Effects of chronic portal hypertension on small heat-shock proteins in mesenteric arteries

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Chen, Xuesong, Hai-Ying Zhang, Kristin Pavlish, and Joseph N. Benoit. Effects of chronic portal hypertension on small heat-shock proteins in mesenteric arteries. Am J Physiol Gastrointest Liver Physiol 288: G616–G620, 2005. First published October 28, 2004; doi:10.1152/ajpgi.00439.2004.—Previous studies have shown that impaired vasoconstrictor function in chronic portal hypertension is mediated via cAMP-dependent events. Recent data have implicated two small heat-shock proteins (HSP), namely HSP20 and HSP27, in the regulation of vascular tone. Phosphorylation of HSP20 is associated with vasorelaxation, whereas phosphorylation of HSP27 is associated with vasoconstriction. We hypothesized that alterations in the expression and/or phosphorylation of small HSPs may play a role in impaired vasoconstriction in portal hypertension. A rat model of prehepatic chronic portal hypertension was used. Studies were conducted in small mesenteric arteries isolated from normal and portal hypertensive rats. Protein levels of HSP20 and HSP27 were detected by Western blot analysis. Protein phosphorylation was analyzed by isoelectric focusing. HSP20 mRNA expression was determined by RT-PCR. To examine the role of cAMP in the regulation of small HSP phosphorylation and expression, we treated both normal and portal hypertensive vessels with a PKA inhibitor Rp-cAMPS. We found both an increased HSP20 phosphorylation and a decreased HSP20 protein level in portal hypertension, both of which were restored to normal by PKA inhibition. However, PKA did not change HSP20 mRNA expression. We conclude that decreased HSP20 protein level is mediated by cAMP-dependent pathway and that impaired vasoconstrictor function in portal hypertension may be partially explained by decreased expression of HSP20. We also suggest that the phosphorylation of HSP20 by PKA may alter HSP20 turnover.

vascular smooth muscle; mesenteric artery; adenosine 3',5'-cyclic monophosphate; protein kinase A

AN INTRIGUING VASCULAR CONSEQUENCE of chronic portal hypertension is a decreased ability of blood vessels to respond to vasoconstrictor stimuli. This finding has been attributed to postreceptor defect in vascular smooth muscle excitation contraction coupling (22). Additional evidence supports the contention that prolonged elevation of cyclic nucleotide-dependent vasodilators leads to this impaired vasoconstrictor function inasmuch as vasoconstrictor effectiveness in portal hypertensive arteries can be restored by PKA inhibition (27). However, the nature of the cellular mechanism is still not clear).

Recent studies have shown that two small heat-shock proteins (HSPs), namely HSP20 and HSP27, are involved in regulation of vascular smooth muscle contraction. Both are constitutively expressed in vascular smooth muscle (10). In response to cyclic nucleotide, HSP20 is phosphorylated on residue Ser-16 (1), which may induce dissociation of oligomer of this protein to dimer. HSP20 has been shown to be an actin-associated protein, which also depends on its phosphorylation status (6, 23). Although considerable evidence supports the contention that HSP20 phosphorylation plays a regulatory role in smooth muscle contraction, the exact mechanism of this regulation remains unclear. Phosphorylation of HSP27 is the result of activation of p38 MAP-kinase cascade downstream of PKC-α and/or activation of RhoA/Rho-kinase (14, 15, 26). The phosphorylation of HSP27 has been shown to favor actin polymerization and promote smooth muscle contraction (21, 16).

In this study, we examined the expression and phosphorylation profile of both HSP20 and HSP27 in mesenteric arterial smooth muscle during chronic portal hypertension. We show a PKA-dependent increase in HSP20 phosphorylation but a decrease in HSP20 protein level in portal hypertension. We suggest that phosphorylation of HSP20 by PKA may alter HSP20 turnover.

MATERIALS AND METHODS

Production of chronic prehepatic portal hypertension and vessels preparation. Portal hypertension was surgically produced as previously described by our lab (2, 3). Eighty-two Sprague-Dawley rats (~300 g) were anesthetized with isoflurane, and a midline abdominal incision was made. The common portal vein was dissected free of surrounding tissue, and a loose ligature of 3–0 silk suture was guided around it. A 20-gauge blunt-end needle was placed alongside the portal vein, and the suture was tied snugly around the portal vein and needle. The needle was subsequently removed to yield a calibrated stenosis of the portal vein. The abdominal contents were moved back in place, and the abdominal incision was closed in layers with suture and metal wound clips. Anesthesia was discontinued, the suture lines were treated with antibiotic ointment, and the animals were allowed to recover. A single dose of buprenorphine (0.25 mg/kg) was given subcutaneously preoperatively to alleviate any postsurgical pain. The animals were returned to the vivarium, and portal hypertension was allowed to develop. Fourteen days postsurgery, the animals were ready for experimental use. All animal procedures were approved by the University of North Dakota Animal Care and Use Committee.

Rats were anesthetized with isoflurane and euthanized. A segment of mid-small intestine and adjacent mesentery was excised and placed in ice-cold physiological salt solution (PSS; in mM: 119 NaCl, 5 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 25 NaHCO₃, 1.2 NaH₂PO₄, 0.027 EDTA, and 5.5 glucose, pH 7.4). Small mesenteric arteries (diameter ~250 μm) were dissected free of surrounding tissue. Vessels were then frozen in liquid nitrogen for biochemical analysis with or without incubation in 50 μM of the PKA inhibitor adenosine 3',5'-cyclic monophosphothioate (Rp-cAMPS, Alexis Biochemicals) for 30 min.
Detection of HSP20 and HSP27 phosphorylation. The phosphorylation status of HSP20 and HSP27 (20) was determined by isoelectric focusing. Frozen tissues were thawed in a 10% TCA/10 mM DTT/acetone slurry. Samples were then homogenized in 0.5 ml of 10% TCA/10 mM DTT/H2O. After centrifugation (5,000 g for 5 min at 4°C), pellets were washed three times with 1 ml of 10 mM DTT in acetone for three times and air-dried for 30 min. Sample pellets were resuspended in 60 μl urea sample buffer (8 M urea, 100 mM DTT, and 0.5% Triton X-100). Samples were shaken for complete protein solubilization and then centrifuged at 12,000 g for 40 min. Supernatants were collected and stored at −70°C until use.

Fifty micrograms of each sample were loaded onto IEF gels (Bio-Rad, PI ranges from 3 to 8), and isoelectric focusing was conducted (600 volt hours). The gels were then transferred to PVDF membrane for 60 volt hours. The blots were dried and blocked with 3% BSA for 2 h and then probed with 1:500 anti-HSP20 (mouse anti-HSP20 from Biodesigen or rabbit anti-HSP20 from Upstate) or 1:1,000 anti-HSP27 (rabbit anti-HSP25 antibody from Stressgen or mouse-anti-HSP27 from Research Diagnostics) antibodies overnight at 4°C. The blots were probed with secondary antibodies conjugated with HRP for 2 h at room temperature, reacted with luminal reagent, exposed to film, scanned, and quantified.

Immunoblotting. Vessels were homogenized with a ground glass homogenizing tube in ice-cold lysis buffer (100 mM K2HPO4, 1 mM phenylmethylsulfonyl, and 0.2% Triton X-100, pH 7.4) plus 1:100 protein inhibitor cocktail. Homogenates were centrifuged at 14,000 g for 20 min at 4°C, and supernatants were collected for protein concentration analysis. Twenty micrograms of proteins were loaded onto 12.5% polyacrylamide gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride membrane and then blocked with 3% BSA for 1 h. The membranes were treated with 1:1,000 anti-HSP20 antibodies (mouse anti-HSP20 from Biodesigen or rabbit anti-HSP20 from Upstate) or 1:1,000 anti-HSP27 (rabbit anti-HSP25 antibody from Stressgen or mouse-anti-HSP27 from Research Diagnostics) antibodies overnight at 4°C. After the membranes were washed with TBS-T, the blots were probed with secondary antibodies conjugated with HRP for 2 h at room temperature, reacted with luminal reagent, exposed to film, scanned, and quantified.

HSP20 mRNA expression. Total RNA was isolated from mesenteric resistance arteries using TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized using Oligo(dT)12–18 primer according to the instruction of SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). The following primers were used in PCR: HSP20 (GeneBank accession no. NM_138887) forward primer, 5′-ATTTTTCTGTGCTGG-3′; HSP20 reverse primer, 5′-CAGGAGACAGGTGCAGAGG-3′; GAPDH forward primer, 5′-TCCCTCAAGATTGTGCAGCAA-3′; GAPDH reverse primer, 5′-AGATCACAACCGATACATT-3′. The expected sizes of the PCR products for HSP20 and GAPDH were 198 and 308 bp, respectively. Twenty-eight cycles of PCR products were separated with electrophoresis in 2% agarose gel. The gels were then stained with ethidium bromide and photographed under ultraviolet light. Data were expressed as the ratio of HSP20 to GAPDH.

Statistical analysis. All data are expressed as means ± SE. Comparisons between normal and portal hypertension were performed with an unpaired Student’s t-test. Statistical significance of multiple treatments was determined by ANOVA and a Tukey’s post hoc test. P < 0.05 was considered to be significant.

RESULTS

Protein levels and phosphorylation status of HSP20 and HSP27 in portal hypertension. Inasmuch as an impaired vasoconstrictor dysfunction in mesenteric arteries from portal hypertension and both HSP20 and HSP27 have been shown to be involved in smooth muscle contraction, we examined the protein levels of both HSP20 and HSP27 in resistance mesenteric arteries from portal hypertension by immunoblotting. We also detected their phosphorylation status by isoelectric focusing. We found both an increased diphotorylated form of HSP20 and a decreased protein level of HSP20 in vessels from portal hypertension (Figs. 1A and 2A), but no changes in protein level and phosphorylation status of HSP27 (Figs. 1B and 2B).

PKA inhibition restored both increased HSP20 phosphorylation and decreased HSP20 protein expression back to normal. Previous data (27) have shown that impaired vasoconstrictor function in chronic portal hypertension is a PKA-dependent event. Therefore, we tested the hypothesis that changes in HSP20 protein levels and phosphorylation status in portal hypertension are mediated through the activation of PKA. Small mesenteric arteries were preincubated with the PKA inhibitor Rp-cAMPS (50 μM for 30 min). We found that Rp-cAMPS returned both decreased HSP20 protein levels (Fig. 3) and increased diphotorylated HSP20 (Fig. 4) in portal hypertension to normal. The response was specific in that...
Rp-cAMPS did not alter HSP20 level or phosphorylation in normal vessels.

**mRNA level of HSP20 remained unchanged in portal hypertension.** We examined the effects of PKA inhibition on HSP20 mRNA to determine whether the observed changes occurred at the transcriptional or posttranscriptional level. HSP20 mRNA expression as detected by RT-PCR was not changed in portal hypertension. Furthermore, PKA inhibition did not change HSP20 mRNA expression in either normal or portal hypertensive vessels (Fig. 5).

**DISCUSSION**

Chronic portal hypertension is an end-state clinical symptom associated with cirrhosis and other diseases that elevate portal vascular resistance. The condition, when fully developed, is characterized by an elevated portal pressure and a hyperdynamic circulatory state with increased cardiac output and decreased peripheral vascular resistance (2, 3). Another in-
TRIGGING VASCULAR CONSEQUENCE OF PORTAL HYPERTENSION IS IMPAIRED VASOCONSTRICTOR FUNCTION. PREVIOUS FINDINGS (22) HAVE SHOWN THAT THIS VASOCONSTRICTOR DYSFUNCTION IS DUE TO A POSTRECEPTOR DEFECT. ADDITIONAL EVIDENCE SUGGESTS THAT PROLONGED ELEVATION OF CYCLIC NUCLEOTIDE-DEPENDENT VASODILATORS LEADS TO THIS VASOCONSTRICTOR DYSFUNCTION, BECAUSE PKA INHIBITION BY Rp-cAMPS RESTORES IMPAIRED VASOCONSTRICTOR FUNCTION BACK TO NORMAL (27). HOWEVER, THE NATURE OF THIS PKA-DEPENDENT VASOCONSTRICTOR DYSFUNCTION IS STILL NOT CLEAR. THE PRESENT STUDY USED THE SAME CONCENTRATION OF Rp-cAMPS (50 μM) THAT HAS PREVIOUSLY BEEN SHOWN TO RESTORE VASCULAR CONTRACTILE FUNCTION IN PORTAL HYPERTENSIVE INTESTINAL ARTERIOLES (27).

RECENTLY, TWO SMALL HSPS, NAMELY HSP20 AND HSP27, HAVE BEEN SHOWN TO BE INVOLVED IN THE REGULATION OF VASCULAR SMOOTH MUSCLE CONTRACTION. BOTH ARE ACTIN-ASSOCIATED PROTEINS (6, 16, 21, 23) CONSTITUTIVELY EXPRESSED IN SMOOTH MUSCLE (10). TO DATE, 10 SMALL HSPS HAVE BEEN IDENTIFIED IN MAMMALS, WITH MOLECULAR WEIGHTS IN THE RANGE OF 12–43 kDa (18). ALL OF THESE PROTEINS CONTAIN A HIGHLY CONSERVED α-CRYSTALLIN DOMAIN OF 80–100 RESIDUES LOCATED, AS A RULE, IN THE COOH-TERMINAL PORTION OF THESE PROTEINS AND A POORLY CONSERVED NH2-TERMINAL REGION, THE WDPF DOMAIN (4, 13). SMALL HSPS TEND TO FORM LARGE OLIGOMERIC COMPLEXES, EITHER HOMOOLIGOMERS COMPOSED OF SAME SMALL HSPS OR HETEROOLIGOMERS COMPOSED OF DIFFERENT SMALL HSPS, AND THESE OLIGOMERIC COMPLEXES ARE IMPORTANT IN THEIR MOLECULAR CHAPERONE FUNCTION (11, 12, 17).

IT IS INTERESTING THAT HSP20 CONTAINS A cAMP/cGMP-DEPENDENT PROTEIN KINASE CONSENSUS PHOSPHORYLATION SITE (RRAS) (10). IN RESPONSE TO CYCLIC NUCLEOTIDE-INDUCED VASORELAXATION, HSP20 IS PHOSPHORYLATED AT SER-16 (1), WHICH MAY TRIGGER THE DISSOCIATION OF OLIGOMERS INTO DIMERS. AS EXPECTED, IN THIS STUDY, PHOSPHORYLATION OF HSP20, ESPECIALLY DI-PHOSPHORYLATED HSP20, WAS INCREASED IN THE PORTAL HYPERTENSIVE STATE (FIG. 2A). THESE EVENTS APPEAR TO BE MODULATED BY A cAMP-DEPENDENT PATHWAY IN THAT PKA INHIBITION RESTORED INCREASED DI-PHOSPHORYLATED HSP20 BACK TO NORMAL VALUES (FIG. 4). THESE RESULTS ARE CONSISTENT WITH SHORT-TERM EXPERIMENTS THAT INDICATE DI-PHOSPHORYLATED HSP20 IS PKA-DEPENDENT (1).

SEVERAL HYPOTHESES HAVE BEEN PROPOSED TO EXPLAIN HOW HSP20 REGULATES VASCULAR SMOOTH MUSCLE CONTRACTION (5, 23). MOST OF THESE HYPOTHESES FOCUS ON THE REGULATION OF HSP20 PHOSPHORYLATION. HOWEVER, THE EXACT MECHANISM OF HSP20 IN REGULATION OF SMOOTH MUSCLE CONTRACTION IS STILL UNCLEAR. IN THIS STUDY, WE FOUND THAT IN THE CHRONIC PORTAL HYPERTENSION CONDITION, HSP20 PROTEIN LEVELS WERE SIGNIFICANTLY REDUCED (FIG. 1A). MOST IMPORTANTLY, THIS DECREASED HSP20 PROTEIN LEVEL WAS RESTORED BY PKA INHIBITOR Rp-cAMPS (FIG. 3). THIS RESULT IS CONSISTENT WITH OTHERS FINDING THAT HSP20 PROTEIN LEVEL WAS DECREASED BY PROLONGED TREATMENT OF CARDIAC MYOCYTE WITH β-ADRENERGIC AGONIST (8), WHICH ALSO INCREASES THE CYTOSOLIC cAMP LEVEL. THIS OBSERVATION COUPLED WITH THE FACT THAT Rp-cAMPS RESTORED VASOCONSTRICTOR FUNCTION IN CHRONIC PORTAL HYPERTENSION LEADS US TO SUGGEST THAT REDUCED HSP20 LEVELS MAY BE PRIMARILY RESPONSIBLE FOR THE LOSS OF VASCULAR FUNCTION.

BECAUSE PKA REGULATES THE EXPRESSION OF A VARIETY OF PROTEINS AT THE TRANSCRIPTIONAL LEVEL BY PHOSPHORYLATING DIFFERENT TRANSCRIPTION FACTORS (7, 9, 24), WE WANTED TO KNOW WHETHER DECREASED HSP20 EXPRESSION IS REGULATED BY PKA AT THE TRANSCRIPTIONAL LEVEL. THEREFORE, WE DETECTED HSP20 mRNA EXPRESSION IN MESENTERIC RESISTANCE ARTERIES FROM BOTH NORMAL AND PORTAL HYPERTENSIVE RATS WITH OR WITHOUT PKA INHIBITION. WE FOUND NO CHANGES IN HSP20 mRNA EXPRESSION IN Portal hypertension (FIG. 5). INHIBITION OF PKA DID NOT CHANGE HSP20 mRNA LEVEL IN EITHER NORMAL OR PORTAL HYPERTENSIVE VESSELS (FIG. 5). INASMUCH AS mRNA LEVELS WERE NOT CHANGED IN PORTAL HYPERTENSIVE VESSELS, WE INTERPRET OUR RESULTS TO INDICATE THAT PKA REGULATES HSP20 EXPRESSION AT POSTTRANSCRIPTIONAL LEVELS.

BECAUSE PKA INDUCED BOTH INCREASED HSP20 PHOSPHORYLATION AND DECREASED HSP20 PROTEIN LEVELS, WE FURTHER SUGGEST THAT THE PHOSPHORYLATION OF HSP20 MAY TRIGGER THE TURNOVER OF HSP20.

THE EXACT MECHANISM WHEREBY DECREASED HSP20 EXPRESSION IS INVOLVED IN CYCLIC NUCLEOTIDE-INDUCED VASORELAXATION STILL REMAINS UNCLEAR. HOWEVER, RECENT STUDIES HAVE SHOWN THAT THE OLIGOMERIC STATE OF SMALL HSPS IS IMPORTANT IN FACILITATING PROTEIN FOLDING, AND THAT PHOSPHORYLATION MAY TRIGGER THE DISSOCIATION OF OLIGOMERS TO DIMERS, WHICH LOSSE THEIR CHAPERONE ABILITY (11, 12, 17). THIS IS CONSISTENT WITH OUR IDEA THAT PHOSPHORYLATION OF HSP20 MAY PROMOTE ITS TURNOVER. AS THE MOST CONCENTRATED PROTEIN IN CELLS, ACTIN FOLDING MAY BE CRITICAL TO THE ACTIN POLYMERIZATION PROCESS, AN ESSENTIAL STEP IN THE REGULATION OF SMOOTH MUSCLE CONTRACTION (19, 25). WE HYPOTHEZIZE THE TRIGGER OF HSP20 IS THE PHOSPHORYLATION, WHICH REDUCES THE ACTIN POLYMERIZATION AND DECREASES THE CONTRACTILE FUNCTION OF VASCULAR SMOOTH MUSCLE.

IN SUMMARY, THE PRESENT STUDY PRESENTS THE FIRST TO DEMONSTRATE BOTH AN INCREASED HSP20 PHOSPHORYLATION AND DECREASED HSP20 PROTEIN LEVEL IN SMALL MESENTERIC ARTERIES FROM PORTAL HYPERTENSION ANIMALS. PHOSPHORYLATION AND FUNCTIONAL PROTEIN CONCENTRATION WOULD BE RESTORED BY PKA INHIBITION. HOWEVER, PKA INHIBITION DID NOT CHANGE HSP20 mRNA EXPRESSION, NOR DID THE PORTAL HYPERTENSION STATE. WE CONCLUDE THAT DECREASED HSP20 PROTEIN LEVEL IN PORTAL HYPERTENSION IS PKA DEPENDENT AND THAT DECREASED HSP20 EXPRESSION MAY PARTIALLY EXPLAIN THE IMPAIRED VASOCONSTRICTOR FUNCTION IN PORTAL HYPERTENSION. WE ALSO SUGGEST THAT PHOSPHORYLATION OF HSP20 BY PKA MAY ALTER HSP20 TURNOVER. FUTURE STUDIES AIMED AT FURTHER EXAMINING THE DYNAMICS OF HSP REGULATION OF VASCULAR SMOOTH MUSCLE CONTRACTION IN CHRONIC PORTAL HYPERTENSION CONDITIONS ARE WARRANTED.

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