Effects of chronic portal hypertension on agonist-induced actin polymerization in small mesenteric arteries

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Chen, Xuesong, Kristin Pavlish, Hai-Ying Zhang, and Joseph N. Benoit. Effects of chronic portal hypertension on agonist-induced actin polymerization in small mesenteric arteries. Am J Physiol Heart Circ Physiol 290: H1915–H1921, 2006. First published December 9, 2005; doi:10.1152/ajpheart.00643.2005.—The ability of arterial smooth muscle to respond to vasoconstrictor stimuli is reduced in chronic portal hypertension (PHT). Additional evidence supports the existence of a postreceptor defect in vascular smooth muscle excitation contraction coupling. However, the nature of this defect is unclear. Recent studies have shown that vasoconstrictor stimuli induce actin polymerization in smooth muscle and that the associated increase in F-actin is necessary for force development. In the present study we have tested the hypothesis that impaired actin polymerization contributes to reduced vasoconstrictor function in small mesenteric arteries derived from rats with chronic prehepatic PHT. In vitro studies were conducted on small mesenteric artery vessel rings isolated from normal and PHT rats. Isometric tension responses to incremental concentrations of phenylephrine were significantly reduced in PHT arteries. The ability to polymerize actin in portal hypertensive mesenteric arteries stimulated by phenylephrine was attenuated compared with control. Inhibition of cAMP-dependent protein kinase (PKA) restored agonist-induced actin polymerization of arteries from PHT rats to normal levels. Depolymerization of actin in arteries from normal rats reduced maximal contractile force but not myosin phosphorylation, suggesting a key role for the dynamic regulation of actin polymerization in the maintenance of vascular smooth muscle contraction. We conclude that reductions in agonist-induced maximal force development of PHT vascular smooth muscle is due, in part, to impaired actin polymerization, and prolonged PKA activation may underlie these changes.

vascular smooth muscle; protein kinase A; myosin; phenylephrine; isometric tension

One of the most intriguing vascular consequences of chronic portal hypertension is the decreased ability of blood vessels to respond to vasoconstrictor stimuli (2, 3). The observed vasoconstrictor dysfunction is systemic in nature in that it is not limited to the splanchic vascular territory (13, 18, 19). This finding has been attributed to a postreceptor defect in vascular smooth muscle excitation contraction coupling (21, 24). Additional evidence supports the contention that prolonged elevation in cyclic nucleotide-dependent vasodilators plays an important role in this defect inasmuch as vasoconstrictor effectiveness in portal hypertension can be restored by protein kinase A (PKA) inhibition (31). However, the cellular mechanism whereby cyclic nucleotide-dependent pathways impair vasoconstriction in chronic portal hypertension is still unclear.

Most previous investigations suggested that cyclic nucleotide-dependent relaxation occurs through inhibition or reversal of the Ca\(^{2+}\)-dependent phosphorylation of the regulatory myosin light chain (MLC\(_{20}\)). However, prolonged activation of cyclic nucleotide-dependent signaling pathways inhibits the development of force in response to contractile agonists but does not inhibit the increases in MLC\(_{20}\) phosphorylation or energy consumption (27, 30). This uncoupling of force from myosin light chain phosphorylation (8, 22) suggests that mechanisms other than thick filament regulation are operative in cyclic nucleotide-dependent relaxation of smooth muscle.

Emerging evidence has focused on the potential role of actin in the regulation of smooth muscle contraction. Specifically, it has been proposed that formation of filamentous actin is regulated and that change in the ratio of F-actin to G-actin (F/G-actin ratio) is a key determinant of force development in smooth muscle (7). Indeed, regulation of actin filament dynamics is known to be essential for cell motility, cytokinesis, muscle contraction, and control of cell shape and polarity (26). Recent studies indicate that the F/G-actin ratio is increased in agonist-stimulated smooth muscle and suggest that actin polymerization per se is an important cellular event during smooth muscle activation (29).

In the present study we tested our hypothesis that agonist-induced actin polymerization in small mesenteric arteries was reduced under chronic portal hypertensive conditions. The results of this study support our hypothesis and further demonstrate that this impaired actin polymerization is a PKA-dependent event.

METHODS

Production of chronic prehepatic portal hypertension. Portal hypertension was surgically produced as previously described by our laboratory (6). Thirty male Sprague-Dawley rats (∼300 g, Charles River Laboratories, Wilmington, MA) were anesthetized with isoflurane, and a midline abdominal incision was made. The 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 line was treated with antibiotic ointment, and the animals were allowed to recover. A single preoperative dose of buprenorphine (0.25 mg/kg) was given subcutaneously to alleviate any postsurgical pain. The animals were returned to the vivarium, and portal hyper-

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tension was allowed to develop. Fourteen days postsurgery, the animals were ready for experimental use. All animal procedures were approved by the Animal Care and Use Committee of the University of North Dakota.

**Isolated vessel preparation.** 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, pH = 7.4). Small mesenteric arteries (diameter ~250 μm) were dissected free of surrounding tissue (23). Two mesenteric artery segments, 3–4 mm in length, from each animal were used for contractile studies; the remaining vessels were incubated in different concentrations of latrunculin A (0.01, 0.03, and 0.1 μM) in PSS for 45 min or 50 μM adenosine 3′,5′-cyclic monophosphothioate (Rp-cAMPS, Alexis Biochemicals) for 30 min followed by 10^{-4} M phenylephrine stimulation for 1 min, and then frozen in liquid nitrogen for biochemical analysis.

**Contractile function studies of small mesenteric arteries.** Isometric tension responses of small mesentery arteries were evaluated on a small vessel myograph (23). Once the vessels were mounted on the myograph, they were normalized to internal circumference of 0.9L_{100} where L_{100} is the circumference that the vessels would reach if fully relaxed and under an internal pressure of 100 mmHg. At this internal circumference, the vessels develop near maximal active wall tension. Normalization was performed by distending the vessel in step increments while measuring force. After normalization, the vessels were primed three times with 10 μM phenylephrine. The tension responses of the vessels to incremental concentrations of phenylephrine were then measured. After washout of the phenylephrine with PSS and return of the vessel to baseline conditions, different concentrations of the actin depolymerizing agent latrunculin A (0.01, 0.03, and 0.1 μM) or the selective myosin light chain kinase (MLCK) inhibitor ML-7 (0.3, 1.0, and 3.0 μM) were added for 45 or 30 min, respectively; a second phenylephrine dose response was then measured.

**Detection of MLC20 phosphorylation.** Small mesenteric arteries were thawed and homogenized in acetone containing 10% (wt/vol) trichloroacetic acid and 10 mM DTT. After centrifugation (4,600 g, 5 min), samples were washed three times with acetone-DTT. The pellet was resuspended in 8 M urea, 20 mM Tris base, 22 mM glycerine, and 10 mM DTT and incubated at room temperature for 1 h. The sample was then centrifuged (10,000 g, 5 min), and the supernatant was saved for electrophoretic analysis. The MLC20 was separated by gel electrophoresis and transferred to nitrocellulose. The membranes were blocked with 5% milk and incubated with mouse anti-MLC20 antibodies (a generous gift from Dr. Kristine Kamm, Univ. of Texas Southwestern Medical Center) overnight at 4°C. The primary antibody was reacted with alkaline phosphatase-conjugated anti-mouse IgG. Unphosphorylated and phosphorylated bands of MLC20 were visualized by chemiluminescence and quantified by densitometry. MLC20 phosphorylation was calculated as the ratio of phosphorylated MLC20 to total MLC20.

**Analysis of F/G-actin ratio.** F-actin and G-actin in smooth muscle tissue were isolated by fractionation and differential centrifugation and quantified by electrophoresis. (F-actin/G-actin in vivo assay kit, Cytoskeleton, Denver, CO). Briefly, each of the mesenteric artery samples was homogenized in F-actin stabilization buffer [50 mM piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.9, 50 mM NaCl, 5 mM MgCl2, 5 mM EGTA, 5% glycerol, 0.1% Triton X-100, 0.1% Nonidet P-40, 0.1% Tween 20, 0.1% β-mercaptoethanol, 0.001% antifoam, 1 mM ATP, 1 μg/μl pepstatin, 1 μg/μl leupeptin, 10 μg/μl benzamidine, and 500 μg/ml tosyl arginyl methyl ester]. Supernatants of the protein extracts were collected after ultracentrifugation (100,000 g, 1 h at 37°C). The pellets were resuspended in ice-cold distilled water plus 1 μM cytochalasin-D and then incubated on ice for 1 h to dissociate F-actin. The resuspended pellets were gently mixed every 15 min. Supernatant (G-actin) and pellet (F-actin) fractions were diluted 50 times and analyzed by immunoblotting using mouse anti-α-smooth muscle actin antibody. The F/G-actin ratio was determined by scanning densitometry.

**Statistical analysis.** All data are expressed as means ± SE. Comparisons between normal and portal hypertension were performed with a Student’s unpaired t-test. Statistical significance of multiple treatments was determined by one-way ANOVA and a Tukey post hoc test or by two-way ANOVA with a Bonferroni post hoc test. Nonlinear regression was performed by stepwise best fit regression. P < 0.05 (2-tailed) was considered to be significant.

**RESULTS**

**Impaired vasoconstrictor function of small mesenteric arteries in portal hypertension.** The dose-response curves to phenylephrine (from 10^{-8} to 10^{-4} M) in small mesenteric
arteries from normal and portal hypertensive rats are shown in Fig. 1. There was a significant reduction in vascular responsiveness in portal hypertensive vessels as evidenced by a decreased maximal tension and an increased EC_{50}. These findings are consistent with previous reports of reduced vasoconstrictor function in portal hypertension (15, 28).

Effect of MLCK inhibition on force development and maintenance of small mesenteric arteries. The effect of MLCK inhibition on isometric force development of normal small mesenteric arteries is shown in Fig. 2. At a ML-7 concentration of 0.3 μM, no effects on the EC_{50} or maximal tension were observed. At a concentration of 1.0 μM, ML-7 did not affect maximal tension (control, 4.48 ± 0.16 mN/mm; 1.0 μM ML-7, 4.38 ± 0.21 mN/mm) but did increase the EC_{50} from 1.34 ± 0.13 μM in control to 4.13 ± 0.84 μM. At a ML-7 concentration of 3.0 μM, maximal tension was reduced compared with control (3.48 ± 0.14 mN/mm). A disproportionately larger increase in the EC_{50} was observed at ML-7 concentrations of 3.0 μM (EC_{50} = 11.3 ± 2.63 μM).

Effects of portal hypertension on agonist-induced actin polymerization in small mesenteric arteries. The effects of portal hypertension and PKA inhibition on actin polymerization in phenylephrine-stimulated small mesenteric arteries are shown in Fig. 3. The actin polymerization was indicated by F/G-actin ratio. The basal level of F/G-actin ratio in normal and portal hypertensive vessels is ~1. Phenylephrine at 10^{-4} M increased F/G-actin by 12-fold in normal vessels but only 8-fold in portal hypertensive vessels. Pretreatment of vessels with PKA inhibitor Rp-cAMPS (50 μM for 30 min) restored actin polymerization ability in portal hypertension to normal value.

Fig. 2. Effects of ML-7 on dose-response curves to PE (from 10^{-8} to 10^{-4} M) in NL small mesenteric arteries (A). At a ML-7 concentration of 0.3 μM, no effects on the EC_{50} or maximal tension were observed. At a concentration of 1.0 μM, ML-7 did not affect maximal tension but did increase the EC_{50}. At a ML-7 concentration of 3.0 μM, maximal tension was reduced compared with control (B). A disproportionately larger increase in the EC_{50} was observed at ML-7 concentrations of 3.0 μM (C). n = 4. *P < 0.05 vs. NL.

Fig. 3. Effects of chronic PHT and protein kinase A (PKA) on PE-induced F-actin-to-G-actin (F/G-actin) ratio changes in small mesenteric arteries. Quantitative immunoblotting shows that PE significantly increases F/G-actin ratio in both NL and PHT vessels. However, the degree of F/G-actin increases in PHT was significantly lower than that of NL. This PHT-associated decrease in PE-induced actin polymerization was restored by adenosine 3',5'-cyclic monophosphothioate (Rp-cAMPS). Below the graph are representative Western blots of F-actin and G-actin expression in NL and PHT mesenteric arteries at the baseline or in response to 10^{-4} M PE stimulation for 1 min with or without PKA inhibition (50 μM Rp-cAMPS for 30 min); n = 5. *P < 0.05 vs. NL.
Effects of actin depolymerization on force development and maintenance of small mesenteric arteries. The effects of pretreatment of latrunculin A on phenylephrine dose-response curve in small mesenteric arteries are shown in Fig. 4. Pretreatment of latrunculin A significantly reduced the maximal tension induced by phenylephrine in a dose-dependent manner in small mesenteric arteries derived from both normal and portal hypertensive rats (Fig. 4, A2 and B2). The pretreatment of latrunculin A significantly decreased the EC50 of phenylephrine dose-response curves at the concentration above 0.03 μM in both normal and portal hypertensive vessels (Fig. 4, A3 and B3). At latrunculin A concentration of 0.1 μM, no difference in maximal tension was found between normal and portal hypertensive rats. Although the difference in EC50 between normal and portal hypertensive rats was still present at high concentration of latrunculin A, there was no statistical significance.

Effects of latrunculin A on agonist-induced actin polymerization and MLC20 phosphorylation. The effects of the pretreatment of latrunculin A on actin polymerization and MLC20 phosphorylation in phenylephrine-stimulated small mesenteric arteries from both normal and portal hypertensive rats are shown in Fig. 5. The pretreatment of latrunculin A significantly decreased the phenylephrine-induced F/G-actin ratio in a dose-dependent manner in both normal and portal hypertensive vessels (Fig. 5, A1 and A2), but latrunculin A did not change MLC20 phosphorylation status (Fig. 5, B1 and B2).

Relationship between F/G-actin ratio and maximal tension. The relationship between F/G-actin ratio and maximal tension is shown in Fig. 6. Stepwise best-fit regression, based on the data from Figs. 4 and 5, shows that the best-fit curve for the relationship between maximal tension and the F/G-actin ratio is a hyperbola (Fig. 6A). Figure 6B shows a log/linear transformation of the hyperbolic function. There was no statistical difference between the relationships derived from normal and portal hypertensive rats. The equation defining the normal relationship in Fig. 6B is as follows: maximal tension = 1.09 × log (F/G-actin ratio) − 0.11. The equation defining the portal hypertensive relationship in Fig. 6B is as follows: maximal tension = 0.95 × log(F/G-actin ratio) − 0.17.

DISCUSSION

The critical findings from this study are that agonist-induced maximal tension in vascular smooth muscle is directly related
to the F/G-actin ratio and that there is a decreased capacity of agonist-induced F/G-actin ratio in small mesenteric arteries from chronic portal hypertension. Consistent with previous reports (15, 28, 31), we observed a characteristic vasoconstrictor dysfunction of small mesenteric arteries in chronic portal hypertension, with a decreased maximal tension and an increased EC50 in dose-response curves to phenylephrine (Fig. 1). An earlier study by Wu and Benoit (31) suggests that this impaired vasoconstrictor function is mediated via cAMP-dependent events in that vasoconstrictor effectiveness in portal hypertension can be restored by the PKA inhibitor Rp-cAMPS. However, our earlier work did not provide insight into the mechanism(s) underlying these events.

As phosphorylation of MLC20 is the key event in the regulation of vascular smooth muscle contraction (11, 12), we examined the effects of MLCK inhibition by its selective inhibitor ML-7 on dose-response curves to phenylephrine in normal mesenteric arteries. The present studies showed that ML-7 significantly increased EC50 to phenylephrine in a dose-dependent manner, and there is a significant change in EC50 at relatively low concentration of ML-7 (1 μM). However, ML-7 only impaired the maximal tension at relatively high concentration (3 μM), where there was a ninefold increase in EC50 (Fig. 2). One possible explanation for the relatively low concentration of ML-7 (1 μM) increasing EC50 without changing maximal tension is that the amount of myosin phosphorylated during agonist-induced contraction far exceeds that which is necessary for maximal force development. Our findings are not surprising given the data of Asano and Nomura (1) who reported that inhibition of Rho-kinase, which promoted myosin dephosphorylation, increased EC50 without significantly changing maximal tension. Data from the present study suggest that vascular sensitivity but not maximal force development is largely dependent on the phosphorylation of myosin. This is consistent with the notion that the balance of MLCK:MLCP (myosin light chain phosphatase) activities determine the calcium sensitization or desensitization (25). Under conditions of partial MLCK inhibition, maximal force can still be achieved by increasing the agonist concentration (25).

The inhibition of MLCK by a relatively low concentration of ML-7 (1 μM) only increased the EC50 in dose-response curves to phenylephrine but did not change the maximal force. However, there is both a decreased maximal tension and an increased EC50 in portal hypertensive vessels. Moreover, the MLCK inhibition impaired maximal tension to the same degree as that in portal hypertension only at high concentration of...
DECREASED ACTIN POLYMERIZATION IN PORTAL HYPERTENSION

Although decreased actin polymerization is associated with impaired vascular function in portal hypertension, the exact role of actin polymerization is not clear. To define the role of actin polymerization in vascular contractile events in portal hypertension, we evaluated the effects of latrunculin A on force generation. As latrunculin A binds to monomeric G-actin in a 1:1 ratio and decreases actin polymerization capacity, the use of latrunculin A would be expected to decrease force generation in a concentration-dependent manner. We observed that the pretreatment of vessels with latrunculin A decreased phenylephrine-induced maximal tension (Fig. 4) and the F/G-actin ratio (Fig. 5) in both normal and portal hypertensive mesenteric arteries in a dose-dependent manner. We further observed that the highest concentrations of latrunculin A rendered the phenylephrine-induced maximal tension of normal and portal hypertensive vessels indistinguishable. Although the difference in EC50 between normal and portal hypertension at the high concentration of latrunculin A is still present, there is no statistical significance. This also supports the concept that the limited capacity of agonist-induced actin polymerization plays an important role in the vascular deficit in portal hypertension. The finding that latrunculin A decreases maximal force generation without changing MLC20 phosphorylation (Fig. 5) suggests that the regulation of MLC20 plays a minimal role in reduced maximal tension development in portal hypertensive vessels. These data are also consistent with our data (Fig. 2) showing that a low concentration of ML-7 (1 μM) decreased phenylephrine sensitivity but not maximal tension in normal vessels.

Most importantly, our data showed that agonist-induced maximal tension was directly related to actin polymerization (Fig. 6) in both normal and portal hypertensive vessels, and there was no difference in the shape of the curves between the two groups. The only difference was that portal hypertensive arterial smooth muscle could not generate the same amount of F-actin as normal smooth muscle. This evidence clearly demonstrates that the reduced capacity of agonist-induced actin polymerization is a key defect in vascular dysfunction of portal hypertension. This means that enhanced capacity of agonist-induced actin polymerization would restore the reduced maximal force generation in portal hypertensive vessels. Our data that PKA inhibition restored the agonist-induced maximal actin polymerization (Fig. 3) is consistent with our previous studies showing that PKA inhibition restored the impaired vasoconstrictor function in portal hypertension to normal (31). Thus reduced agonist-induced actin polymerization capacity is possibly the cause of impaired maximal tension generation in portal hypertensive vasculature.

The exact mechanism whereby PKA mediates decreases in agonist-induced actin polymerization in arterial smooth muscle in chronic portal hypertension still remains unclear. However, our recent study has demonstrated a decreased level of small heat shock protein with molecular mass of 20 kDa (HSP20) in mesenteric arteries from portal hypertensive rats (6). We further suggested that the decreased HSP20 protein level was associated with a PKA-mediated increase in HSP20 phosphorylation (6). Although the exact mechanism whereby alterations in HSP20 phosphorylation relate to reductions in arterial smooth muscle force generation in portal hypertension is not clear, we are tempted to speculate that changes in actin monomer integrity (proper folding or breakdown) are involved. It is

Fig. 6. Relationship between F/G-actin ratio and maximal tension. Points of maximal tension corresponding to F/G-actin ratio were plotted on the basis of the data from Figs. 4 and 5. Stepwise best-fit regression shows the best-fit curve is a hyperbola (A). Linear transformation of the upper curve by semilog plot was used to statistically compare the relationships. There was no statistical difference in the slope of these relationships (B).

ML-7 (3 μM), where there was a ninefold increase in EC50. These observations prompt us to speculate that mechanisms other than MLC20 phosphorylation are also impaired in portal hypertension. Inasmuch as actin polymerization is dynamically regulated and is essential for vascular smooth muscle contraction, we hypothesized that agonist-induced actin polymerization was impaired in portal hypertension. Supporting our hypothesis are data showing that 10−4 M phenylephrine induced a 12-fold increase in actin polymerization in normal mesenteric arteries but only an 8-fold increase in actin polymerization in chronic portal hypertensive vessels (Fig. 3). The possibility of actin polymerization being a second messenger-regulated event is supported by our data showing that the impaired capacity of agonist-induced actin polymerization in chronic portal hypertensive vessels was restored by the PKA inhibitor Rp-cAMPS (Fig. 3). In this study, we used the same concentration of Rp-cAMPS (50 μM) that has previously been shown by our laboratory to restore vascular contractile function in portal hypertensive intestinal arterioles (31). This finding demonstrated that impaired capacity of agonist-induced actin polymerization in portal hypertension was related to increased PKA actions. This is consistent with other reports that cAMP decreased actin polymerization (9).
well known that the small heat shock proteins are important in facilitating protein folding and that phosphorylation of small heat shock proteins induces the dissociation of oligomers to dimers, which lose their chaperone ability (4, 10, 16, 17, 20). Because HSP20 is an actin-associated protein (5) and its expression in smooth muscle is not induced by heat shock (14), we suggest that HSP20 may have role in the housekeeping of the integrity of actin monomer, the most concentrated protein within the cell. Thus we propose a general hypothesis that prolonged activation of PKA in portal hypertension impairs agonist-induced actin polymerization in arterial smooth muscle by limiting the proper folding of actin monomer asserted by changes in phosphorylation and/or expression of HSP20. The physiological consequence is decreased G-actin pool available for actin polymerization and subsequently reduced force generation.

In summary, the present study is the first to demonstrate that the capacity of agonist-induced actin polymerization in portal hypertensive mesenteric arteries is impaired, and the decreased actin polymerization is a PKA-dependent event. Future studies investigating the detailed mechanisms governing impaired capacity of agonist-induced actin polymerization in vascular smooth muscle from portal hypertension are warranted.

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GRANTS

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REFERENCES