Transduction of phosphorylated heat shock-related protein 20, HSP20, prevents vasospasm of human umbilical artery smooth muscle

Charles R. Flynn, Colleen M. Brophy, Padmini Komalavilas, Deron Tessier, Jeffrey Thresher, and Lokesh Joshi

The Biodesign Institute, Arizona State University, Tempe; Department of Surgery, Mayo Clinic Scottsdale; Scottsdale; and Carl T. Hayden Veterans Affairs Medical Center, Phoenix, Arizona

Submitted 21 September 2004; accepted in final form 22 December 2004

Flynn, Charles R., Colleen M. Brophy, Padmini Komalavilas, Deron Tessier, Jeffrey Thresher, and Lokesh Joshi. Transduction of phosphorylated heat shock-related protein 20, HSP20, prevents vasospasm of human umbilical artery smooth muscle. J Appl Physiol 98: 1836–1845, 2005; doi:10.1152/japplphysiol.01043.2004.—Activation of cyclic nucleotide-dependent signaling pathways inhibits agonist-induced contraction of most vascular smooth muscles except human umbilical artery smooth muscle (HUASM). This impaired vasorelaxation may contribute to complications associated with preeclampsia, intrauterine growth restriction, and preterm delivery. Cyclic nucleotide-dependent signaling pathways converge at the phosphorylation of the small heat shock-related protein HSP20, causing relaxation of vascular smooth muscle. We produced recombinant proteins containing a protein transduction domain linked to HSP20 (rTAT-HSP20). Pretreatment of HUASM with in vitro phosphorylated rTAT-HSP20 (rTAT-pHSP20) significantly inhibited serotonin-induced contraction, without a decrease in myosin light chain phosphorylation. rTAT-pHSP20 remained phosphorylated upon transduction into isolated HUASM as demonstrated by two-dimensional gel electrophoresis. Transduction of peptide analogs of phospho-HSP20 containing the phosphorylation site on HSP20 and phosphate-resistant mimics of the phosphorylation site (S16E) also inhibited HUASM contraction. These data suggest that impaired relaxation of HUASM may result from decreased levels of phosphorylated HSP20. Protein transduction can be used to restore intracellular expression levels and the associated physiological response. Transduction of posttranslationally modified substrate proteins represents a proteomic-based therapeutic approach that may be particularly useful when the expression of downregulated in full-term human umbilical artery smooth muscle (HUASM), which is refractory to cyclic nucleotide-dependent relaxation (4, 5). These findings implicate phosphorylated HSP20 as a point in which the cyclic nucleotide signaling pathways converge to prevent contraction or cause relaxation. This pathway is of significant pharmacological interest with numerous drugs in clinical use that activate various aspects of the pathway (Fig. 1).

One relatively new modality for delivering cargo (i.e., proteins, nucleic acids, liposomes, gold particles) into mammalian cells employs the use of positively charged protein transduction domains (PTDs) found in a few select proteins such as the human immunodeficiency virus-1 transcriptional activator TAT (10, 12, 35), the Drosophila homeoprotein antennapedia transcription protein (17, 21), or the herpes simplex virus structural protein (9). Transduction efficiency does not appear to be limited by the size or type of protein being transduced, and the range of tissues and cells amenable to transduction is extensive (29). Although the mechanism of protein transduction is unclear, recent reports suggest that the mechanism of entry into the cytoplasm may be through macropinocytosis and that the programmed disruption of macropinosomes via alteration of pH effectively increases transduction efficiency (35). Most studies using protein transduction domains have focused on delivering fluorescently labeled cargos or delivering proteins that lead to apoptosis (33). In contrast, HUASM presents a unique model to determine whether protein transduction can be used to restore a physiologically relevant response (relaxation) by transducing phosphorylated HSP20. Although impaired vasorelaxation may contribute to the postpartum vasospasm necessary for the separation of the maternal-fetal circulation, the impaired relaxation in umbilical artery may contribute to pregnancy complications such as preeclampsia, intrauterine growth restriction, and preterm delivery. It has been shown that abnormal Doppler waveforms in the umbilical...
artery preceded signs of fetal hypoxemia in more than 90% of cases (3). In another study, a majority of patients with abnormal umbilical waveforms deliver small-for-gestational-age infants, require cesarean section for fetal distress, and delivered preterm (7).

METHODS

Construction of bacterial expression vectors. Standard techniques were used for DNA manipulations. Base sequences for all DNAs were confirmed by nucleotide sequence analysis performed with an Applied Biosystems 377 automated sequencer (ASU Bioresources Facility). Heavy- and light-chain cDNAs encoding TAT-HSP20 and (5′) primer (5′/H11032 CTAC GGCCGCAAGAAACGCCGCCAGCGCCGCCGCGGCCT-3′) were amplified from pAS2-1 (Clontech, Palo Alto, CA) by using a mutagenic forward mutagenic primer (5′/H11032 GATGAGAACCCCGCATAT-3′). Complementary oligonucleotide sets (CCA-3′/H11032 GATGAGAACCCCGCATAT-3′) and HSP20 were used for DNA manipulations. Base sequences for all DNAs were confirmed by nucleotide sequence analysis performed with an Applied Biosystems 377 automated sequencer (ASU Bioresources Facility).

Expression and purification of HSP20 proteins. Protein was expressed in Escherichia coli as previously described by Panitch et al. (24a). Briefly, single colonies of Rosetta(DE3)pLysS (Novagen) containing recombinant pET14b-rTAT-HSP20, pET14b-rTAT-HSP20 S16A, or pET14b-rHSP20 were used to inoculate 4 liters of minimal media {KH2PO4 13.3 g/l, (NH4)HPO4 4.0 g/l, citric acid 1.7 g/l, MgSO4 7H2O 1.2 g/l, a trace metal solution 10 ml/l containing [6 g/l iron(III) citrate, 1.5 g/l MnCl2·4H2O, 0.8 g/l Zn(CH3COO)2·H2O, 0.3 g/l H3BO3, 0.25 g/l Na2MoO4·2H2O, 0.25 g/l CuCl2·6H2O, 0.15 g/l CuCl2·2H2O], thiamine hydrochloride 4.5 mg/l, glucose 27.5 g/l, antifoam 0.1 ml/l, feed solution glucose 500 g/l, and NH2OH 25%}, and cultures were grown in a New Brunswick fermentor. Cultures were induced with 2 mM isopropyl-1-thio-β-D-galactopyranoside when the optical density at 600-nm wavelength reached 25–30. After 5 h, cells were harvested by centrifugation (6,000 g, 10 min) and stored at −20°C. Frozen cells were thawed and thoroughly resuspended in 1× TNE buffer (50 mM NaCl, 1 mM EDTA, and 500 mM Tris, pH 8.0) and incubated at 4°C for 30 min. After sonication on ice, the inclusion bodies containing recombinant protein were harvested by centrifugation (19,000 g, 10 min). Metal chelation affinity chromatography was used to purify recombinant HSP20 analogs. Briefly, inclusion bodies were resuspended in binding buffer (20 mM Na2HPO4, 0.5 M NaCl, 50 mM imidazole, pH 7.4, 8 M urea). The sample was then added to Ni2+-charged Chelating Sepharose Fast Flow (Pharmacia Biotech, Peapack, NJ) and incubated for 30 min at room temperature. The resin was then loaded in a water-chilled XK-26 column and washed extensively with binding buffer on an AKTA fast-performance liquid chromatography system (Pharmacia Biotech, Peapack, NJ).
Protein was refolded by using an overnight linear gradient of urea from 8 to 0 M and eluted with binding buffer containing 500 mM imidazole. The eluate was concentrated by using a stirred ultrafiltration cell (8200, Millipore, Bedford, MA) with a 10,000-molecular weight limit filter under 75 psi nitrogen.

**Peptide synthesis.** Peptides containing an 11-amino-acid modified TAT protein transduction domain (YARAAQRQA-ascribed PDT) (15) were fused to either the functional 13-amino-acid sequence (WLRRaSAPLPLGKL, where P denotes phosphoserine) of HSP20 (PTD-pHSP20, a 24-amino-acid peptide) or a phosphomimetic HSP20 sequence (WLRRAEAPLPLGKL, PTD-HSP20 S16E, also a 24-amino-acid peptide) that were synthesized at Arizona State University. Peptides were synthesized by using standard solid-phase peptide synthesis, which uses standard Fmoc chemistry. Crude peptide was purified by using reverse-phase fast protein liquid chromatography (Akta Explorer, Amersham Biosciences, Piscataway, NJ). A linear gradient of water/acetone/tetrahydrofuran containing 0.1% trifluoroacetic acid was used with a C18 column (Grace Vydac, Hesperia, CA). Identification of peptides was confirmed with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) or electrospray ionization mass spectrometry (Waters, Milford, MA), and purity was evaluated by analytical reverse phase Alliance HT HPLC (Waters, Milford, MA). Only peptides with molecular masses identical to the predicted mass and >95% purity were used. The peptides were stored as the lyophilized powder or prepared as 20 mM stock solutions in sterile, deionized water and aliquots stored at −20°C until use.

**In vitro phosphorylation of TAT-HSP20.** Recombinant TAT-HSP20 was phosphorylated in a 1-mL reaction containing physiological saline solution (PSS) buffer (20) (in mM: 140 NaCl, 5 KCl, 1.6 CaCl₂, 1.2 MgCl₂, 1.2 Na₂HPO₄, 5.6 glucose, 2 MOPS, and 0.02 EDTA, pH 7.4) supplemented with 1 mM dithiothreitol (DTT), 20 mM MgCl₂, 0.2 mM ATP, and 500 units of the catalytic subunit of protein kinase A (PKA) (Promega, Madison, WI). The reaction was incubated for 30 min at 30°C and stopped by incubation at 50°C for 10 min.

**MALDI mass spectrometric analysis.** Samples were analyzed at the Protein Chemistry Core Facility at Arizona State University in a Voyager-DE STR MALDI-TOF Biospectrometry Workstation operating in the positive linear mode. For tryptic digests, proteins (∼4 mg) were added to 50-μL reaction containing 50 mM ammonium bicarbonate buffer pH 8.0 and 0.5 μM G-(tosylamido-2-phenyl) ethyl chloromethyl ketone-treated trypsin (Worthington Scientific, Lakewood, NJ). Samples were digested overnight at 37°C. Protein samples were combined 1:1 or 1:10 with a saturated sinnapinic acid matrix (intact proteins) or a saturated α-cyano-4-hydroxy-cinnamic acid matrix solution (tryptic digests) in 50% acetonitrile-water and 0.1% trifluoroacetic acid. Samples were deposited on the analysis plate, air-dried, then subjected to laser pulses with an acceleration voltage of 25,000 V h. Samples were overlaid with ∼2 ml of mineral oil and allowed to equilibrate for 16 h at room temperature before isoelectric focusing for 20,000 V h. Samples were subsequently equilibrated for 15 min first in an SDS equilibration buffer (50 mM Tris·Cl pH 8.8, 6.0 M urea, 2% CHAPS, 10 mM DTT, 0.02% bromophenol blue) containing 5% ampholines (pH 3–10) for 15 min. Protein (75 μg) in a 120-μL volume was applied to the entire length of one channel in an isoelectric focusing sample tray (Bio-Rad) to which an immobilized pH gradient strip (linear pH gradient range 3–10, 7-cm length) was applied gel side down. Samples were overlaid with ∼2 ml of mineral oil and allowed to equilibrate for 16 h at room temperature before isoelectric focusing for 20,000 V h. Samples were subsequently equilibrated for 15 min first in an SDS equilibration buffer (50 mM Tris·Cl pH 8.8, 6.0 M urea, 2% glycerol, 2% lauryl sulfate, and 0.02% bromophenol blue) containing 1% DTT and second (15 min) in equilibration buffer containing 2.5% iodoacetamide. After equilibration, samples were separated in the second dimension on precast 4–20% polyacrylamide mini-gels in 1× electrophoresis buffer (25 mM Tris·Cl pH 8.3, 192 mM glycine, 0.1% wt/vol SDS) at 120 V for 1.5 h. Electrophoretic transfer of proteins from the gels onto polyvinylidene difluoride membranes was carried out in 1× electrophoresis buffer (25 mM Tris·Cl pH 8.3, 192 mM glycine, 0.1% wt/vol SDS) at 120 V for 1.5 h. Immunoblotting of the PVDF membranes was performed by using a standard chemiluminescence-based system (Bio-Rad) with the appropriate secondary antibody (1:2,000 dilution) and visualized using an Immunolight Western blot detection system (Li-Cor Biosciences, Lincoln, NE). The densities of the bands were quantified by using software packaged with the instrument.

**Determination of MALDI phosphorylation.** Rings of porcine coronary artery and human umbilical artery were treated as described above, then frozen immediately in liquid nitrogen while still attached to the force transducer. The frozen samples were pulverized and placed in a frozen slurry of precipitating solution consisting of 90% acetone, 10% trichloroacetic acid, and 10 mM DTT and then allowed to melt to room temperature. The precipitating solution was removed, and the tissues were washed three times with 90% acetone and 10 mM DTT.
The samples were dried, and the pellets were suspended in urea-CHAPS buffer consisting of 9 M urea, 2% CHAPS, and 100 mM DTT and then vortexed to solubilize the proteins. Protein (10 µg) was diluted with 10 µl of urea sample buffer (6.7 M urea, 18 mM Tris, 20 mM glycine, 9 mM DTT, 4.6% saturated sucrose, and 0.004% bromophenol blue) and separated on glycerol-urea minigels (40% glycerol, 10% acrylamide, 0.5% bisacrylamide, 20 mM Tris, and 22 mM glycine) (25). Electrophoretic transfer of proteins from the gels onto polyvinylidene difluoride membranes was carried out in a buffer containing 10 mM Na2HPO4 (pH 7.6) at 25 V for 1 h at 20°C. The blot was incubated with rabbit anti-bovine tracheal smooth muscle MLC 20 (MLC20; 1:10,000 dilution in TBST) and IRDye700DX-conjugated affinity purified anti-rabbit secondary antibody (1:5,000 dilution in TBST). Membranes were scanned and processed as described above.

Statistical analysis. Data are reported as means ± SE. The comparison between variables was analyzed by Student’s t-test for paired data. Significance was accepted at the P < 0.05 level.

RESULTS

Vasorelaxation is impaired in umbilical artery. We used a muscle perfusion system to gauge physiological responses of isolated smooth muscle tissues. Human umbilical arteries were obtained from normal term umbilical cords. Porcine coronary arteries were obtained from an abattoir and were used as control smooth muscles (i.e., muscles that relaxed in response to activation of cyclic nucleotide-dependent signaling pathways).

The muscle rings were treated with 0.5 µM serotonin (5-HT) to determine submaximal contractile force, then washed and equilibrated with physiological saline solution (PSS) buffer until tension returned to basal levels. Umbilical arteries pretreated with PSS contracted in response to 0.5 M serotonin (5-HT) in a manner similar to that observed with porcine coronary arteries (Fig. 2A). Pretreatment with 1 µM SNP inhibited contraction of porcine coronary artery to 33.02% of maximal levels, whereas umbilical smooth artery pretreated with the same dose of SNP contracted to 99.13 ± 10.29% of the baseline maximal levels (Fig. 2B). Isoproterenol pretreatment (10 µM) completely inhibited 5-HT-induced contraction of porcine coronary artery but not umbilical artery (97.24% ± 11.22 baseline maximal levels). 5-HT-induced contraction also was not inhibited in umbilical artery smooth muscle by other known vasorelaxants (1 µM forskolin, 0.1 µM atrial natriuretic peptide, and 10 µM papaverine; traces not shown). These data confirm that full-term HUASM is uniquely refractory to inhibition of contraction in a manner similar to impaired relaxation of agonist-contracted muscle (4).

Recombinant HSP20 production and characterization. To determine whether restoration of intracellular levels of phosphorylated HSP20 levels would attenuate vasospasm, arterial strips were treated with recombinant fusion proteins composed of a PTD fused to human HSP20. The DNA sequences encoding a histidine tag in tandem with or without the PTD from the human immunodeficiency virus TAT protein were appended downstream of the HSP20 start methionine (Fig. 3A). Site-directed mutagenesis was performed to encode an alanine for serine 16 nonphosphorylatable control (tTAT-HSP20 S16A). Each of these constructs was introduced independently into E. coli, and protein expression was induced with isopropylthiogalactoside. A protein having an apparent molecular mass of 24 kDa increased in abundance after induction and was recognized by anti-HSP20 antibodies (Fig. 3, B and C). Recombinant proteins from each of three constructs were purified by urea solubilization and metal chelation affinity binding and refolded by the gradual removal of urea during a linear urea
Recombinant HSP20 migrated with an apparent molecular mass of ~21 kDa, whereas the transducible rTAT-HSP20 and rTAT-HSP20 S16A migrated at ~24 kDa.

To examine the apparent discrepancy between the calculated molecular mass and the observed mass on the basis of electrophoretic mobility in SDS-PAGE, MALDI-TOF spectroscopy was employed. Representative spectra obtained from each purified protein are shown (Fig. 3E). The dominant ion of rTAT-HSP20 was at 19,885.23 m/z with a minor ion species at 20,063.30 m/z, both of which differed slightly from the expected molecular mass of 20,043.81 Da. rTAT-HSP20 S16A exhibited a dominant ion at 19,870.16 m/z and a minor ion species at 20,046.22 m/z. There was an observed 15.07-Da mass difference between the major ion species in rTAT-HSP20 and rTAT-HSP20 S16A, a difference similar to the expected mass difference of 16 Da. Recombinant HSP20 (with an appended histidine tag) exhibited a dominant ion at 18,524.6 m/z and a minor ion species at 18,703.3 m/z, in contrast to the expected molecular mass of HSP20, which was 18,544.06 Da. These findings suggest that the migration behavior of the major proteins of interest (i.e., migrating with a mass greater than the contaminating band) was likely due to the positively charged residues at the amino terminus and that there were some alterations in the predicted amino acid sequences of HSP20 variants.

The nature of the differences between expected and observed masses of the purified recombinant proteins was investigated by trypsin digestion and MALDI-TOF mass spectrometry. The resulting ionization spectrum of the rTAT-HSP20 tryptic digest is shown (Fig. 4A). The resulting masses were compared with the expected peptide masses and amino acid sequences obtained after in silico digestion (Fig. 4B). A reasonable correlation of the fragmentation data with the expected mass data was observed for 8 of the 11 peptide fragments with lengths longer than two residues. Three expected peptide fragments with lengths greater than two residues (fragments 1, 13, and 20) were not accounted for in the spectrum. For fragment 1, however, a dominant ion of 1,475.53 m/z corresponding to the mass of fragment 1 void of the start methionine (bold) (1,475.65 m/z) was observed. Other bacterially produced recombinant proteins lacking an initiation methionine have been described previously (14, 27, 39). For fragment 13, a minor ion corresponding to the amino acid sequence encoded by the rTAT-HSP20 cDNA was not readily identified. However, a dominant ion of 2,915.88 m/z likely attributable to a posttranslationally modified fragment 13 was observed. The ion at 3,836.13 m/z (fragment 20) corresponds to a peptide resulting from one missed cleavage at R146 (fragment 20 + 21 = 3,837.05 m/z). Similar results were obtained for rTAT-HSP20 S16A (data not shown). Trypsin-digest analysis of recombinant HSP20 revealed the presence of an initiating methionine (data not shown) and the lack of an ion corresponding to fragment 13. Collectively, these results suggest that the rTAT-HSP20 and rTAT-HSP20 S16A analogs expressed and purified from
Bacteria lack the initiating methionine and, in addition, harbor an alteration of (or modification to) at least one amino acid in fragment 13 (FGEGLLEAELAALCPTTLAPYYLR). Recombinant HSP20 without a PTD possessed an intact initiating methionine as well as an alteration in the peptide fragment corresponding to fragment 13 (data not shown).

HSP20 transduction restores expression and prevents contraction. Purified and biochemically characterized recombinant proteins were assessed for physiological activity. A fraction of the rTAT-HSP20 was phosphorylated in vitro using the catalytic subunit of cAMP-dependent protein kinase yielding rTAT-pHSP20. Rings were washed with PSS buffer and challenged with submaximal dose of 5-HT and the contractile responses recorded. Rings were washed and equilibrated with PSS buffer and then treated with PSS buffer (control), rTAT-HSP20 or rTAT-pHSP20 (7.5 μM for 10 min). After addition of submaximal doses of 5-HT, the contractile response was recorded (Fig. 5A). Pretreatment with nonphosphorylated rTAT-HSP20 did not significantly attenuate the magnitude of the 5-HT-induced contraction (12.22 ± 8.73%, \( P > 0.05 \)); however, the contractile force was not sustained over time. The mutant rTAT-HSP20 S16A did not inhibit 5-HT-induced contraction (5.3 ± 3.00% inhibition, \( P > 0.05 \); tracing not shown), and the contraction was sustained in a manner similar to 5-HT alone. In all these cases, removal of the agonist (5-HT) led to passive force reduction back to baseline. Pretreatment with rTAT-pHSP20 significantly inhibited 5-HT-induced smooth muscle contraction (81.99 ± 6.83% inhibition, \( P < 0.05 \), Fig. 5B).

To determine that relatively physiological levels of HSP20 were restored with protein transduction, immunoblots of treated and untreated arteries were probed with anti-HSP20 antibodies. After indicated treatments, the rings were rinsed extensively with PSS buffer, snap frozen in liquid nitrogen, and total protein extracted. HSP20 was detected in all tissues treated with recombinant HSP20 analogs but was not detected in untreated umbilical rings or those treated with 5-HT alone (Fig. 5C). In porcine coronary rings, endogenous HSP20 migrated at a lower molecular mass than rTAT-HSP20. Recombinant TAT-HSP20 migrated similar to HSP20 from extracts of treated tissue. In untreated rings treated with recombinant protein and different than that observed in untreated pig coronary extracts.
These results suggest that the protein detected in extracts from recombinant protein-treated tissues migrated at a higher relative mass owing to the appended protein transduction domain. Taken together, these results suggest that transduction of phosphorylated HSP20 can restore physiological amounts of HSP20 and attenuate umbilical artery vasospasm.

To verify that HSP20 phosphorylation at serine 16 was important in mediating inhibition of contraction in HUASM, we employed isoelectric focusing followed by SDS-PAGE (two-dimensional gel electrophoresis) of treated HUASM homogenates. Monoclonal anti-HSP20 antibodies did not detect endogenous HSP20 in control (buffer-treated HUASM) or 0.5 μM 5-HT-treated tissues (Fig. 5D, top two panels). However, in rTAT-HSP20-treated HUASM a single spot was detected at a pI of ~9.00 and a molecular mass of ~25 kDa corresponding to the transduced rTAT-HSP20. The location of this spot on the gel was in agreement with the mass observed for rTAT-HSP20 shown in Fig. 2 and in agreement with the projected pI of 9.25. In contrast, tissues treated with rTAT-pHSP20 revealed a second, more acidic spot of similar mass representing the phosphorylated rTAT-HSP20. These findings suggest that phosphorylated rTAT-pHSP20 is present in HUASM after transduction.

A phosphopeptide analog of HSP20 that contains a short fragment surrounding serine 16 has been shown to relax bovine carotid artery, porcine coronary artery, and human saphenous vein smooth muscle (7). This analog, PTD-pHSP20 (YARAAARQARAWLRRApSAPLPGLK, where pS denotes phosphoserine), is composed of an optimized protein transduction domain (underlined) fused to sequences encoding the active portion [phosphorylation site, serine 16 (1)] of HSP20. A second analog also was synthesized that was identical in sequence to PTD-pHSP20 except the phosphoserine was replaced with a nonhydrolyzable glutamate residue (PTD-pHSP20 S16E). HUASM was precontracted with 5-HT, and the peptide analogs were added to the bath at increasing concentrations (Fig. 6). Both PTD-pHSP20 and PTD-HSP20 S16E led to dose-dependent relaxation of agonist precontracted HUASM. PTD alone or a peptide composed of the same amino acids in the pHSP20 domain arranged in a scrambled configuration (PTD-pHSP20scr) did not induce relaxation (data not shown). These findings,
Mechanisms that activate force generation. Inhibits muscle contraction by a mechanism independent of the 5-HT. Collectively, these data suggest that rTAT-pHSP20 inhibits force generation, but not MLC20 phosphorylation in response to 5-HT, regardless of HSP20 pretreatment. This transient permeabilization of intact rings of vascular smooth muscle or cardiac myocytes and treatment with phosphopeptide analogs of HSP20 alters physiological responses (1, 26). However, these approaches have significant limitations. Intracellular delivery via protein transduction represents an additional tool to characterize the functional relevance of proteins in that physiological levels of postranslationally modified proteins can be restored.

In this study we demonstrate that treatment of term human umbilical artery smooth muscle with rTAT-pHSP20 restores the physiological response of inhibiting agonist-induced contraction. Treatment with nonphosphorylated rTAT-HSP20 did not inhibit agonist-induced contraction; however, the contractions were transient (Fig. 5A, middle). It is possible that the rTAT-HSP20 was phosphorylated by endogenous PKA or PKG. This possibility is supported by the fact that mutated rHSP20 in which the serine 16 was replaced with an alanine did not inhibit agonist-induced contraction and the contractions were not sustained. Relatively physiological amounts of the protein were restored and the proteins remained phosphorylated in the tissues (Fig. 5, C and D, respectively).

Phosphopeptide analogs of HSP20 containing protein transduction domains relaxed agonist precontracted HUASM (Fig. 6). However, much higher concentrations of the peptides were required compared with the recombinant fusion proteins (∼10⁻³ vs. 10⁻⁶ M, respectively). In addition, the peptides did not inhibit agonist-induced contractions (data not shown). This effect is not surprising given the more extensive tertiary structure of the recombinant protein compared with the peptide analogs.

It has been proposed that PKG mediates vasorelaxation through phosphorylation and activation of the regulatory chain of the myosin phosphatase (31). However, the MLC20/phospho-MLC20 molar ratio was similar in 5-HT-contracted tissues in the presence or absence of rTAT-HSP20, despite the combined with the detection of rTAT-pHSP20 that remained phosphorylated after transduction into tissue, support the premise that phosphorylation of HSP20 is at least one mediator of inhibiting contraction of HUASM. Because increases in the phosphorylation of the regulatory MLCs have been implicated in the initiation of contraction, we determined the effect of rTAT-pHSP20 on MLC phosphorylation. Umbilical artery rings were snap frozen in liquid nitrogen while still connected to the force transducer, and total protein extracts were prepared as described previously (20). The measured contraction in all treatments was similar to that shown previously. Antibodies specific for MLC20 were used to probe total protein extracts of pretreated and untreated (basal) umbilical arteries that were separated on glycerol-urea gels (18, 34) (Fig. 7A). The intensity values of densitometric scans were used to calculate phosphoMLC20/MLC20 molar ratios (Fig. 7B). MLC20 phosphorylation in umbilical artery increased upon stimulation with 5-HT, regardless of HSP20 pretreatment or relaxation. Thus pretreatment with rTAT-pHSP20 inhibited force generation, but not MLC20 phosphorylation in response to 5-HT. Collectively, these data suggest that rTAT-pHSP20 inhibits muscle contraction by a mechanism independent of the mechanisms that activate force generation.

**Discussion**

HUASM is an ideal model for vasospasm in that it is refractory to agonists that normally elicit relaxation. This impaired cyclic nucleotide-dependent relaxation is associated with decreased expression and phosphorylation of HSP20 (2, 4). In this study, we used protein transduction to restore both expression of HSP20 at physiologically relevant levels and physiological responsiveness (i.e., inhibition of agonist-induced contraction). Previous studies have used genetic manipulation or permeabilization to alter intracellular levels of specific proteins. For example, genetic manipulation of mesangial cells to overexpress HSP20 inhibits contractile responses (37).

![Graph](image)

**Fig. 6.** Phosphopeptide analogs of HSP20 relax agonist precontracted muscles. HUASM was precontracted with 5-HT (0.5 μM) followed by treatment with a PTD-pHSP20 phosphopeptide-mimetic (YARAARQARWLRRApSAPLGLK) denoted ■ (solid line) or a phosphopeptide-mimetic possessing a S16E substitution (YARAARQARWLRRRESAPLGLK) denoted PTD-HSP20 S16E (▲; dashed line) at increasing concentrations (Conc).

**Fig. 7.** HSP20 attenuates vasospasm independent of myosin light chain phosphorylation. A: umbilical artery was treated as indicated in Figs. 2 and 5 and was snap frozen after 2 min while attached to the force transducer. Immunoblot analyses using anti-myosin light chain (MLC) 20 (MLC20) antibody to probe HUASM homogenates shows that MLC20 phosphorylation levels for all treated tissues are comparable. B: as expected, quantified MLC20 phosphorylation molar ratios (mol P/mol MLC20) confirm that MLC phosphorylation for 5-HT-treated tissues is significantly greater than that for basal. In addition, MLC phosphorylation remains elevated for HSP20 treatments, despite the relaxation observed with rTAT-pHSP20 pre-treatment (*P < 0.05, compared with the respective 5-HT phosphorylation, n = 3).
significantly inhibited force production with rTAH-pHSP20 pretreatment. These results support the findings of other investigators, which suggest that HSP20-induced relaxation is dissociated from changes in MLC20 phosphorylation (11, 28, 38). Although the precise mechanisms of HSP20-induced relaxation are not known, small heat shock proteins have been shown to modulate actin filament dynamics (1, 2, 6, 32). Treatment of cultured cells with phosphopeptide analogs of HSP20 leads to loss of actin stress fibers (7). In addition, phosphorylated HSP20 contains a 14–3–3 consensus sequence and binds to 14–3–3 proteins (7). Phosphocoflin, an actin-depolymerizing protein, also binds to 14-3-3 proteins. One possible mechanism by which pHSP20 mediates relaxation is via disrupting protein-protein interactions (cofilin:14-3-3), leading to actin depolymerization. Thus HSP20 may mediate relaxation via a thin filament regulatory process.

One limitation of this study is that the umbilical artery smooth muscle had high levels of basal MLC phosphorylation, consistent with other reports using this tissue (38). Another limitation of this study was the comparison of vascular smooth muscles from different vascular beds and different species. Nevertheless, we show that transduced bioactive molecules can attenuate smooth muscle contraction. An advantage of this system was that immunoblots of tissue homogenates could be performed to correlate the relative amounts of intracellular HSP20 with physiological responses. In addition, this physiological activity was dependent on a phosphorylated bioactive protein. Finally, this approach bypasses receptors, signaling molecules, and gene expression and allows for a functional assessment of the activity of a protein in human tissue. The inhibition of contraction associated with transduction of phosphorylated HSP20 provides more evidence supporting a role for HSP20 in mediating relaxation. Taken together, these data suggest that protein transduction of posttranslationally modified molecules represents a “proteomic”-based approach to the development of therapeutics.

ACKNOWLEDGMENTS

We thank Dr. Dan Brune and John Lopez for assistance with mass spectrometry. We extend special thanks to Dr. Jim Stull for providing antibodies to 20-kDa MLC.

GRANTS

This work was supported by the National Institutes of Health (C. Brophy) and the American Heart Association (L. Joshi).

REFERENCES


