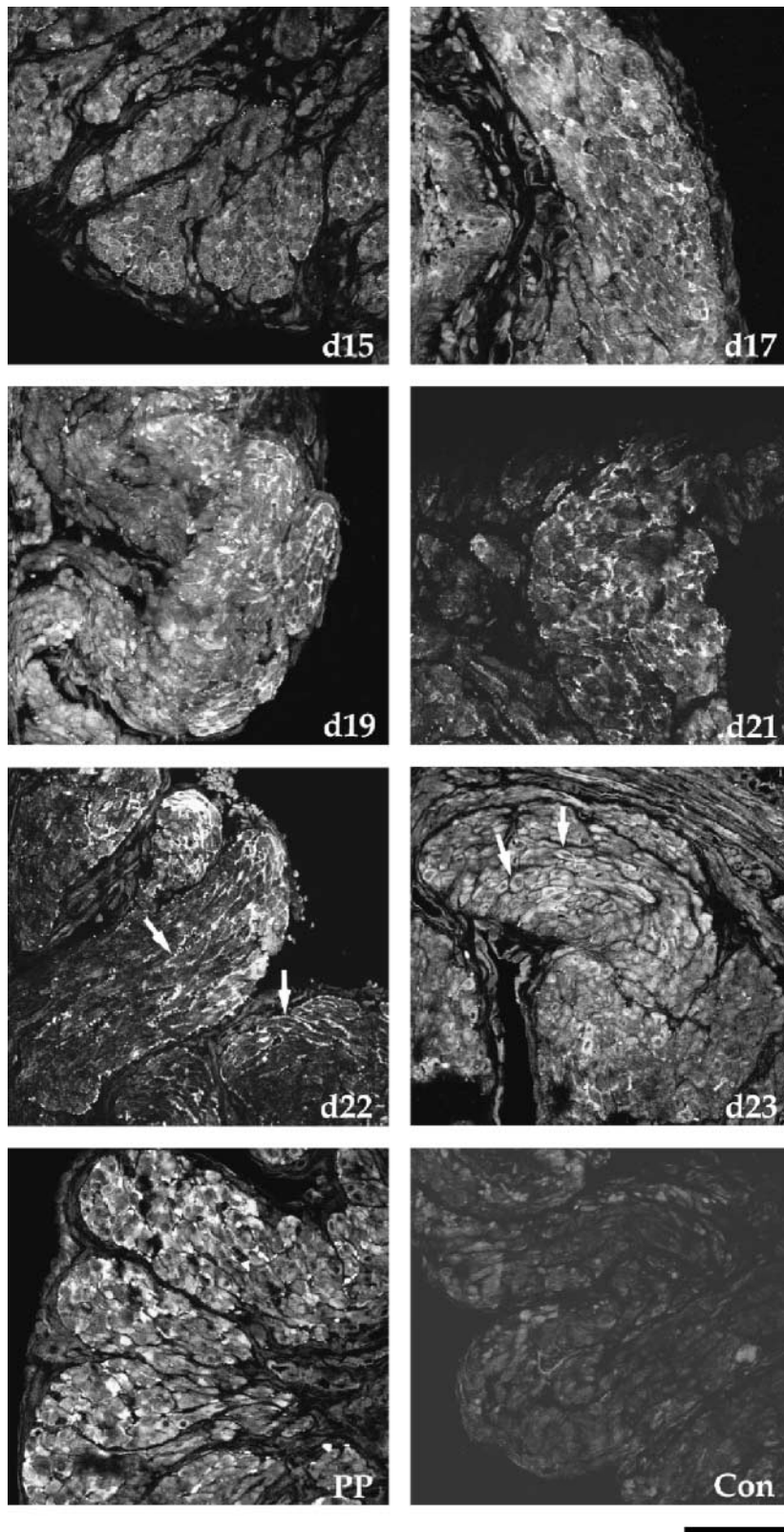


Figure 6 Immunocytochemical analysis of Ser-15 phosphorylated Hsp27 protein expression in longitudinal muscle layers of pregnant rat myometrium between day 15 of pregnancy (d15) and 1 day post-partum (PP) using phosphorylation state-specific polyclonal anti-human Hsp27 antisera (S15). Con, pre-immune serum control. Arrows on d22 indicate membrane localization while arrows on d23 demonstrate cytoplasmic localization. Scale bar = 50 μ m.

protection require phosphorylated Hsp27 (Liang & MacRae 1997, Mounier & Arrigo 2002) and as a chaperone, phosphorylated Hsp27 may coat actin filaments to prevent access to actin-severing proteins and help

maintain their organization in myometrial cells during gestation.

Our immunocytochemistry experiments showing that Ser-15 phosphorylated Hsp27 was detectable by S15

antisera in both circular and longitudinal muscle layers during labour and PP, was in stark contrast to Hsp27 detected with the SRQL antisera. In addition, immunoblot analysis of total Hsp27 with the CT antisera also demonstrated detection of Hsp27 at labour and PP compared with the immunocytochemistry results with the SQRL antisera. The reason(s) for the lack of detection of Hsp27 at labour and PP by the SQRL antisera is unknown; however, we cannot discount the possibility that SRQL antisera specificity is sensitive to conformational changes in Hsp27 structure. In addition to phosphorylation, for example, sHSP can undergo deamidation, acylation, oxidation and glycation (Gaestel 2002). Therefore, specific post-translational modifications during labour and PP might cause a conformational change in the Hsp27 molecule that could prevent the SQRL antisera from efficiently recognizing its epitope *in situ*.

Our data also indicate that Hsp27 is detectable at much higher levels in the longitudinal muscle layer than in the circular muscle layer during late pregnancy. The functional consequences of this expression pattern are unknown, but the two muscle layers do have different embryological origins and exhibit different contractile and physiological characteristics (Osa & Katase 1975, Kawabayashi & Osa 1976, Chow & Marshall 1981, Mlynarczyk *et al.* 2003). Therefore, it is possible that Hsp27 has muscle layer-specific roles during pregnancy and labour.

Hsp27 and myometrial contractility

Temporal-specific differences in uterine contractility have been reported during pregnancy in humans, sheep, rats and mice (Harding *et al.* 1982, Bengtsson *et al.* 1984, Buhimschi & Garfield 1996, Buhimschi *et al.* 1997, 1998, Hennan & Diamond 1998, Mackler *et al.* 1999). These reports have consistently provided evidence that uterine activity significantly increases just prior to labour. Specifically in the rat, Buhimschi and Garfield (1996) and Buhimschi *et al.* (1998) used abdominal and myometrial surface electromyographic analysis as a means to assess uterine contractions. Only within the last 24 h before active labour occurred did significant contractile activity become evident. Reports of increased myometrial contractility just prior to labour coincide well with documented activation of the myometrium.

Myometrial activation is thought to be necessary for the myometrium to properly respond to contractile stimuli and is marked by the increase in expression of contraction-associated proteins (Challis *et al.* 2000, 2002). Interestingly, two families of heat shock proteins, Hsp70 and Hsp90, are already considered contraction-associated proteins in the myometrium of the ewe (Wu *et al.* 1996). Although we cannot yet determine the specific role(s) of Hsp27 in the rat myometrium during pregnancy, labour and PP, our results demonstrating that Hsp27 expression is highly upregulated during late pregnancy and labour, and documented evidence of roles for Hsp27 in smooth

muscle contraction and actin polymerization, suggest Hsp27 is a potential contraction-associated protein.

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