Impaired agonist-dependent myosin phosphorylation and decreased RhoA in rat portal hypertensive mesenteric vasculature

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Zhang, Hai-Ying, Yuichi Shirasawa, Xuesong Chen, Hong Yu, and Joseph N. Benoit. Impaired agonist-dependent myosin phosphorylation and decreased RhoA in rat portal hypertensive mesenteric vasculature. Am J Physiol Gastrointest Liver Physiol 288: G603–G608, 2005. First published October 28, 2004; doi:10.1152/ajpgi.00116.2004.—The purpose of the present study was to examine the effects of portal hypertension on agonist-induced myosin phosphorylation and RhoA expression in vascular smooth muscle. A possible link to cAMP-dependent events was also examined. Portal hypertension was produced by stenosis of the portal vein. Vessel segments were treated with or without 50 μM of the PKA inhibitor Rp-cAMPS for 30 min and subsequently stimulated with 10^{-4} M phenylephrine. Myosin regulatory light-chain phosphorylation was detected by immunoblotting. Total RNA from first-order mesenteric arteries and portal veins was isolated and amplified by RT-PCR using RhoA and GAPDH primers. RhoA protein expression was also measured in first-order mesenteric arteries using Western blot analysis. Myosin phosphorylation in maximally stimulated first-order mesenteric arteries was significantly lower in portal hypertensive animals (19.9 ± 2.86%) when compared with sham-operated control (43.8 ± 3.53%). Inhibition of PKA selectively increased myosin phosphorylation to 34.7 ± 4.18%. Rp-cAMPS did not affect the phosphorylation of the portal veins or superior mesenteric arteries. RhoA mRNA and membrane-associated RhoA protein expression in portal hypertensive first-order mesenteric arteries were significantly lower when compared with controls. Acute inhibition of PKA had no effect on RhoA mRNA expression. However, it restored membrane-associated RhoA protein expression in portal hypertensive vessels to control levels. The results suggest that reductions in membrane-associated RhoA expression, which appear to be regulated by cAMP-dependent events, lead to reduced myosin phosphorylation and may underlie the reduced vasoconstrictor effectiveness in the resistance vasculature of portal hypertensive intestine.

myosin regulatory light chain; vascular smooth muscle; protein kinase A; Rp-cAMPS

Previous studies by our laboratory demonstrated that chronic portal hypertension leads to a significant reduction of vasoconstrictor effectiveness in the mesenteric resistance vasculature (10, 11, 20). The exact mechanism of portal hypertension-induced vascular contractile dysfunction remains controversial, although early studies (22) provided evidence that impaired vasoconstrictor responses in cirrhosis were not limited to agonist-receptor interaction and suggested a postreceptor defect exists. Functional studies by our laboratory have provided direct evidence for an impaired receptor as well as nonreceptor responses of intestinal arterioles in portal hypertensive conditions (36).

Subsequent studies from our laboratory established a link between glucagon and the loss of vasoconstrictor effectiveness in portal hypertension and further demonstrated that the altered vascular responses in portal hypertension were primarily attributable to cAMP- but not cGMP-dependent events (37). Although the relaxation of vascular smooth muscle by cAMP is known to involve reduced Ca^{2+} influx via L-type calcium channels and increased uptake of Ca^{2+} by the sarcoplasmic reticulum (13, 23), little is known about the consequences of chronic vasodilator stimuli on phosphorylation of myosin, which is arguably the single most important biochemical event leading to smooth muscle contraction.

Recent studies by Taylor et al. (34) have provided evidence that cyclic nucleotide-mediated attenuation of agonist-induced arterial constriction results not only from the regulation of pathways of Ca^{2+} mobilization but also from a reduction in Ca^{2+} sensitivity of the contractile apparatus. Studies by Adelstein et al. (1) provided evidence that acute elevations in cAMP promote the PKA-catalyzed phosphorylation of inactive myosin light-chain kinase (MLCK) and prevent the Ca^{2+}/calmodulin-mediated conversion of inactive MLCK to active MLCK.

Recent reports (12) suggest that phosphorylation of myosin light-chain phosphatase (MLCP) by Rho-kinase leads to the inhibition of MLCP. This event required activation of Rho-kinase by the small GTPase RhoA. In an effort to more precisely define the signaling events leading to vasoconstrictor impairment in portal hypertension, the present study examines possible relationships among the 20-kDa myosin regulatory light-chain (RLC_{20}) phosphorylation, cAMP-dependent events, and RhoA expression in resistance arteries and portal veins (PV).

Materials and Methods

Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), weighing 210–320 g, were studied. All animals were housed in an environmentally controlled vivarium, where they were allowed free access to a standard pellet diet and water. Procedures involving animals were reviewed and approved by the University of North Dakota Institutional Animal Care and Use Committee. Animals were divided into two groups: prehepatic portal hypertension by PV stenosis. Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), weighing 210–320 g, were studied. All animals were housed in an environmentally controlled vivarium, where they were allowed free access to a standard pellet diet and water. Procedures involving animals were reviewed and approved by the University of North Dakota Institutional Animal Care and Use Committee. Animals were divided into two groups: prehepatic portal hypertension by partial PV stenosis and sham-operated controls.

PV stenosis. Rats were anesthetized with isoflurane, and a midline abdominal incision was made. The PV was surgically isolated and stenosed by tying a 3-0 silk suture around the vein and a 20-gauge needle that had been placed next to the vein. The needle was then

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removed, and the vein was allowed to reexpand to yield a calibrated constriction of the PV. The ligature was located between the porta heptis and the coronary vein and constricted PV to ~30% of its original diameter. The abdomen was closed in layers with suture and metal wound clips. To minimize postoperative discomfort, buprenorphine (0.25 mg/kg body wt) was intramuscularly injected. Animals were returned to the vivarium and allowed to recover for 10 days. Sham-operated rats, in which the PV was surgically isolated but not stenosed, served as controls.

Measurement of RLC20 phosphorylation. Ten days after the initial surgery, rats were anesthetized and euthanized and segments of the superior mesenteric artery, first-order mesenteric artery, and PV were removed for study. Vessel segments were divided into two physiological groups, incubated with 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) with or without 50 μM Rp-cAMPS for 30 min at 37°C. The vessel segments were then stimulated with 10−4 M phenylephrine for 1 min and snap-frozen in liquid nitrogen.

Frozen tissues were thawed in a 10% TCA/10 mM DTT/acetone slurry. Each sample was transferred to a homogenizing tube (Kontes Duall Round Glass Tissue Grinders, Fisher) and 0.5 ml of 10% TCA/10 mM DTT/water were added. Samples were homogenized for 20 s at 70 rpm. After centrifugation (3,000 g for 2 min), sample pellets were washed three times with 500 μl ethyl ether for 5 min each and air-dried in a fume hood. Sample pellets were resuspended in 50 μl urea sample buffer (in mM: 18.5 Tris, 20.4 glycine, 9.2 DTT, and 0.18 EDTA) containing 8 M urea, 4.6% saturated sucrose, and 0.004% bromophenol blue. Urea pellets were added directly to each sample to saturation. Samples were shaken for complete protein solubilization and stored at −70°C until use.

After preelectrophoresis at 400 V for 60 min, 20 μl of each sample were loaded to the glycerol polyacrylamide gel using a minigel system (Bio-Rad, Hercules, CA). Electrophoresis was performed at 400 V for 100 min. Gels were gently rinsed in the transfer buffer (192 mM glycine, 25 mM Tris, 0.05% SDS, and 20% methanol). After activation of the polyvinylidene fluoride (PVDF; Immobilon-P, Millipore, Bedford, MA) membrane in methanol, proteins were transferred to membrane at 25 V for 1 h in ice-cold transfer buffer. Membranes were fixed in 0.4% glutaraldehyde (8% stock grade I, Sigma) for 30 min at room temperature and washed in PBS. Membranes were blocked in 5% liquid block (Amersham Pharmacia Biotech, Piscataway, NJ) for 60 min. Membranes were then incubated overnight at 4°C with 1:10,000 goat anti-mouse horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. After the membranes were washed with PBS, we incubated them with 1:10,000 goat anti-mouse horseradish peroxidase (Santa Cruz Biotechnology) at room temperature for 1 h. Immunoreactivity was assessed with an enhanced chemiluminescent Western blot detection system. The bands were scanned and quantified by UN-SCAN-IT gel quantification software (Silk Scientific, Orem, UT).

Western blot analysis of RhoA. Fractionation was done according to the previous report (32). In brief, vessels were homogenized with a glass homogenizing tube in ice-cold buffer (in mM: 250 sucrose, 10 Tris, pH 7.5). Homogenates were centrifuged at 700 g for 10 min at 4°C. The supernatant was centrifuged at 17,000 g for 45 min at 4°C. The resulting pellet was enriched in plasma membrane, and the supernatant was enriched in cytosolic proteins. The pellet was recovered in PBS with protease inhibitor cocktail. Forty micrograms of proteins of each sample were loaded in each lane of a gel of sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, proteins were transferred to PVDF membranes and then blocked with 5% liquid block (Amersham Pharmacia Biotech) at room temperature for 1 h. The membranes were treated with 1:100 anti-RhoA monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. After the membranes were washed with PBS, we incubated them with 1:10,000 goat anti-mouse horseradish peroxidase (Santa Cruz Biotechnology) at room temperature for 1 h. Immunoreactivity was assessed with an enhanced chemiluminescent Western blot detection system. The bands were scanned and quantified by UN-SCAN-IT gel quantification software. Results are expressed as the ratios to control values.

Statistics. Differences in phosphorylated RLC20 ratios and RhoA protein levels among four subgroups were analyzed by one-way ANOVA. Newman-Keuls multiple comparisons post hoc test was used to identify statistical differences between subgroups. Differences in RhoA mRNA expression between two groups were analyzed by Student’s t-test. Data are shown as means ± SE, and P < 0.05 is considered a significant level of difference.

RESULTS

Table 1 summarizes RLC20 phosphorylation and dephosphorylation levels of first-order mesenteric arteries, superior mesenteric arteries and PV for control and portal hypertensive rats. There was a significant reduction in agonist-induced RLC20 phosphorylation of portal hypertensive first-order mesenteric arteries when compared with control. There was no change in the phosphorylation responses of superior mesenteric arteries and PVs in chronic portal hypertension. Inhibition of the cAMP-dependent protein kinase with Rp-cAMPS restored the RLC20 phosphorylation of first-order mesenteric arteries from portal hypertensive levels to normal levels (Fig. 1). Rp-cAMPS did not affect any phosphorylation levels of the other vessels.

RLC20 can be mono- or diphosphorylated. The monophosphorylation is the phosphorylation only at Ser19, whereas the diphosphorylation is the phosphorylation at both Ser19 and Thr18. In the present study, most vessel samples show only a monophosphorylated band. The phosphorylation level accounts for this phenomenon. Previous study by Kitazawa et al. (14) indicated only when the phosphorylation level is more than ~40%, RLC20 can be diphosphorylated. This is consistent with our rat smooth muscle cell (A7r5 cell line) data (not shown in the present study).
DISCUSSION

Active relaxation of vascular smooth muscle is generally mediated through elevations in cyclic nucleotides (cAMP and cGMP). These substances trigger several events that favor reductions in cytosolic calcium and dephosphorylation of myosin. Many receptor-linked vasoactive substances including glucagon, β-agonists, and nitric oxide can cause active vasorelaxation, through cyclic nucleotide-dependent pathways (19). Whereas the short-term actions of these agents have been widely studied, the long-term consequences of chronically vasodilated conditions on vascular smooth muscle signaling remain largely unstudied. Chronic portal hypertension represents a condition whereby the vascular smooth muscle is exposed to vasodilators for extended periods of time. One of the vascular sine qua non of portal hypertension is the hyperdynamic circulation that is characterized by reduced vascular resistance and increased cardiac output. The reduced peripheral vascular resistance has been linked to the increased circulating vasodilators and a reduced arterial vasoconstrictor effectiveness. Studies from our laboratory have provided evidence that the reduced vasoconstrictor effectiveness is a consequence of the portosystemic shunting of vasodilator substances rather than a consequence of elevated portal venous pressure (10, 11, 35). Furthermore, we have provided direct evidence that cAMP- but not cGMP-dependent events underlie the impaired responsiveness to norepinephrine (37). Despite considerable functional data supporting our contention, there is little evidence regarding the biochemical events that govern vascular smooth muscle activation in the portal hypertensive mesenteric vasculature. In the present study, we report a decreased ability of portal hypertensive resistance arteries to phosphorylate 20-kDa RLC20 during agonist-induced constrictor stimuli. Impairment of this key regulatory event is consistent with previously published functional data demonstrating an impaired vasoconstrictor responsiveness in this condition. In addition to this observation, we provide the first direct evidence linking cAMP reductions to agonist-induced myosin phosphorylation observed in portal hypertension. These observations provide direct support of our earlier functional work in which norepinephrine-induced vasoconstrictor effectiveness was restored to normal in portal hypertensive animals treated with Rp-cAMPS, an inhibitor of PKA (37). Rp-cAMPS is a competitive inhibitor

Table 1. Myosin regulatory light-chain phosphorylation and dephosphorylation levels of vessels stimulated with phenylephrine

<table>
<thead>
<tr>
<th></th>
<th>CTL</th>
<th>CTL + Rp-cAMPS</th>
<th>PHT</th>
<th>PHT + Rp-cAMPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLC20</td>
<td>56.2 ± 3.53%</td>
<td>59.0 ± 5.00%</td>
<td>80.1 ± 2.86%*†‡</td>
<td>65.3 ± 4.18%</td>
</tr>
<tr>
<td>RLC20-P</td>
<td>43.8 ± 3.53%</td>
<td>41.0 ± 5.00%</td>
<td>19.9 ± 2.86%*†‡</td>
<td>34.7 ± 4.18%</td>
</tr>
<tr>
<td>SMA</td>
<td>63.0 ± 6.13%</td>
<td>60.6 ± 5.31%</td>
<td>66.6 ± 7.24%</td>
<td>64.4 ± 2.61%</td>
</tr>
<tr>
<td>PV</td>
<td>37.0 ± 6.13%</td>
<td>39.4 ± 5.31%</td>
<td>33.4 ± 7.24%</td>
<td>35.6 ± 2.61%</td>
</tr>
<tr>
<td>RLC20</td>
<td>63.4 ± 4.38%</td>
<td>67.3 ± 3.95%</td>
<td>60.4 ± 3.79%</td>
<td>60.1 ± 2.56%</td>
</tr>
<tr>
<td>RLC20-P</td>
<td>36.6 ± 4.38%</td>
<td>32.7 ± 3.95%</td>
<td>39.6 ± 3.79%</td>
<td>39.9 ± 2.56%</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of subjects. CTL, sham-operated control; CTL + Rp-cAMPS, vessels from sham-operated control treated with Rp-cAMPS; PHT, portal hypertension; PHT + Rp-cAMPS, vessels from portal hypertensive rats treated with Rp-cAMPS; A1, first-order mesenteric arteries; SMA, superior mesenteric arteries; PV, portal veins; RLC20, dephosphorylated myosin regulatory light chain; RLC20-P, phosphorylated myosin regulatory light chain. *P < 0.01 compared with CTL; †P < 0.05 compared with CTL + Rp-cAMPS; ‡P < 0.05 compared with PHT + Rp-cAMPS.
of PKA I and II (2, 26). It discriminates between PKA and other cAMP receptors, e.g., catabolite gene activator protein (cAMP receptor protein) or cyclic nucleotide-gated channels (16, 27). Therefore, it is generally accepted that Rp-cAMPS is a specific inhibitor of PKA. Previous studies by independent research teams have demonstrated that treatment of smooth muscle with forskolin, the adenylate cyclase activator, decreased myosin light-chain phosphorylation (30, 31, 33). The fact that RLC\textsubscript{20} phosphorylation in control arteries was not altered by Rp-cAMPS leads us to conclude that inhibition of cAMP-dependent events does not further increase RLC\textsubscript{20} phosphorylation in normal conditions. To this end, the activation of cAMP-dependent events, such as in portal hypertensive condition (9), accounts for the decreased myosin light-chain phosphorylation in smooth muscle.

It is not surprising that our results did not show a reduced RLC\textsubscript{20} phosphorylation in portal hypertensive superior mesenteric artery. Previous studies by Joh et al. (10, 11) showed that the reduced vasoconstrictor function in portal hypertension was more pronounced in the smaller resistance vessels. To this end, our present findings corroborate an earlier functional observation.

For the myosin phosphorylation in PV, we had similar findings. The behaviors of the PV in portal hypertension are more complicated. An earlier study showed an increased vascular responsiveness of PV to 5-HT in portal hypertensive conditions (4). Thus mechanisms other than cAMP-dependent myosin phosphorylation, such as myosin light-chain phosphatase regulatory subunit (MYPT1) isoform switching (24), caldesmon phosphorylation (7), actin polymerization, and small heat-shock protein phosphorylation (3), may account for the vascular behavior changes in the superior mesenteric artery and PV in portal hypertension.

There are several possible mechanisms whereby elevations in cAMP could lead to reductions in vascular smooth muscle responsiveness to vasoconstrictor stimuli. Previous studies by Adelstein et al. (1) have suggested that PKA-mediated phos-
phorylation of MLCK can prevent smooth muscle activation. A subsequent study by Stull et al. (29) failed to support the hypothesis of Adelstein and showed that PKA does not affect smooth muscle contractility by phosphorylating site A in MLCK in vivo. In view of these findings, one must consider alternative pathways when examining a mechanism of cAMP-dependent modulation of vascular smooth muscle contraction in portal hypertension. In the present study, we report a restoration of RLC\textsubscript{20} phosphorylation following short-term inhibition of PKA. Because RLC\textsubscript{20} phosphorylation is determined by the balance of MLCK/MLCP activity, one possible explanation for our findings is that MLCP is activated in portal hypertension, thereby favoring RLC\textsubscript{20} dephosphorylation. We suggest that changes in RhoA, a monomeric GTPase, may explain cAMP-dependent changes in vascular smooth muscle myosin phosphorylation in portal hypertensive conditions. It is well established that RhoA binding with GTP can activate Rho-kinase (a serine/threonine kinase), which phosphorylates the regulatory subunit of MLCP (MYPT1) and inhibits MLCP activity (12). G\textsubscript{o} and other trimeric G proteins can activate RhoA (8). In the present study, RhoA mRNA expression in portal hypertensive first-order mesenteric arteries was significantly lower when compared with controls. These findings are consistent with the idea that MLCP activity may also be altered in portal hypertension. The inability of PKA inhibition to return RhoA mRNA levels to normal is most likely due to the short period of Rp-cAMPs treatment, which did not allow for RhoA transcriptional events to be realized. Inasmuch as membrane-associated RhoA-GTP is the active RhoA (28) and the fact that RhoA activity is consistent with membrane-associated RhoA protein level (32), we further measured RhoA protein expression levels in both membrane and cytosolic fractions. Acute inhibition of PKA had no effect on cytosolic RhoA levels of portal hypertensive resistance arteries. However, PKA inhibition restored membrane-associated RhoA protein levels to normal. We interpret these findings to indicate that RhoA translocation, but not the transcriptional expression level, is responsible for the restoration of myosin phosphorylation following short-term inhibition of PKA.

Evidence from studies by other laboratories supports our findings. PKA phosphorylates RhoA on Ser188 in natural killer cells, and RhoA translocates toward the cytosol (17). Ht31/ Rt31, the first PKA-anchoring protein (AKAP) that has the potential to integrate Rho and PKA signaling pathways, has been identified (15). Moreover, studies in lymphoid and endothelial cells have shown that PKA inhibits RhoA activation (18, 25). Furthermore, cAMP-specific phosphodiesterase 4 inhibitor rolipram, which blocks cAMP degradation, led to activation of PKA, reduction of active RhoA, as well as decreased RLC\textsubscript{20} phosphorylation at Ser19 (5). In view of these facts, we suggest a possible link among RhoA, AKAPs, and elevations in cAMP-dependent vasodilators.

We propose that reduced RhoA expression in portal hypertensive resistance vasculature is regulated by cAMP-dependent events and contributes to the reduced myosin phosphorylation by altering MLCP activity. Future studies examining the effects of portal hypertension on the MLCK/MLCP axis are warranted.

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GRANTS

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