Vascular contractile response and signal transduction in endothelium-denuded aorta from cirrhotic rats

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Abstract

AIM: The mechanism of decreased vascular reactivity to vasoconstrictors in portal hypertension is still unclear. In addition to nitric oxide, defects in post-receptor signal transduction pathway have been suggested to play a role. However, substantial evidences observed equivocal changes of vascular reactivity following different agonists that challenged the hypothesis of the post-receptor defect. The current study was to evaluate the vascular reactivity to different agonists and the inositol trisphosphate (IP$_3$) changes in signal transduction cascade from cirrhotic rats with portal hypertension.

METHODS: The endothelial denuded aortic rings from cirrhotic and sham-operated rats were obtained for ex viva tension study and measurement of the corresponding [3H] IP$_3$ formation following different receptor and nonreceptor-mediated agonists’ stimulation. Additionally, iNOS protein expression was measured in thoracic aorta. The contractile response curves to phenylephrine were performed in endothelial denuded aortic rings with and without preincubation with a specific iNOS inhibitor (L-N (6)-(1-iminoethyl)-lysine, L-NIL).

RESULTS: In endothelial denuded aortic rings of cirrhotic rats, the vascular responses were reduced with phenylephrine and arginine vasopressin (AVP) stimulation but were normal with U-46619, NaF/AlCl$_3$, and phorbol ester dibutyrate (PdBU) stimulation. Compared to the corresponding control groups, the degree of the increment of [3H] IP$_3$ formation from basal level was also decreased with phenylephrine and AVP stimulation, but was normal with U-46619 and NaF/AlCl$_3$ stimulation. The preincubation with L-NIL did not modify the hyporesponsiveness to phenylephrine. Additionally, the iNOS protein expression in thoracic aorta was not different in cirrhotic and sham-operated rats.

CONCLUSION: Without the influence of nitric oxide, vascular hyporeactivity to vasoconstrictors persisted in cirrhotic rats with portal hypertension. However, the decreased vascular reactivity is an agonist-specific phenomenon. In addition, G-protein and phospholipase C pathway associated with the IP$_3$ productions may be intact in cirrhotic rats with portal hypertension.

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Key words: Cirrhosis; Portal hypertension; Inositol trisphosphate; Vascular reactivity; Protein kinase C

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INTRODUCTION

Peripheral arterial vasodilatation is a characteristic hemodynamic derangement observed in cirrhosis with portal hypertension[1-4]. Increased circulating vasodilators and endothelial-related vasodilatory activity have been suggested to play a role for the development of arterial vasodilatation[5-11]. Additionally, the decreased vascular reactivity to vasoconstrictors also contributes to the arterial vasodilatation in portal hypertension[12-19]. The vasoconstrictors used in those studies to evaluate vascular reactivity were mainly $\alpha_1$-adrenoceptor agonists, arginine vasopressin (AVP), and angiotensin II. Although increased nitric oxide (NO) production has been shown to play a role, the mechanisms of decreased vascular reactivity have not been completely established and the data on vascular reactivity in portal hypertensive humans and animals are conflicting (for reviews see Hadoke and Hayes[19]). It has been hypothesized that the presence of a defect in the post-receptor signal transduction cascade plays, in part, a role for the vascular hyporeactivity[14,16,20,21]. Theoretically, if the post-receptor signal transduction cascade is generally impaired in portal hypertension, any vasoconstrictor that leads to a receptor and G-protein-linked stimulation of vascular contraction should lead to a decreased vascular
contractility. However, two recent studies in portal hypertensive patients and animals showed a normal vascular reactivity to the agonist of thromboxane A2 (TXA2) receptor[24,25]. In addition, an increased vascular response to 5-hydroxytryptamine stimulation was found in cirrhotic patients[26]. The results from these studies do not support the presence of a defect in the post-receptor signal transduction cascade in portal hypertension. Therefore, the current study was undertaken in cirrhotic rats with portal hypertension to measure the vascular contractile response, without the influence of NO, following administration of different agonists. The inositol triphosphate (IP3) formations following agonists’ stimulation and the responses to protein kinase C (PKC) activator were also measured to evaluate the post-receptor mechanism of vascular reactivity in cirrhotic portal hypertension.

**MATERIALS AND METHODS**

**Animals**

Adult male Sprague-Dawley rats (250-350 g) were used in all experiments. Cirrhosis with portal hypertension was produced by common bile duct ligation (CBL), as previously described[25]. Sham-operated rats had their bile duct exposed but not ligated. All rats were caged at 24 °C, with a 12-h light-dark cycle, and allowed free access to food and water. Animal studies were approved by the Animal Experiment Committee of the University and conducted humanely.

**Hemodynamic measurements**

Four to five weeks after surgery, rats were anesthetized with ketamine, 150 mg/kg. The first set of eight cirrhotic and six sham-operated rats were included in this experiment. The ileocolic vein was cannulated with PE-10 tubing for measuring portal venous pressure (PP) and the femoral artery was cannulated with PE-50 tubing for arterial blood pressure (MAP). Pressure and heart rate were monitored by a polygraph (RS 3400, Gould, Valley View, OH, USA) via strain-gauge transducers (P23XL, Viggo-Spectramed, Oxnard, CA, USA). Cardiac output was measured by thermodilution (Columbus Instruments, OH, USA), and cardiac index (CI) and systemic vascular resistance (SVR) were calculated as previously described[26-27].

**Ex vivo tension experiments**

The second set of CBL and sham-operated rats were used in this study. Rats were killed by an overdose of sodium pentobarbital. Then the thoracic aorta (above the diaphragm and below the aortic arch) was excised and put into aerated (mixture of 95% O2, 5% CO2 and maintained at 37 °C with an outer water jacket and circulating heat pump. One wire was fixed to the chamber and the other was attached to a force displacement transducer (FT 03, Grass Instrument Co., Quincy, MA, USA) with a basic tension of 1.8 g. Tension was recorded on a physiograph (RS 3400, Gould, Valley View, OH, USA). The readiness of tissue indicated by consistent responses induced by three consecutive tests with KCl (30-90 mmol/L) in each group was obtained. Tissue was then rinsed and allowed to recover for 45 min. Segments of aorta from each animal were allocated into five subsets. Then, the cumulative concentration-response curves to PEP (10⁻¹⁰ to 10⁻⁴ mol/L), AVP (10⁻¹⁰ to 10⁻⁴ mol/L), synthetic TXA2 analog U-46619 (10⁻⁹ to 10⁻⁵ mol/L), receptor-independent G-protein activator by NaF (10⁻⁵ to 1 mol/L)/ AlCl₃ (30 µmol/L), direct activation of PKC by phorbol ester dibutyrate (PdB) (10⁻⁸ to 3×10⁻⁷ mol/L) were obtained, respectively.

**[3H]InsP₃ assay**

The third set of endothelial-denuded aortic segments were weighted (4 mm in length) and incubated in 0.5 ml KRBS containing LiCl (20 mmol/L) and imipramine (4 µmol/L) at 37 °C under a mixture of 95% O₂, 5% CO₂ for 10 min. Then, agonists [PEP (10⁻¹⁰ to 10⁻⁴ mol/L), AVP (10⁻¹⁰ to 10⁻⁴ mol/L), U-46619 (10⁻⁹ to 10⁻⁵ mol/L), NaF/AlCl₃ (0.1 and 1 mol/L)] or vehicle (0.1% ascorbic acid) were added into the tissue bath for 20 min. These concentrations of agonists were chosen according to the concentration-response contractile curve. We used the highest two or three concentrations of agonist to assess their corresponding IP₃ formations under the same experimental condition. The reaction was stopped with 2 mL of CH₃OH-CHCl₃-HCl (40:20:1, v/v). The assay method was essentially the same as previously reported[29-30]. To extract inositol phosphates, the tissue was sonicated for 45 min and a mixture of 1.26 mL of H₂O and 0.63 mL of CHCl₃ was added to separate the organic and aqueous phases. Tubes were centrifuged at 2 500 r/min for 10 min. The supernatant (aqueous phase containing extracted inositol phosphates) was removed, neutralized to pH 6.8 and 7.2. The neutralized supernatant was added to 300-500 µL of a 1:1 (v/v) mixture of Freon (1, 1, 2-trichlorotrifluoroethane) and tri-n-octylamine, and vortex-mix the separate phases. This neutralized mixture was centrifuged for 10 min at 2 000 r/min. Then three separated phases can be found. These three phases are upper phase: neutralized sample plus water soluble components; middle phase: tri-n-octylamine perchlorate; lower phase: Freon plus unrecalled tri-n-octylamine. Then the upper phase was removed and stored in -70 °C for subsequent analysis. The IP₃ formations with or without stimulation of agonist were measured in all samples by the commercial kit of radioimmunoassay (RIA) (PerkinElmer™ Life Sciences, Inc., Boston, MA, USA). The radioactivity of these samples after RIA was counted in a β-counter (LS 6500, Beckman Instruments, Fullerton, CA, USA).

**Vascular reactivity with and without [L-N(6)-(1-iminoethyl)-lysine, L-NIL] preincubation and iNOS protein measurement**

In order to clarify the role of iNOS on the decreased vascular
reactivity, the fourth set CBL and sham-operated rats were killed by an overdose of sodium pentobarbital. The endothelium-denuded aorta segments were prepared as described above. Then, the cumulative concentration-response curves to PEP (10\(^{-10}\) to 10\(^{-4}\) mol/L) were obtained with and without preincubation with L-NIL (10\(^{-6}\) mol/L) in CBL and sham-operated rats. In addition, the iNOS protein expression was measured in the thoracic aorta from CBL and sham-operated rats by Western blot analysis.

### Statistical analysis

The data are given as mean±SE. Contractile force is expressed in grams (g). Cumulative concentration-response curves to PEP, AVP, U-46619, NaF/AlCl\(_3\), and PdBU were obtained. pEC50 (negative logarithm of the concentration producing the half maximum effect) values were calculated from the sigmoid logistic curves in each vessel preparation. Statistical analysis was performed by two-way ANOVA. Additionally, values for maximal contraction (R\(_{max}\)) were calculated in absolute values. Statistical analysis of the differences between R\(_{max}\) of the aortic rings from rats with cirrhosis and normal reactivity was performed with paired or unpaired Student’s t test when appropriate.

### RESULTS

#### Hemodynamic studies

About 4 wk after CBL, cirrhotic rats showed jaundice, splenomegaly, mesenteric edema and variable amount of ascites. In Table 1, CBL rats had significantly lower SVR associated with higher CI and PP than sham-operated rats.

#### Ex vivo contractile responses in thoracic aorta

Both PEP (10\(^{-10}\) to 10\(^{-4}\) mol/L) and AVP (10\(^{-10}\) to 10\(^{-4}\) mol/L) induced concentration-dependent contractions in the aorta from CBL and sham-operated rats. Our results demonstrated that the R\(_{max}\) but not pEC50, to PEP and AVP was significantly decreased in the aorta of CBL rats compared to those of sham-operated rats (Table 2). These results showed a decreased vascular reactivity and a normal vascular sensitivity to PEP and AVP of aorta in CBL rats (Figure 1 and Table 2). Similarly, U-46619, NaF/AlCl\(_3\), and PdBU also induced the concentration-dependent contractions curve in both study groups (Figure 2). However, neither the R\(_{max}\) nor its pEC50 values differed between the two vessel groups (Table 2).

#### \[^{[3]}\text{H}\]1,4,5 Inositol trisphosphate (IP\(_3\)) formation in aortic rings

The basal IP\(_3\) formation in aortic rings was similar between the CBL and sham-operated rats (385±6 and 402±10 cpm/mg tissue weight, n = 8 in each group with different agonist stimulation). Both PEP (10\(^{-10}\) to 10\(^{-5}\) mol/L) and AVP (10\(^{-7}\) to 10\(^{-5}\) mol/L) induced dose-dependent increases of \[^{[3]}\text{H}\]IP\(_3\) in both groups. In the presence of PEP and AVP, the increments of \[^{[3]}\text{H}\]IP\(_3\) (% of basal levels) in the CBL rats were significantly lower than that in the sham-operated rats at the concentration of 10\(^{-6}\) and 10\(^{-5}\) mol/L of PEP and 10\(^{-7}\) to 10\(^{-5}\) mol/L of AVP, respectively (Figure 3). In contrast, the percentage of increases after U-46619 (10\(^{-7}\) and 10\(^{-5}\) mol/L) and NaF/AlCl\(_3\) (0.1 and 1 mol/L) stimulation were similar between the two groups (Figure 4).

#### Vascular reactivity with and without L-NIL preincubation and iNOS protein measurement

The effects of selective iNOS inhibition on vascular responses
to PEP are shown in Figure 5A. In cirrhotic aortic rings, the vascular hyporeactivity to PEP stimulation still persisted after preincubation with L-NIL. In addition, the iNOS protein expression in the thoracic aorta of BDL rats was not different from sham-operated rats (Figure 5B). These results indicated that iNOS did not play a role in the decreased vascular contractility in portal hypertension.

**DISCUSSION**

The mechanism of agonists-induced contraction of vascular smooth muscle cells involves both membrane and intracellular changes. Agonists bind to surface G-protein-linked transmembrane receptors may activate phospholipase C (PLC). This enzyme hydrolyzes the lipid precursor phosphatidylinositol (4,5)-biphosphate stored in the plasma membrane to give IP₃ and 1,2-diacylglycerol. The IP₃ binds to its receptor located in the membrane of the sarcoplasmic reticulum that lead to open the calcium channels and increases the cytosolic calcium concentration. The agonists have also shown to open L-type calcium channel located on the plasma membrane to increase cytosolic calcium concentration. On the other hand, diacylglycerol activates PKC with regulation of intracellular calcium concentration. The cytosolic free calcium then binds to calmodulin to form calcium-calmodulin complex, which in turn interact with the contractile proteins that lead to smooth muscle cell contraction. Therefore, any alteration in the G-proteins receptor coupling, transmembrane receptor, or the signal transduction downstream from the transmembrane receptor may play a role in the vascular hyporesponsiveness in portal hypertension.

The mechanisms responsible for the decreased vascular reactivity in cirrhosis are not completely understood. In experimental portal hypertension, the increased nitric oxide production has been suggested to play an important role for the vascular hyporeactivity. Although Vallance and Moncada proposed that the increased activity of iNOS led to NO overproduction and subsequent hyperdynamic circulation, substantial studies showed a lack of iNOS expression in the vessels from portal hypertensive animals despite the enhanced NO production in the hemodynamic derangements in portal hypertension (for review see Wiest and Groszmann). The role of eNOS is further supported in a recent study using gene-knockout mice. Nevertheless, a persistent vascular hyporeactivity to vasoconstrictor stimulation in portal hypertensive human and animals was still observed in endothelium-free aortic ring or following pre-incubation with NOS inhibitor. This observation was further supported in endothelial denuded hepatic artery from cirrhotic patients. Therefore, in addition to nitric oxide, other factors are also involved in the pathogenesis of vascular hyporeactivity in portal hypertension. A number of studies have found an attenuated vascular reactivity to vasoconstrictors (such as angiotensin II, vasopressin, and α₁-adrenoreceptor agonists), but these receptors were not down regulated or the receptor numbers and affinities were...
unchanged\(^{[15-18,41]}\). These studies lead to a hypothesis of a defect in signal transduction cascade in portal hypertension. Accordingly, if the post-receptor defect does exist in portal hypertension, a generalized hyporesponsiveness to any receptor and G-protein linked vasoconstrictors should be observed in cirrhotic human and animals with portal hypertension.

In the current study, we found an expected decrease in contractile responses to PEP (a \(\alpha_1\)-adrenoceptor agonist) and AVP (a \(V_1\)-AVP receptor agonist) in the endothelium-denuded aortic ring from cirrhotic rats compared to those from sham-operated rats. By contrast, we found a normal contractile response to U-46619 (a TXA\(_2\) receptor agonist) in the aortic rings from cirrhotic rats compared to those from the sham-operated rats. TXA\(_2\) receptor is a G-protein-coupled and PLC-linked transmembrane receptor as well as those of \(\alpha_1\), \(V_1\)-AVP or angiotensin II receptors. Van Obbergh \textit{et al}, and Schepke \textit{et al}, have demonstrated similar observations of a normal contractile response to U-46619 in cirrhotic rats and humans\(^{[22,23]}\). In addition, another study in cirrhotic patients also showed an increased vascular response following 5-hydroxytryptamine stimulation\(^{[24]}\). Taken together, it is conceivable that the vascular hyporeactivity to vasoconstrictors in portal hypertension is a selective agonist-mediated phenomenon. In other words, decreased vascular reactivity in portal hypertension may occur in some agonists but do not occur in others. Please note that the vascular reactivity in the current study was performed in endothelium-denuded aortic rings from cirrhotic rats. Similar to previous observations, we also observed a lack of the role of iNOS on the vascular hyporesponsiveness in portal hypertension evidenced by both \textit{ex vivo} vascular tension study with L-NIL preincubation and aortic iNOS protein expression\(^{[35,36]}\). Therefore, the role of NO on vascular reactivity has been minimized to the least extent. Moreover, we found a normal contractile response by NaF/AlCl\(_3\) from the aortic ring of cirrhotic rats compared to those from sham-operated rats.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure3.png}
\caption{(A) Phenylephrine (PEP) (10\(^{-7}\) to 10\(^{-5}\) mol/L) and (B) AVP (10\(^{-7}\) to 10\(^{-5}\) mol/L)-induced \(^{[3]}\)H]IP\(_3\) formation in the aortic ring from CBL (black bars) and sham (white bars) rats. \(^{[3]}\)H]IP\(_3\) formation was expressed as the percentage of counts (cpm) per minute in the presence of agonist divided by the counts without agonist (basal formation). The data are expressed as mean±SE. *P<0.05 vs sham rats, \(n=8\) in each group with different agonist stimulation. Sham: sham-operated rats; CBL: common bile duct-ligated rats.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4.png}
\caption{(A) U-46619, a synthetic TXA\(_2\) analog (10\(^{-7}\) and 10\(^{-5}\) mol/L), (B) NaF/AlCl\(_3\) (0.1 and 1 mol/L)-induced \(^{[3]}\)H]IP\(_3\) formation in the aortic ring from CBL (black bars) and sham (white bars) rats. \(^{[3]}\)H]IP\(_3\) formation was expressed as the percentage of counts (cpm) per minute in the presence of agonist divided by the counts without agonist (basal formation). The data are expressed as mean±SE, \(n=8\) in each group with different agonist stimulation.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure5.png}
\caption{(A) Maximum cumulative concentration-response curves to phenylephrine (PEP) (10\(^{-10}\) to 10\(^{-4}\) mol/L) in thoracic aorta from CBL (●) and sham (□) rats with and without preincubation with L-NIL (L-NIL) (1-iminoethyl)-lysine, a selective iNOS inhibitor). \(n=10\) in each group with different agonist stimulation. CBL: common bile duct-ligated rats; sham: sham-operated rats. (B) Western blot analysis of inducible nitric oxide synthase (iNOS) protein expression from thoracic aorta in CDL and sham-operated rats. Lanes A and B: CBL rats; lanes C and D: sham rats.}
\end{figure}
rats. A similar observation showing a normal contractile response to NaF/AlCl₃ in the endothelium-denuded hepatic artery in cirrhotic patients was reported by Schepke et al.²⁵. Previously, an impairment of contractile response to NaF/AlCl₃ had been reported in the intestinal microcirculation of portal hypertensive rats.³⁴ In that study, vascular contractility was evaluated by perfusion of mesenteric beds with intact endothelium. It is possible that the different vascular preparation and the presence of NO may contribute to such discrepancy. Because NaF/AlCl₃ is a direct G protein activator, it produces smooth muscle cells’ contraction independent to the receptor and G-protein coupling.³⁵ However, the current study cannot exclude the minor G-protein-related RhoA/Rho kinase pathway. Further studies are needed to clarify this point.

Phosphoinositide metabolism is important in the signal transduction cascade for receptor-coupled vasoconstriction.³⁶ Its hydrolyzed products, IP₃ and 1,2-diacylglycerol, are crucial second messengers for triggering and maintaining the contractile response of the vascular smooth muscle cell.³⁷,³⁸ In the current study, we observed that the increased formation of IP₃ after PEP and AVP stimulation in cirrhotic rats was significantly lower than that in sham-operated rats. In contrast, the increased formation of IP₃ after U-46619 and NaF/AlCl₃ stimulation was similar between the two experimental groups. The changes in IP₃ formation following different agonists’ stimulation were parallel to the corresponding changes of vascular contractile response between cirrhotic and normal rats. For instance, in our previous study, we observed that, in portal hypertensive animals, the PEP-stimulated [³²P]inositol phosphate formation in tail artery was attenuated, whereas the NaF/AlCl₃-mediated [³²P]inositol phosphate formation in portal vein was unaltered.³⁹ On the other hand, Hartleb et al.⁴⁰ observed a lack of vascular hyporesponsiveness to L-type calcium channel activator in cirrhotic rats. Moreover, Castro et al.⁴¹, found that the intracellular calcium mobilization pathway is preserved in the vascular smooth muscle cells from cirrhotic rats. Together these results strengthened the presence of an intact signal transduction cascade in cirrhosis with portal hypertension that challenged the hypothesis of the post-receptor defect.

PKC plays an essential role in the regulation of vascular smooth muscle cell contraction.⁴² In the current study, the PdBu-mediated contractile response was not different between the two experimental groups. The role of PKC in vascular contractility in portal hypertension is controversial. Previous studies have reported that the PdBu-mediated contractile response remains unchanged in aortic rings and mesenteric vascular bed of portal vein stenosed rats, which in line with the findings of the present studies in cirrhotic rats.³⁵,⁴³ In contrast, other studies have demonstrated that the alteration of PKC plays a partial role for the decreased vascular reactivity in portal hypertension.³⁴,⁴⁵ However, it has been suggested that overproduction of NO may contribute, in part, for the alteration of PKC activity in portal hypertension.³⁶ Therefore, in the current study, it is possible that a normal PdBu-induced contractile response was observed in endothelial-denuded aortic ring in cirrhotic rats, which suggested a normal PKC activity in cirrhotic rats. All together, the current study suggested that the G-protein and PLC pathway associated with the IP₃ and 1,2-diacylglycerol actions were not impaired in cirrhotic rats with portal hypertension. The decreased vascular reactivity observed in the specific receptors may probably result from a defect in receptor-G-protein coupling. Further studies are needed to clarify this phenomenon.

In summary, this study demonstrated that, without the influence of NO, vascular hyporeactivity persisted in CBL rats with portal hypertension. However, the decreased vascular reactivity is an agonist-specific phenomenon. In addition, the current study is against the presence of a post-receptor defect in vascular hyporeactivity observed in portal hypertension.

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