Phosphorylation of the Small Heat Shock-related Protein, HSP20, in Vascular Smooth Muscles Is Associated with Changes in the Macromolecular Associations of HSP20*

Colleen M. Brophy‡‡, Mary Dickinson‡, and David Woodrum§ §‡‡

From the Departments of ✪Surgery, ✪Medicine (Institute for Molecular Medicine and Genetics), ✪Cell Biology and Anatomy, Medical College of Georgia, Augusta, Georgia 30912 and the |Augusta Veterans Administration Medical Center, Augusta, Georgia 30910

Cyclic nucleotide-dependent vasorelaxation is associated with increases in the phosphorylation of a small heat shock-related protein, HSP20. We hypothesized that phosphorylation of HSP20 in vascular smooth muscles is associated with alterations in the macromolecular associations of HSP20. Treatment of bovine carotid artery smooth muscles with the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine, and the adenylate cyclase activator, forskolin, led to increases in the phosphorylation of HSP20 and dissociation of macromolecular aggregates of HSP20. However, 3-isobutyl-1-methylxanthine and forskolin treatment of a tissue that is uniquely refractory to cyclic nucleotide-dependent vasorelaxation, human umbilical artery smooth muscle, did not result in increases in the phosphorylation of HSP20 or to dissociation of macromolecular aggregates. HSP20 can be phosphorylated in vitro by the catalytic subunit of cAMP-dependent protein kinase (PKA) in both carotid and umbilical arteries and this phosphorylation of HSP20 is associated with dissociation of macromolecular aggregates of HSP20. Activation of cyclic nucleotide-dependent signaling pathways does not lead to changes in the macromolecular associations of another small heat shock protein, HSP27. Interestingly, the myosin light chains (MLC20) are in similar fractions as the HSP20, and phosphorylation of HSP20 is associated with changes in the macromolecular associations of MLC20. These data suggest that increases in the phosphorylation of HSP20 are associated with changes in the macromolecular associations of HSP20. HSP20 may regulate vasorelaxation through a direct interaction with specific contractile regulatory proteins.

* This work was supported in part by an American Health Association Clinician Scientist Award (to C. M. B.), a Veterans Administration Merit Review Award, and National Institutes of Health Grant RO1 HL58027–01. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be identified as such in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Institute for Molecular Medicine and Genetics, Medical College of Georgia, 1120 15th St., Augusta, GA 30912. Tel.: 706-721-4761; Fax: 706-823-2269; E-mail: colleenb@mail.mcg.edu.

†† Supported by the M.D./Ph.D. program at the Medical College of Georgia.

†‡‡ Supported by the M.D./Ph.D. program at the Medical College of Georgia.

† The abbreviations used are: HSP, heat shock protein; IBMX, 3-isobutyl-1-methylxanthine; CHAPS, 3-[3-chloro(2-propyl)]dimethylammonio]-1-propanesulfonic acid; Temed, N,N,N’N’-tetramethylethylenediamine; MLC20, myosin light chain.

EXPERIMENTAL PROCEDURES

Materials—The catalytic subunit of cAMP-dependent protein kinase was purchased from Promega (Madison, WI). Hesperidin was obtained from American Bioanalytical (Natick, MA). Urea, sodium dodecyl sulfate, glycerine, and Tris were from Research Organics (Cleveland, OH). Coomassie Brilliant Blue was from ICN Biomedicals Inc. (Aurora, OH). [γ-32P]ATP and [32P]orthophosphate were from Amersham (Arlington Heights, IL). [γ-32P]Protein A from NEN Life Science Products (Boston, MA). Forskolin and 3-isobutyl-1-methylxanthine (IBMX) were from Calbiochem (La Jolla, CA). Column calibration standards were obtained from Pharmacia (Uppsala, Sweden). Piperazine diacylamide and other electrophoresis reagents were from Bio-Rad. CHAPS, EGTA, EDTA, Tween 20, HEPES, and all other reagent grade chemicals were from Sigma. Rabbit polyclonal anti-MLC20 antibodies were from Dr. James Stull (University of Texas, Dallas, TX), mouse anti-HSP27 antibodies were from Dr. Michael Welsh (University of Michigan, Ann Arbor, MI) (13), rabbit anti-HSP20 antibodies were from Dr. Kanefusa Kato (5). Goat anti-mouse and anti-rabbit secondary antibodies were from Jackson Immunochemical (West Grove, PA). All other reagents were of analytical grade. Protein concentrations were determined by the modified Bradford assay (Pierce).

Preparation of Vascular Smooth Muscle Strips—Intact bovine carotid arteries were obtained from an abattoir (Shapiro’s meatpackers, Augusta, GA), and umbilical cords were from the labor and delivery suite.
of the Medical College of Georgia and placed directly in cold HEPEPS buffer (140 mM NaCl, 4.7 mM KCl, 1.0 mM MgSO4, 1.0 mM Na2HPO4, 1.5 mM CaCl2, and 10 mM glucose, and 10 mM Hepes, pH 7.4). The adventitia was dissected from the carotid arteries and the endothelial lining was denuded with a cotton-tipped applicator. Human umbilical arteries were dissected free of Wharton’s jelly. The arteries were opened longitudinally, the endothelium denuded with a cotton-tipped applicator, and thin (1 mm in diameter) transverse strips were cut. Vessel viability was determined by concurrent muscle bath experiments as described previously (3). The strips were equilibrated for at least 60 min in Krebs bicarbonate buffer (KRB, 120 mM NaCl, 4.7 mM KCl, 1.0 mM MgSO4, 1.0 mM Na2HPO4, 1.0 mM KH2PO4, 10 mM glucose, 1.5 mM CaCl2, and 25 mM Na2HCO3) at 37 °C bubbled with 95% O2, 5% CO2 to maintain a pH of 7.4. The strips were left in buffer alone (unstimulated) or treated with IBMX (1 mM) and FSK (10 μM) for 10 min.

In Vitro Phosphorylation of Vascular Smooth Muscle Homogenates— Strips of bovine carotid artery smooth muscle were homogenized in 10 mM Tris, 10 mM EDTA, 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 100 mM sodium fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, pH 7.4, and centrifuged 10,000 × g. The supernatants (1.2 mg of protein) were phosphorylated in a reaction mixture containing 40 mM Tris, pH 7.4, 20 mM magnesium acetate, 10 mM K2HPO4, and 40 mM of the catalytic subunit of cAMP-dependent protein kinase. The reaction was initiated with the addition of 200 μCi (γ-32P)ATP (800 cpm/μmol) and incubated for 30 min at 30 °C. The reaction was stopped by the addition (1:1, v/v) of 6.25 mM Tris, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.025% bromphenol blue and boiling for 5 min. The proteins were acetone precipitated and re-suspended in 9 M urea, 2% CHAPS, and 100 mM dithiothreitol for 24 h at room temperature.

In Situ Phosphorylation—Strips of bovine carotid artery smooth muscle were incubated in low phosphate KRB containing 150 μM/ml (β)-phosphohistidine at 37 °C, for 4 h. After stimulation, the vessels were snap frozen in liquid nitrogen and then allowed to slowly return to room temperature. The suspension was centrifuged (10,000 × g) and washed three times in acetone. The pellets were dried under a stream of nitrogen and then allowed to slowly return to room temperature. The supernatants (10,000 × g) were dissolved in 100 mM dithiothreitol for 24 h at room temperature. The samples were then adjusted to 9 μl urea, 2% CHAPS, 100 mM dithiothreitol, 15% glycerol, and 5% ampholines (5 parts 6–8, 3 parts 5–7, and 2 parts 3–10).

Gel Electrophoresis—Two-dimensional gel electrophoresis was performed on strips of carotid artery smooth muscle (Panels A and B) and umbilical artery smooth muscle (Panels C and D) that were unstimulated (Panels A and C) or stimulated with forskolin and 3-isobutyl-1-methylxanthine (Panels B and D). Homogenates of carotid (Panels E and F) and umbilical (Panels G and H) were phosphorylated in vitro in the absence (Panels E and G) or presence of cAMP-dependent protein kinase (Panels F and H). The arrowsheads refer to the relative mobility of HSP27 (12) and the arrows to the relative mobility of HSP20 (1).

cAMP-dependent Phosphorylation—Treatment of strips of bovine carotid artery smooth muscle with the adenyl cyclase activator, forskolin (10 μM), and the phosphodiesterase inhibitor, IBMX (1 mM), for 10 min leads to increases in the phosphorylation of HSP20 (Fig. 1 and Table I). In a muscle that is uniquely refractory to cyclic nucleotide-dependent relaxation, human umbilical smooth muscle, treatment with forskolin (10 μM) and IBMX (1 mM) did not lead to significant increases in the phosphorylation of HSP20 (Fig. 1, Table I). HSP20 can be phosphorylated in vitro by the catalytic subunit of cAMP-dependent protein kinase using homogenates of either bovine carotid artery smooth muscle or human umbilical artery smooth muscle (Panels A and B) and umbilical artery smooth muscle (Panels C and D) that were unstimulated (Panels A and C) or stimulated with forskolin and 3-isobutyl-1-methylxanthine (Panels B and D). Homogenates of carotid (Panels E and F) and umbilical (Panels G and H) were phosphorylated in vitro in the absence (Panels E and G) or presence of cAMP-dependent protein kinase (Panels F and H). The arrowsheads refer to the relative mobility of HSP27 (12) and the arrows to the relative mobility of HSP20 (1).
phorylated isoform of HSP20 in umbilical artery smooth muscle, with a pI of 5.9. Other human vascular tissues contain only the isoform with a pI of 5.9 (3). Thus, the isoform with a pI of 5.9 is likely the most important isoform for cyclic nucleotide-dependent vasorelaxation.

Activation of cyclic nucleotide-dependent signaling pathways by treatment of carotid or umbilical artery smooth muscles with IBMX and forskolin did not lead to increases in the phosphorylation of HSP27 (Fig. 1, Table I). The in vitro phosphorylation of HSP27 with PKA led to increases in the phosphorylation of HSP27 in umbilical but not carotid arterial homogenates. Overall, there was more phosphorylated HSP27 in umbilical compared with carotid artery smooth muscles.

Subcellular Fractionation—To determine if HSP20 and HSP27 were predominantly in cytosolic fractions of vascular smooth muscles, subcellular fractionation was performed on bovine carotid artery smooth muscles. Both HSP20 and HSP27 were in the 10,000 and 100,000 × g supernatant fractions (Fig. 2). There was some immunoreactive HSP20 and HSP27 in the 10,000 × g pellet from the unstimulated umbilical but not carotid artery homogenates (data not shown). There was no immunoreactive HSP20 in the particulate fraction from umbilical artery homogenates after IBMX/FSK stimulation (data not shown). The distribution of HSP20 and HSP27 did not change with IBMX/FSK treatment of carotid arteries.

Macromolecular Aggregates of Heat Shock Proteins and Contractile Elements—Bovine carotid or human umbilical artery smooth muscles were treated with buffer alone or with forskolin (10 μM) and IBMX (1 mM), for 10 min. The strips were homogenized and cytosolic supernatants were subjected to gel filtration as described previously (18). Immunoblots of homogenates of carotid or umbilical artery smooth muscles using specific polyclonal antibodies against HSP20 (5) or specific monoclonal antibodies against HSP27 (13) produce single bands using either antibody. Thus, the fractions from the gel filtration column were dot blotted and the amount of immunoreactive protein was analyzed using a PhosphorImager. In unstimulated carotid artery homogenates, the fraction containing the peak amount of immunoreactive HSP20 was again fraction 30 (Fig. 3, Panel A). Stimulation with forskolin and IBMX led to a shift in the peak amount of immunoreactive HSP20 to fraction 35 (Fig. 3, Panel A). In unstimulated human umbilical artery homogenates, the fraction containing the peak amount of immunoreactive HSP20 was again fraction 30 (Fig. 3, Panel B). However, in homogenates from umbilical arteries stimulated with forskolin and IBMX, which does not lead to relaxation of these muscles (3) or to significant increases in the phosphorylation of HSP20 (Fig. 3), the peak amount of immunoreactive HSP20 remained in fraction 30 (Fig. 3, Panel C). The in vitro phosphorylation of HSP20 using homogenates of bovine carotid arteries also led to a shift of the peak amount of immunoreactive HSP20 from fraction 30 to 35 (Fig. 3, Panel C). HSP20 can be phosphorylated in vitro using homogenates of umbilical artery smooth muscle (Fig. 1) and this was associated with a shift in the peak amount of immunoreactivity from fraction 30 to 35 (Fig. 3, Panel D).

In unstimulated carotid artery homogenates, the fraction containing the peak amount of immunoreactive HSP27 was fraction 35 (Fig. 4, Panel A). Stimulation with forskolin and IBMX did not lead to a shift in the peak amount of immunoreactive HSP27 (Fig. 4, Panel A). In unstimulated human umbilical artery homogenates, there were two fractions with immunoreactive HSP27, fractions 27 and 35 (Fig. 4, Panel B), and stimulation with forskolin and IBMX did not lead to a shift in the amount of immunoreactive HSP27 (Fig. 4, Panel C). The in vitro phosphorylation of homogenates of either carotid or umbilical arteries by PKA did not lead to a change in the fractions containing immunoreactive HSP27 (Fig. 4, Panels B and D).

To determine if the aggregates of the small heat shock proteins associated with specific elements of the contractile machinery, immunoblotting with antibodies against α-actin and the myosin light chains (MLC20) was performed on column fractions. In unstimulated bovine carotid artery homogenates, there was immunoreactive MLC20 in fraction 30, but not in fraction 35 (Fig. 5A). After stimulation with forskolin and IBMX, immunoreactive MLC20 was in fraction 35, but not in fraction 30. In the human umbilical homogenates, the immunoreactive MLC20 was present in fraction 35 in both unstimulated and IBMX/forskolin-treated muscles (Fig. 5B). Immunoblots of samples from fractions 30 and 35 were also probed with antibodies against α-actin. Immunoreactive α-actin was present in fractions 30 and 35 from homogenates of both carotid and umbilical arteries (data not shown).

To determine the apparent molecular weight of the macromolecular aggregates, a calibration column was performed using proteins of known molecular weight (Fig. 6). The catalase standard (232 kDa) eluted from the column just after fraction 30 and the aldolase standard (158 kDa) eluted just before fraction 35.

### Table I

**Cyclic AMP-dependent phosphorylation in vascular smooth muscle**

Intact strips of carotid (BCA) and umbilical (HUA) artery smooth muscles were phosphorylated in situ and left unstimulated (CONTROL) or stimulated with IBMX and FSK. The proteins were separated by two-dimensional electrophoresis and the extent of phosphorylation of the isoform of HSP20 with a pI of 5.9 was determined with a PhosphorImager and the results are reported as relative densitometric units. The extent of phosphorylation of the three isoforms of HSP27 were also determined and the total amount of phosphorylated HSP27 is reported. A total of three experiments is reported. The results are expressed as the mean ± S.E.; *p < 0.05 umbilical compared to carotid; #p < 0.05 stimulated compared to control.

<table>
<thead>
<tr>
<th></th>
<th>IBMX/FSK</th>
<th></th>
<th>PKA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CONTROL</td>
<td></td>
</tr>
<tr>
<td>BCA HSP20</td>
<td>48.7 ± 5.0</td>
<td>314.6 ± 39.5*</td>
<td>0</td>
</tr>
<tr>
<td>BCA HSP27</td>
<td>162.2 ± 36.2</td>
<td>101.6 ± 19.0</td>
<td>0</td>
</tr>
<tr>
<td>HUA HSP20</td>
<td>16.8 ± 8.1</td>
<td>48.0 ± 20.1</td>
<td>0</td>
</tr>
<tr>
<td>HUA HSP27</td>
<td>332.7 ± 77.9*</td>
<td>325.6 ± 82.0</td>
<td>0</td>
</tr>
</tbody>
</table>

![Fig. 2. Subcellular fractionation of HSP20 and HSP27.](image-url)
Cyclic nucleotide-dependent relaxation of vascular smooth muscle is associated with increases in the phosphorylation of a small heat shock-related protein, HSP20 (1). In this investigation, we demonstrate that this increase in phosphorylation is associated with a dissociation of macromolecular aggregates of HSP20 in a muscle that displays normal cyclic nucleotide-dependent vasorelaxation, bovine carotid artery. The non-phosphorylated HSP20 eluted from the column just before the catalase standard (232 kDa) and the phosphorylated HSP20 just before the aldolase standard (158 kDa). Thus, the macromolecular aggregates of non-phosphorylated HSP20 are in an aggregate with a molecular mass greater than 232 kDa and the phosphorylated HSP20 in an aggregate with a molecular mass less than 158 kDa. Since the phosphorylated HSP20 was clearly in an aggregate with a molecular mass greater than 67 kDa, it appears that the HSP20 aggregates do not dissociate completely into monomers after maximal activation of cyclic nucleotide-dependent signaling pathways with IBMX and forskolin.

In a muscle that is uniquely refractory to cyclic nucleotide-dependent vasorelaxation, human umbilical artery smooth muscle, HSP20 is not phosphorylated (3, 4). The macromolecular aggregates of HSP20 from umbilical smooth muscle do not dissociate with activation of cyclic nucleotide-dependent signal pathways. However, HSP20 is present in umbilical smooth muscle and can be phosphorylated in vitro with the catalytic subunit of cAMP-dependent protein kinase (PKA). The in vitro phosphorylation of HSP20 is associated with disaggregation of macromolecular aggregates.

Another small heat shock protein, HSP27, is a predominant protein in vascular smooth muscle and is phosphorylated by receptor-initiated signaling cascades involving MAPKAP kinase-2 in smooth muscles (12). Both HSP20 and HSP27 are predominantly cytosolic proteins in vascular smooth muscles. HSP27 also exists in macromolecular aggregates in bovine carotid artery.
Molecular Associations of HSP20

The following standards were applied to the Superose 6 column: thyroglobulin (669 kDa, 5 mg/ml, peak 1), catalase (232 kDa, 5 mg/ml, peak 2), aldolase (158 kDa, 5 mg/ml, peak 3), and bovine serum albumin (67 kDa, 8 mg/ml, peak 4) were applied to the column. The elution of each standard was confirmed by the relative mobility of the proteins in each fraction on gel electrophoresis (data not shown).

The data using these two muscles suggests that the small heat shock proteins, HSP20 and HSP27, are predominantly cytosolic proteins in vascular smooth muscles. Cyclic nucleotide-dependent increases in the phosphorylation of HSP20 leads to dissociation of macromolecular aggregates of HSP20. In addition, activation of cyclic nucleotide-dependent signaling pathways in vascular smooth muscles leads to changes in the macromolecular associations of MLC20. Thus, HSP20 may be an important downstream signaling mediator of cyclic nucleotide-dependent vasorelaxation and may mediate relaxation through a direct interaction with contractile elements.

Acknowledgments—We thank James Stull, Kanefusa Kato, and Mike Welsh for generously supplying antibodies, the labor and delivery nurses at the Medical College of Georgia, for umbilical cords, Shapiro’s Meatpackers for bovine carotid arteries, and Shannon Lamb for technical assistance.

REFERENCES


Carotid artery smooth muscle, however, the aggregates do not dissociate with activation of cyclic nucleotide-dependent signaling pathways. In carotid artery smooth muscle, HSP27 is in a similar fraction as the HSP20 after stimulation with IBMX and forskolin, suggesting that phosphorylated HSP20 may exist in a complex with HSP27. In the umbilical artery smooth muscle immunoreactive HSP27 was in two fractions and did not change after stimulation of the tissues with IBMX and forskolin.

The initiation of vascular smooth muscle contraction is associated with increases in the phosphorylation of the MLC20 (19). In this investigation, we determined that the MLC20 were in the same fraction as non-phosphorylated HSP20 in cytosolic homogenates of unstimulated bovine carotid artery smooth muscle. Stimulation of strips of carotid artery smooth muscle with IBMX and forskolin led to a shift of the MLC20, to fraction 35. This is the same fraction as the HSP20 was in after IBMX and forskolin treatment. However, in both unstimulated and IBMX/forskolin-treated umbilical artery smooth muscle, the MLC20 were in fraction 35, suggesting that phosphorylated HSP20 may exist in an aggregate with the MLC20. Immunoreactive α-actin was present in both fractions 30 and 35 from both tissues. Using an actin co-sedimentation assay, we have recently demonstrated that phosphorylated recombinant HSP20 is associated with globular actin whereas non-phosphorylated recombinant HSP20 is associated with filamentous actin (20). In addition, α-actin co-immunoprecipitates with HSP20 (20). Thus, while the precise mechanism by which HSP20 modulates vasorelaxation are not known, these data suggest that HSP20 is associated with specific elements of the contractile machinery, α-actin and MLC20, and may modulate vasorelaxation through a direct interaction with contractile elements.

While these studies examine vascular smooth muscles from different arterial beds and different species, these two tissues display marked differences in physiologic responses to activation of cyclic nucleotide-dependent signaling pathways. Thus, these two different tissues represent a useful model to compare the events associated with cyclic nucleotide-dependent vasorelaxation. In addition, the doses of IBMX and forskolin used to activate cyclic nucleotide-dependent cellular signaling pathways are essentially maximal doses of each agent. These doses of IBMX and forskolin lead to rapid and complete relaxation of agonist pre-contracted carotid, but not umbilical artery smooth muscles (3). Treatment of umbilical artery smooth muscles with IBMX and forskolin does not lead to significant increases in the phosphorylation of HSP20 and in fact the amount of HSP20 has been shown to be reduced after stimulation of the tissues with IBMX and forskolin.

Fig. 5. The regulatory myosin light chains. Strips of carotid (Panel A) or umbilical (Panel B) artery smooth muscle were not stimulated (solid lines) or stimulated with forskolin and 3-isobutyl-1-methylxanthine (FSK/IBMX, dotted lines). Cytosolic supernatants were separated on a molecular sieving column and immunoreactive myosin light chains (relative densitometric units) was measured in each of the column fractions (fraction number). Representative immunoblots from column fractions 30 (lanes 1 and 3) and 35 (lanes 2 and 4) from unstimulated muscles (lanes 1 and 2) or muscles stimulated with FSK/IBMX (lanes 3 and 4) are depicted in the upper right of each panel. The relative mobility of molecular weight markers is noted on the left of each immunoblot. These results are representative of three separate experiments.

Fig. 6. Calibration of the Superose 6 column. The following standards were applied to the Superose 6 column: thyroglobulin (669 kDa, 5 mg/ml, peak 1), catalase (232 kDa, 5 mg/ml, peak 2), aldolase (158 kDa, 5 mg/ml, peak 3), and bovine serum albumin (67 kDa, 8 mg/ml, peak 4) were applied to the column. The elution of each standard was confirmed by the relative mobility of the proteins in each fraction on gel electrophoresis (data not shown).
Phosphorylation of the Small Heat Shock-related Protein, HSP20, in Vascular Smooth Muscles Is Associated with Changes in the Macromolecular Associations of HSP20

Colleen M. Brophy, Mary Dickinson and David Woodrum

doi: 10.1074/jbc.274.10.6324

Access the most updated version of this article at http://www.jbc.org/content/274/10/6324

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 18 references, 12 of which can be accessed free at http://www.jbc.org/content/274/10/6324.full.html#ref-list-1