Heat shock protein expression in umbilical artery smooth muscle

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Postpartum vasospasm in the umbilical arteries may be due to impaired vasorelaxation secondary to alterations in the expression of heat shock proteins. The contractile responses of pre- and full-term bovine umbilical artery smooth muscles were determined in a muscle bath. Heat shock protein expression was determined in bovine and human arterial tissues using western blotting with specific antisera. Full-term bovine and human umbilical artery smooth muscle was refractory to relaxation induced by the nitric oxide donor, sodium nitroprusside. This impaired vasorelaxation was associated with the expression of the inducible form of the heat shock protein, HSP70i, and increases in the expression of the small heat shock protein, HSP27. Small heat shock proteins have been implicated in modulating contraction and relaxation responses in vascular smooth muscles. Thus, alterations in heat shock protein expression may play a role in umbilical artery vasospasm.

Introduction

Vasospasm in the umbilical vasculature is essential for the conversion from the placental–fetal circulation to the neonatal circulation. Two major hypotheses have emerged to explain vasospasm in the umbilical vessels after parturition: the loss of prostanooids, which sustain vasorelaxation; and the increase in oxygen tension in the arterialized blood passing through the umbilical arteries (Etherington, 1967; Karim *et al.*, 1997). While vasodilating prostanooids induce vascular relaxation by activation of adenylate cyclase, the umbilical artery smooth muscle is refractory to relaxation by adenylate cyclase activators (Renowden *et al.*, 1992; Bergh *et al.*, 1995). Vasospasm must occur in both the umbilical arteries and veins and, although the oxygen tension increases in the arteries after birth (Wulf *et al.*, 1964), it is unlikely that it increases in the vein. Therefore, the specific mechanisms that initiate the intense vasospasm in the umbilical vasculature after birth have not been established.

Heat shock proteins (HSPs) are a group of phylogenetically well conserved proteins that may have pivotal roles in the adaptation to physiological stress (Welch *et al.*, 1986). Although the specific cellular functions of HSPs are not completely understood, some of the HSPs are constitutively present in mammalian tissues, which suggests they play a role in normal physiological processes. Heat shock proteins have been implicated in the mediation of smooth muscle contraction and relaxation responses (Bitar

et al., 1991; Beall *et al.*, in press). Since the final effector cell in any vasospastic process is the vascular smooth muscle, it is hypothesized that HSPs are induced during parturition and, thereby, influence the process of umbilical artery vasospasm.

Materials and Methods

Materials

Electrophoresis reagents and the DC protein assay kit were from BioRad (Hercules, CA). Mouse anti-HSP70 antibody was from Amersham (RPN 1197) and mouse anti-HSP27 was a generous gift from M. Welsh (University of Michigan, Ann Arbor, MI) (Welsh *et al.*, 1996). All other reagents were of analytical grade.

Preparation of vascular smooth muscle strips

Full-term umbilical cords were obtained from the Labor and Delivery suite at the Medical College of Georgia under a protocol approval by the institutional Human Assurance Committee. Only cords obtained from normal deliveries from healthy women were used in this study.

Pre-term bovine umbilical cords were obtained from an abattoir (Shapiro's Meatpackers, Augusta, GA). The gestational age of the calves was approximated by weight and arbitrarily placed into a trimester scale. Calves < 22 kg were considered first trimester; 22–44 kg, second trimester; and > 88 kg, third trimester. Full-term bovine umbilical cords

were obtained from local farmers at assisted births. The umbilical arteries were placed in cold physiological saline solution (10 mmol *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid l^{-1} (Hepes), 140 mmol NaCl l^{-1} , 4.7 mmol KCl l^{-1} , 1.0 mmol $\text{MgSO}_4 \text{l}^{-1}$, 1.0 mmol $\text{NaH}_2\text{PO}_4 \text{l}^{-1}$, 1.5 mmol $\text{CaCl}_2 \text{l}^{-1}$, 10 mmol glucose l^{-1} , pH 7.4) for transport and storage. At the laboratory, the umbilical arteries were dissected from the cords and the endothelial layer was gently denuded with a cotton-tipped applicator. The arteries were opened longitudinally and thin transverse strips were cut.

Contractile physiology

Transverse sections (1 mm) of umbilical artery smooth muscles were mounted in a tissue bath with 3–0 silk suture. One end was anchored to a fixed glass pipette and the other to a force transducer (Grass Instrument Corp., Quincy, MA). Tension was recorded on a strip-chart recorder (Gould Instrument Corp., Norcross, GA). Strips were equilibrated for 60 min in water jacketed chambers at 37°C containing Krebs–bicarbonate buffer (KRB, 120 mmol NaCl l^{-1} , 4.7 mmol KCl l^{-1} , 1.0 mmol $\text{MgSO}_4 \text{l}^{-1}$, 1.0 mmol $\text{NaH}_2\text{PO}_4 \text{l}^{-1}$, 10 mmol glucose l^{-1} , 1.5 mmol $\text{CaCl}_2 \text{l}^{-1}$, and 25 mmol $\text{Na}_2\text{HCO}_3 \text{l}^{-1}$). The strips were gassed with 95% O_2 –5% CO_2 and maintained at pH 7.4. Some strips were gassed with 95% N_2 –5% CO_2 . The maximal contractile length was adjusted with repeated additions of 110 mmol KCl l^{-1} (with equimolar replacement of NaCl). After re-equilibration, the strips were pre-contracted with a submaximal dose of 5-hydroxytryptamine (1 mmol l^{-1}), and the cumulative dose-dependent relaxation response to the NO donor, sodium nitroprusside (SNP), was determined. The data are reported as the percentage of relaxation to each dose of SNP. Serotonin and SNP were diluted in water and added directly to the muscle bath.

Immunoblotting

Umbilical artery smooth muscles were homogenized in 60 mmol Tris l^{-1} , 10 mmol EGTA l^{-1} , 2 mmol EDTA l^{-1} , 10 mmol β -mercaptoethanol l^{-1} , 5 mg Aprotinin ml^{-1} , 250 mmol Leupeptin l^{-1} , 1 mmol benzamidine l^{-1} , pH 7.4. The samples were centrifuged at 10 000 *g* for 10 min and the supernatant was protein normalized. The proteins were separated on SDS-PAGE gels and transferred to Immobilon-P (Millipore, Bedford, MA). The blots were blocked with 5% milk in 10 mmol Tris l^{-1} , 150 mmol NaCl l^{-1} , 0.5% Tween-20, pH 7.4 (TBS) for 1 h. The blots were incubated with mouse anti-HSP70 antibodies (1:5000 dilution in TBS, 5% milk) or mouse anti-HSP27 antibodies (1:4000 dilution in TBS, 5% milk) for 3 h at 4°C. The blots were then washed in TBS, 0.5% Tween 20 (three washes of 5 min). Immunoreactive spots were detected using donkey anti-mouse horse-radish peroxidase for 1 h at 25°C and, after washing (eight washes of 5 min in TBS), western blot chemiluminescence reagent was applied (Dupont NEN, Boston, MA) and the blots were developed with Kodak XAR film.

HSP27 quantitation

Human umbilical artery smooth muscle or bovine carotid artery smooth muscles were homogenized in 25 mmol Hepes l^{-1} , 150 mmol NaCl l^{-1} , 10 mmol EDTA l^{-1} , 1 mmol DTT l^{-1} , 2 mmol benzamidine l^{-1} , pH 7.4. The samples were centrifuged at 10 000 *g* for 10 min and the supernatants were protein normalized. Arterial homogenates containing 10 mg protein were dot blotted onto nitrocellulose. The blots were fixed with 20% methanol, dried, blocked with TBS, 5% milk for

1 h, washed three times with TBS, 0.5% Tween, and then probed with anti-HSP27 antibodies (1:4000 dilution in TBS, 5% milk) for 1 h. The blots were washed six times with TBS, 0.5% Tween and then probed with ^{125}I goat anti-mouse IgG.

Two-dimensional electrophoresis

Strips of carotid and umbilical arteries were equilibrated for 60 min in bicarbonate buffer. The tissue was crushed using a mortar and pestle under liquid N_2 . The powder was resuspended in 9 mol urea l^{-1} , 2% 3-[3-cholamidopropyl dimethylammonio]-1-propanesulfonate (chaps), 100 mmol dithithreitol l^{-1} , and the proteins were separated by two-dimensional gel electrophoresis. Protein (150 μg) was loaded onto an isoelectric focusing (IEF) slab gel with 2% (v/v) ampholytes (1.0 part pH 3.5–10, 1.5 parts pH 5–7, and 2.5 parts pH 6–8, LKB Pharmacia, Uppsala) and electrophoresis was performed for 1 h at 100 V, 1 h at 200 V, and 19 h 24 min at 500 V. The IEF lanes were placed on SDS-PAGE gels (12%) and run for 21 h at 90 V. The gels were transferred to Immobilon and stained with Aurodyne Forte (Amersham, Arlington Heights, IL), a dye for quantitative protein detection.

Statistical analysis

Densitometric analysis of the phosphoproteins was performed using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The results are depicted as means $\pm$ SEM. Statistical analysis was by one-way analysis of variance followed by the Tukey's test using SigmaStat software (Jandel Scientific, San Rafael, CA) and $P < 0.05$ was considered significant.

Results

Full term bovine umbilical artery smooth muscle is refractory to NO-dependent vasorelaxation

Strips of bovine umbilical artery smooth muscle were equilibrated in a muscle bath and, after pre-contraction with 1 μmol serotonin l^{-1} , the cumulative dose-dependent relaxation response to the NO donor, sodium nitroprusside (SNP), was determined. There were no differences in bovine umbilical artery smooth muscle relaxation responses in umbilical cords obtained from animals in the first, second, or

Fig. 1. Nitric oxide-dependent relaxation responses in bovine umbilical artery smooth muscle. Strips of bovine umbilical artery smooth muscle from pre- and full-term bovine umbilical cords were equilibrated in a muscle bath under low (95% N₂-5% CO₂) and high (95% O₂-5% CO₂) O₂ tensions. The strips were pre-contracted with 1 μ mol 5-hydroxytryptamine l⁻¹ and the cumulative dose-dependent relaxation responses to the NO-donor, sodium nitroprusside (SNP), were determined. There were no differences in the relaxation responses of bovine umbilical artery smooth muscles from cords obtained from first (▲), second (●) or third (■) trimester calves. Neither were there differences in the relaxation responses of pre-term bovine umbilical artery smooth muscles equilibrated under low or high O₂ tensions (data not shown). The relaxation response of full-term bovine umbilical artery smooth muscles (▼) to SNP (10⁻⁶-10⁻⁴ mol l⁻¹) was significantly less than the relaxation response of pre-term bovine umbilical artery smooth muscles to SNP ($n = 5-8$, * $P < 0.05$, ANOVA).

third trimesters (Fig. 1). There was also no difference in the relaxation responses of bovine umbilical smooth muscles equilibrated under low O₂ tensions (95% N₂-5% CO₂) compared with high O₂ tensions (95% O₂-5% CO₂) ($n = 5$, $P > 0.05$, ANOVA, data not shown). However, full-term bovine umbilical cords, obtained from spontaneous normal deliveries, displayed a dose-dependent relaxation response that was shifted to the right (Fig. 1). In addition, full-term human umbilical artery smooth muscle did not relax in response to sodium nitroprusside (10⁻⁸-10⁻³ mol SNP l⁻¹, $n = 8$, data not shown).

Inducible form of HSP70 is present in human umbilical artery smooth muscle but not bovine carotid artery smooth muscle

Western blots with antibodies that specifically recognize the inducible form of HSP70 (HSP70i) demonstrated that an immunoreactive band of M_r 70 kDa was present in human umbilical artery smooth muscle (Fig. 2). HSP70i was not present in a vascular smooth muscle that displays normal relaxation physiology, bovine carotid artery smooth muscle (Fig. 2), or in human saphenous vein (data not shown). However, HSP70i was inducible in bovine carotid artery smooth muscle by stressors such as norepinephrine (10 μ mol l⁻¹) or sodium arsenite (500 μ mol l⁻¹), suggesting that the antibodies recognized bovine HSP70i (Fig. 2).

Inducible form of HSP70 is present in full-term bovine umbilical artery smooth muscle but not pre-term bovine umbilical artery smooth muscle

Western blots using anti-HSP70i demonstrated a strongly immunoreactive band of M_r 70 kDa in full term bovine

Fig. 2. Immunoreactive HSP70 in human umbilical artery smooth muscle. Strips of human umbilical artery or bovine carotid artery smooth muscle were homogenized and the 10000 g supernatants were separated on 10% SDS-PAGE gels and transferred to Immobilon (C). Some strips of bovine carotid artery smooth muscle were treated with noradrenaline (NA, 10 μ mol l⁻¹ for 30 min) or arsenite (Ar, 500 μ mol l⁻¹ for 30 min) 6 h before homogenization. Immunoreactive bands of M_r 70 kDa (arrowhead) were present in all human umbilical artery smooth muscles examined ($n = 12$, only three representative samples are shown on the figure). Although immunoreactive bands were not detected in homogenates of bovine carotid artery smooth muscle, stimulation with noradrenaline or arsenite induced a band of M_r 70 kDa in carotid homogenates.

Fig. 3. Immunoreactive HSP70 in full-term bovine umbilical artery smooth muscle. Strips of pre- or full-term bovine umbilical artery smooth muscles were homogenized and the 10000 g supernatants were separated on 10% SDS-PAGE gels and transferred to Immobilon. Immunoreactive bands of M_r 70 kDa (arrowhead) were present in all full-term umbilical artery smooth muscles examined ($n = 4$, only two representative samples are shown on the figure). There were only weakly immunoreactive bands for HSP70i in umbilical artery smooth muscles from pre-term bovine cords ($n = 4$). Densitometric analysis revealed significantly more HSP70i in full-term compared with pre-term cords (2.1 ± 0.5 versus 14.4 ± 1.3 , $P < 0.05$, Mann-Whitney rank sum test, $n = 4$).

umbilical artery smooth muscle homogenates (Fig. 3). In western blots of pre-term umbilical artery smooth muscle, there was only a very weakly immunoreactive band at M_r 70 kDa.

HSP27 in umbilical artery smooth muscle compared with bovine carotid artery smooth muscle

Immunoblotting with antibodies specific for the small heat shock protein, HSP27, demonstrated a single immunoreactive band in homogenates of bovine carotid and human umbilical artery smooth muscle (Fig. 4). Using anti-HSP27 antibodies and a dot blotting technique, HSP27 was quantitated in homogenates of umbilical and carotid arteries. There was significantly more immunoreactive HSP27 in homogenates of human umbilical smooth muscles than in bovine carotid smooth muscles (Fig. 4). The amount of HSP27 protein present on two-dimensional gels was quantitated to confirm this result. There was significantly more HSP27 protein in umbilical artery homogenates than in carotid artery homogenates (28.8 ± 3.3 versus 1.8 ± 0.4 , $n = 4$, $P < 0.05$). Insufficient amounts of full-term bovine umbilical artery smooth muscle were obtained to quantitate differences in HSP27 expression between pre- and full-term bovine umbilical artery.

Discussion

The present study demonstrates the activation of the stress response umbilical artery smooth muscle from human and bovine full-term umbilical cords. The inducible form of HSP70 (HSP70i) was present in full-term umbilical artery smooth muscle from both human and bovine umbilical cords (Figs 2 and 3). In addition, while HSP70i was not present in normal vascular smooth muscle (bovine carotid artery smooth muscle), immunoreactive HSP70i could be induced

Fig. 4. Quantitation of HSP27 expression in human umbilical artery smooth muscle. Human umbilical artery smooth muscle or bovine carotid artery smooth muscles were homogenized and the 10000 g supernatants were protein normalized. (a) Western blots revealed a single immunoreactive band using anti-HSP27 antibodies in both carotid (C) and umbilical (U) homogenates. This led to the use of a dot blot method to quantitate HSP27 immunoreactivity in the homogenates. Arterial homogenates containing 10 μ g total protein were dot blotted onto nitrocellulose and probed with anti-HSP27 antibodies and then with 125 I-labelled goat anti-mouse IgG secondary antibodies. The amount of immunoreactive protein (relative units) was determined using a PhosphorImager. (b) There was significantly more immunoreactive HSP27 in homogenates of human umbilical arteries (U) than in homogenates of bovine carotid arteries (C) ($n = 6-10$, $*P < 0.05$, ANOVA).

by stressors such as noradrenaline and sodium arsenite. Using a limited amount of tissue, it was possible to demonstrate that immunoreactive HSP70i was present in full-term bovine umbilical artery smooth muscle but not pre-term bovine umbilical artery smooth muscle. Finally, there was more immunoreactive HSP27 in human umbilical artery smooth muscle than in bovine carotid artery smooth muscle.

The expression of HSPs in umbilical artery smooth muscle was associated with impaired sodium nitroprusside dependent relaxation responses in full-term umbilical arteries (Fig. 1) (Renowden *et al.*, 1992; Bergh *et al.*, 1995). There were no differences in relaxation responses in umbilical artery smooth muscles from bovine umbilical

cords at different stages of gestation. There were also no differences in the relaxation responses at low (95% N₂-5% CO₂) compared with at high (95% O₂-5% CO₂) O₂ tensions. The exposure of pre-term umbilical artery smooth muscles to high O₂ tensions (> 100 mm Hg) did not render the muscles refractory to sodium nitroprusside-dependent relaxation, suggesting that an event in addition to increased O₂ tension is necessary for umbilical artery vasospasm.

While most of the data pertaining to heat shock protein expression and function has been obtained from tissue cultures, it has recently been demonstrated that physiological restraint and surgical stress induce the expression of HSP70 selectively in vascular and adrenal tissue in rats (Udelsman *et al.*, 1991, 1993). Restraint stress also induced an increase in the expression of HSP27 selectively in vascular tissue, and the physiological expression of HSPs in the vasculature was associated with hypertension (Blake, 1995).

The present study is the first investigation of which we are aware that shows activation of the stress response in human vascular tissue under physiological conditions. Activation of the stress response in the umbilical vasculature may contribute to vasospasm after parturition. HSP27 may mediate sustained vascular smooth muscle contraction in bombesin-stimulated smooth muscles (Bitar *et al.*, 1991). In addition, increases in the phosphorylation of proteins with M_r 27 kDa and similar isoelectric points to HSP27 have been implicated in association with agonist-induced contraction of vascular smooth muscles (Barany *et al.*, 1992). The thrombin-induced contraction of vascular smooth muscle has been shown to be associated with increases in the phosphorylation of HSP27 (Brophy *et al.*, 1998). HSP27 is relatively abundant in muscle tissues and has been shown to modulate actin filament dynamics (Miron *et al.*, 1988). In addition, increases in the phosphorylation of another small HSP, HSP20, have been implicated in mediating vascular smooth muscle relaxation (Beall *et al.*, 1997). HSP20 does not appear to be phosphorylated in umbilical artery smooth muscles treated with sodium nitroprusside (Bergh *et al.*, 1995). Taken together, these data support a role for the small HSPs in modulating umbilical artery vasospasm.

Although the specific stimulus for the induction of the HSPs in umbilical vascular tissue is not known, HSP27 has been identified as an oestrogen-regulated protein in human breast cancer cell lines (Faucher *et al.*, 1993). HSP27 has been immunolocalized to the endometrium and cervix (Ciocca *et al.*, 1983). The expression of HSP27 is regulated by hormones in the endometrium during the menstrual cycle (Ciocca *et al.*, 1983). HSP27 has also been associated with the oestrogen receptor (Faucher *et al.*, 1993). Thus, it has been suggested that HSP27 is transcriptionally induced by oestrogen and that it interacts with the oestrogen receptor. Oestrogen concentrations typically reach peak values at parturition (Tulchinsky *et al.*, 1972). Therefore, the appropriate hormonal milieu needed to precondition this vascular system to induce HSP27 precedes the events of delivery.

The present study was constrained by the limited numbers of full-term bovine umbilical cords available and by the inability to obtain 'normal' pre-term human umbilical cords. However, HSP70 is not typically expressed in normal vascular tissue. Thus, these data suggest that the stress

response is induced in umbilical artery tissue around the time of birth. In addition, the suggestion that HSPs have important roles in smooth muscle contraction and relaxation responses supports a role for the function of these proteins in vasospasm of the umbilical vasculature after parturition.

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