The phosphorylation of eNOS initiates excessive NO production in early phases of portal hypertension

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Phosphorylation of eNOS initiates excessive NO production in early phases of portal hypertension. The phosphorylation of eNOS at Ser1176 was enzyme-specific activity, suggesting posttranslational modifications in eNOS expression but was due to an increase in constrictor. The reduced responsiveness in PVL was not due to production in the PVL group blunted the response to the vasodilator. The two groups was abolished, suggesting that enhanced NO production in early portal hypertension. Male Sprague-Dawley rats were subjected to either sham or portal vein ligation (PVL), and mesenteric arterial beds were used for ex vivo perfusion studies. Mesenteric arterial beds from PVL rats had an approximately 60–70% decrease in response to methoxamine (an α1-agonist) compared with the sham group (P < 0.01). When Nω-monomethyl-L-arginine (a NOS inhibitor) was added to the perfusion, the difference in perfusion pressure between the two groups was abolished, suggesting that enhanced NO production in the PVL group blunted the response to the vasoconstrictor. The reduced responsiveness in PVL was not due to changes in eNOS expression but was due to an increase in enzyme-specific activity, suggesting posttranslational modification of eNOS. The phosphorylation of eNOS at Ser1176 was significantly increased by twofold (P < 0.05) in the PVL group. Furthermore, PVL significantly increased Akt phosphorylation (an active form of Akt) by threefold (P < 0.05). When vessels were treated with wortmannin (10 nM) to block the phosphatidylinositol-3-OH-kinase/Akt pathway, NO-induced vasodilation was significantly reduced. These results suggest that the phosphorylation of eNOS by Akt activates the enzyme and may be the first step leading to an initial increase in NO production in portal hypertension.

Akt; portal vein ligation; in vivo; superior mesenteric artery (NO) synthase (eNOS) and enhances its ability to generate NO (7, 12, 22). eNOS is the NOS isoform that produces endothelium-derived NO, a vasodilator that regulates vascular tone (17). Various forms of stimuli, such as growth factors, cytokines, and mechanical forces by shear stress, stimulate the production of NO by a phosphatidylinositol-3-OH-kinase (PI3K)-dependent mechanism (7, 12, 22). The possible involvement of eNOS phosphorylation in vascular function was demonstrated in the study using gene transfer of an adenovirus vector encoding Akt. Transduction of rabbit femoral arteries with constitutively active Akt increased NO-mediated vasodilation in these arteries. Interestingly, transduction with dominant negative Akt attenuated this effect in these vessels (18). Involvement of eNOS phosphorylation in vascular function was also shown in that the topical application of platelet-activating factor increased eNOS phosphorylation and NO production in the microcirculation of the hamster cheek pouch (8). Collectively, these observations strongly support the idea that eNOS phosphorylation by Akt can be involved in the regulation of vascular tone in vivo. Despite this accumulating evidence, the phosphorylation and activation of eNOS on Ser1179 by Akt have not been shown in vivo.

Vascular NO overproduction plays a central role in both systemic and splanchnic vasodilatation, which is a hallmark of portal hypertension that causes devastating complications in liver cirrhosis (2, 20, 23, 24). Studies have demonstrated that eNOS, but not the inducible isoform of NOS (iNOS), produces excessive NO in systemic and particularly splanchnic vascular beds (20). Increased eNOS expression and enzyme activity are well-established events in the chronic model of portal hypertension (20, 23, 24). However, the mechanism of the early induction of excessive NO production by eNOS remains to be elucidated.

Therefore, we investigated the mechanism of initial eNOS induction in the splanchnic circulation using 1 day postoperative portal vein-ligated (PVL) animals as...
an early or acute model of portal hypertension. In this model, we found that eNOS enzyme activity was first upregulated before the induction of eNOS expression in response to PVL. The posttranslational modification of eNOS is the important control of eNOS catalytic activity. Thus we explored the possibility of eNOS phosphorylation by Akt activation, which was hypothesized as a mechanism of increased eNOS activity in early portal hypertension. Our data strongly suggest the potential involvement of eNOS phosphorylation by Akt in the upregulation of eNOS activity observed in early portal hypertension. To our knowledge, this is the first in vivo evidence of eNOS phosphorylation by Akt regulating vascular function.

MATERIALS AND METHODS

Animals. A total of 82 male Sprague-Dawley rats (Harlan Sprague Dawley; Indianapolis, IN) weighing 275–325 g were studied. The rats were housed in Plexiglas cages in a temperature- and humidity-controlled environment at lowed free access to water and rat chow (Ralston Purina; St. Louis, MO) until the time of experiments. All experimental procedures in this study were conducted in accordance with the standard procedures indicated in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH 86-23, Revised 1985).

Induction of portal hypertension. A prehepatic portal hypertensive animal model extensively studied in our laboratory (5) was used. The animals were divided into two groups. One group included 38 rats in which a sham operation was performed (sham group). The other group included 44 rats in which partial PVL surgery was performed to induce portal hypertension (PVL group). The rats were anesthetized with ketamine hydrochloride (Ketalar, 100 mg/kg body wt, Parke-Davis; Avon, CT). After a midline abdominal incision, the portal vein was freed from the surrounding tissue. A ligature (silk gut 5-0) was placed around a 20-gauge blunt-tipped needle lying along the portal vein. Subsequent removal of the needle yielded a calibrated stenosis of the portal vein. In sham-operated rats, the same operation was performed with the exception that after the portal vein was isolated, no ligature was placed. After the operation, the animals were housed in plastic cages and allowed free access to rat food and water. Studies were performed 1 day after the operation.

In vitro perfusion technique. In vitro mesenteric perfusion studies were performed 1 day after PVL or sham surgery using a modification of methods originally described by McGregor (28, 29). The superior mesenteric artery (SMA) was cannulated with a polyethylene-60 catheter and gently perfused with 15 ml of warm Krebs buffer to eliminate blood. After the SMA was isolated with its mesentery, the gut was dissected near its mesenteric border. The SMA with its associated mesenteric tissue was placed into a 37°C water-jacketed container and perfused at a constant rate (4 ml/min) with oxygenated 37°C Krebs buffer (95% O2-5% CO2) through a roller pump (Masterflex, Cole-Parmer; Barrington, IL). The Krebs buffer had the following composition (in mM): 118 NaCl, 4.7 KCl, 1.2 K2HPO4, 1.2 MgSO4, 2.5 CaCl2, 25 NaHCO3, 0.026 EDTA, and 11 glucose; pH 7.4. Under these conditions, this preparation has been shown to be viable over several hours with unchanged perfusate NO concentration under basal conditions (28, 29) and unaltered pressor responsiveness (28, 29). The effluent from the perfused tissue was removed continuously from the perfusion chamber to prevent exogenous exposure of the tissue to perfusate-containing drugs. The tissue preparation was covered lightly with a piece of Parafilm to prevent tissue drying. Polyvinyl chloride-free circulation tubing (Abbot Laboratories; Abbott Park, IL) was used to avoid NO absorption by the tubes. Perfusion pressure was continuously monitored and recorded using a strain-gauge transducer (Statham; Oxnard, CA).

Methoxamine was dissolved in Krebs solution, and SMA beds were challenged with 100 μM methoxamine for 2 min to study vasoconstrictor response. We used an inhibitor of PI3K, wortmannin (10 nM), which was dissolved in dimethyl sulfoxide and diluted with Krebs solution. The final concentration of dimethyl sulfoxide was <0.1%, and dimethyl sulfoxide at that concentration did not cause any significant changes in perfusion pressure (data not shown). Wortmannin was perfused to SMA for 20 min before the methoxamine challenge.

Determination of nitrite/nitrate concentrations. The nitrite/nitrate (NOx) concentration in the perfusate was measured using a Sievers NO analyzer (Sievers Instruments; Boulder, CO) as previously described (28) with a slight modification in collecting the perfusate. Per fusate was collected for exactly 1 min during the last one-half of 2 min of methoxamine perfusion. Results were expressed as picomoles of NOx per milliliter of the perfusate.

Western blotting. SMA vessels were harvested at 24 h after the PVL (n = 14) or sham surgery (n = 14), immediately frozen in a liquid nitrogen, and kept at −80°C until analyzed. SMA samples were homogenized in a lysis buffer containing 50 mM Tris·HCl, 0.1 mM EGTA, 0.1 mM EDTA, 5 mM sodium fluoride, 1 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, protease inhibitor cocktail tablet (Roche Diagnostics; Mannheim, Germany), 1% (vol/vol) Nonidet P-40, 0.1% SDS, and 0.1% deoxycholate; pH 7.5. Protein content in the supernatants was quantified using the Lowry method with bovine serum albumin as the standard. The supernatants were subjected to SDS-PAGE of proteins (100 μg), and Western blotting was performed as previously described (30) using antibodies that recognize phosphorylated eNOS at Ser1177 PAb, phosphophorylated Akt PAb, Akt PAb (Cell Signaling Technology; Beverly, MA), eNOS MAb, iNOS MAb (Transduction Laboratories; Lexington, KY), and β-actin MAb (Sigma; St. Louis, MO). Enhanced chemiluminescence was used for protein detection. The intensities of the bands corresponding to the proteins of interest were measured using a densitometer. The phosphorylation of eNOS and Akt was evaluated as a ratio of phosphorylated eNOS and phosphorylated Akt against total eNOS and Akt, respectively.

NOS activity assay. The conversion of L-[14C]arginine to L-[14C]citrulline was used to determine NOS activity (15). Briefly, SMA were homogenized in a lysis buffer identical to that described for Western blotting (27). Samples were incubated with buffer containing 1 mM reduced nicotinamide adenine dinucleotide phosphate, 3 μM tetrahydrobiopterin, 100 nM calmodulin, 2.5 mM CaCl2, and 1.4 μM L-[14C]arginine (25.2 nCi) at 37°C. To determine NOS activity, duplicate samples were incubated for 30 min in the presence and absence of Nω-nitro-L-arginine methyl ester (L-NAME; 2 mM). The reaction was terminated by the addition of 1 ml of cold stop buffer (20 mM HEPES, 2 mM EDTA, and 2 mM EGTA; pH 5.5), and the reaction mixture was passed over a Dowex AG 50WX-8 resin-containing column into a vial and analyzed using a liquid scintillation counter. Radiolabeled counts per minute of L-citrulline generation were measured and used to determine L-NAME-inhibitable NOS activity.
RESULTS

In vitro perfusion pressure. To determine whether the hyporesponsiveness to a vasoconstrictor occurs in this acute or early model of portal hypertension (i.e., 24 h of PVL), we performed a measurement of perfusion pressure in mesenteric arterial beds obtained from PVL and sham-operated animals. The resting perfusion pressures were 13.9 ± 1.1 and 14.0 ± 1.3 mmHg in the sham and PVL groups, respectively. PVL significantly reduced the perfusion pressure by 60% and 70% in response to 30 and 100 μM methoxamine (an α-adrenergic agonist), respectively, compared with the sham group (Fig. 1A). The values of perfusion pressures shown in Fig. 1, A and B, are ones after the resting perfusion pressure was subtracted. To determine the involvement of NO in this reduced contractile response to methoxamine, the mesenteric arterial beds were first treated with Nω-monomethyl-L-arginine (L-NMMA; a specific NO inhibitor) to inhibit NO production before methoxamine. After the L-NMMA treatment, the difference in the methoxamine response between the sham and PVL groups was no longer significant (Fig. 1B). Moreover, the NO inhibitor L-NMMA had no effect on the resting perfusion pressure (15.1 ± 0.4 mmHg for sham and 14.9 ± 0.5 mmHg for PVL in the presence of L-NMMA).

Furthermore, we measured the NO concentration in the perfusate after a challenge with methoxamine. There was a significant increase (P < 0.05) in the NO concentration in the perfusate collected from the PVL group (838.0 ± 88.9 pmol/ml) compared with the sham group (526.9 ± 120.9 pmol/ml). These results strongly suggest that the overproduction of NO may impair the contractile response to methoxamine in mesenteric artery beds of PVL animals.

NOS activity and eNOS protein expression. To determine the mechanism of NO overproduction in mesenteric arterial beds in early portal hypertension, we determined the NOS activity to test whether the up-regulation of eNOS catalytic activity was responsible for the early increase in NO production in SMA observed in PVL animals. Interestingly, PVL caused a significant fourfold increase in NOS activity compared with the sham group (Fig. 2A).

We also performed Western blot analysis to determine eNOS protein expression in SMA. Additionally, we tested iNOS expression to check the involvement of iNOS in NO overproduction in the mesenteric artery of PVL animals. PVL did not cause a significant upregulation of eNOS protein expression (Fig. 2B), although there was a trend of increased eNOS expression in the PVL group. In agreement with studies by us and other groups in late portal hypertension (20, 27, 30), no iNOS expression was observed in SMA from both sham and PVL animals (Fig. 2C). In the chronic model of portal hypertension, a significant upregulation of eNOS protein expression has been shown (2, 3, 23, 30, 31). In contrast, in this early model of portal hypertension, no significant upregulation in eNOS protein expression was observed. These results strongly suggest that enhanced eNOS activity played more critical role than eNOS mRNA or protein expression in the early stage of portal hypertension. Furthermore, this observation will raise the possibility that the posttranslational modification of eNOS plays an important role in early upregulation of eNOS in the mesenteric artery of PVL animals. iNOS is also a component of total NOS activity. Considering that there was no iNOS expression observed in SMA by Western blot analysis (Fig. 2C), it is likely that eNOS, not iNOS, was the major component of the NOS activity determined in this study.

Phosphorylation of Akt and eNOS. Recently, studies from our group (12) and others (7, 22) have demonstrated that the phosphorylation of eNOS increases the catalytic activity of eNOS in endothelial cells. We tested the hypothesis that the phosphorylation of
eNOS by Akt plays a role in the increased eNOS activity in PVL animals. First, we investigated phosphorylation of Akt, an active form of Akt. Similar to eNOS, Akt becomes activated when it is phosphorylated. Our data indicate that the phosphorylation of Akt was significantly enhanced in PVL animals compared with sham animals (Fig. 3A).

Next, we tested the phosphorylation of eNOS using phosphorylated eNOS antibody, which recognizes the phosphate group at Ser^{1177} (human) or the rat equivalent (Ser^{1176}), which is known as the phosphorylation site for Akt, AMP-activated protein kinase (AMPK), and cAMP-dependent protein kinase (PKA) (4, 21). As shown in Fig. 3B, PVL significantly increased eNOS phosphorylation in SMA, suggesting that the phosphorylation of eNOS by Akt plays a role in the upregulation of eNOS activity observed in PVL animals. Collectively, these data strongly suggest that Akt was activated and phosphorylated eNOS, which seems to be a mechanism of the initial increase in NO production observed in the early portal hypertension.

Fig. 2. Endothelial NOS (eNOS) activity, but not eNOS expression, is enhanced in PVL rat superior mesenteric arteries (SMA). A: NOS activity was assessed in homogenized SMA from sham (n = 4) and PVL (n = 4) rats as described. NOS activity was significantly enhanced in SMA from PVL animals compared with sham animals (*P < 0.05). B: eNOS protein levels were examined after 1 day of PVL (n = 4) or sham (n = 4) surgery. There were no significant differences in eNOS expression between the PVL and sham groups. Top, representative blot. No inducible NOS (iNOS) expression was observed in SMA from both sham and PVL animals (C).

Fig. 3. Phosphorylation of Akt and eNOS was increased in PVL animals. A: Western blot analysis of homogenized SMA using anti-phosphorylated Akt and total Akt showed that PVL significantly increased phosphorylated Akt, an active form of Akt (*P < 0.05), in SMA isolated 1 day after PVL; n = 3 rats/group. B: Western blot analysis of homogenized SMA indicate that phosphorylated (Phospho) eNOS was significantly increased in the PVL group compared with the sham group (*P < 0.05); n = 4 rats/group.
**Effects of wortmannin on NO production and contractile response.** To test whether inhibition of the PI3K/Akt pathway ameliorates excessive NO production in SMA of PVL animals, we first tested NO production and vascular function in SMA after treatment with wortmannin (10 nM), an inhibitor of PI3K (Fig. 4A). We found that wortmannin treatment of SMA in PVL animals decreased NO production by 50%. We then tested whether the reduction in NO production by wortmannin influenced the contractile response to vasoconstrictor in SMA beds of PVL animals (Fig. 4B). Wortmannin treatment significantly increased the contractile response to methoxamine in SMA of PVL animals. Collectively, these results strongly suggest an involvement of the PI3K/Akt pathway in increased NO production and subsequent reduction in the vasoconstrictor response in SMA observed in PVL animals.

**DISCUSSION**

Our study demonstrates that the upregulation of eNOS enzyme activity is the initial event leading toward the NO overproduction and vasodilatation in portal hypertension. Enhanced phosphorylation of eNOS by Akt may, at least in part, play a role in the upregulation of eNOS catalytic activity responsible for the NO overproduction and the reduced contractile response to methoxamine observed in mesenteric arterial beds in early portal hypertension.

eNOS catalytic activity is highly regulated by complex posttranslational modifications, one of which being phosphorylation by kinases. Akt, also known as protein kinase B, can directly phosphorylate eNOS and increase eNOS enzyme activity, leading to NO production in the cultured endothelial cell (7, 12, 22). Furthermore, a study using rabbit femoral arteries demonstrated that transfection of constitutively active Akt to the arteries significantly increased the resting diameter and blood flow in those vessels. A NOS inhibitor blocked this Akt-mediated vasodilatation, suggesting that Akt causes the NO-mediated vasodilatation (18). Akt can directly phosphorylate recombinant eNOS or eNOS in situ, at Ser1177 (human) or Ser1179 (bovine) (7, 12, 13, 22). In our study, the phosphorylated Ser1177-specific antibody, known as the phosphorylation site of Akt, was used and showed a significant increase in eNOS phosphorylated at this Akt site in the PVL group. In support of this finding, an active form of Akt (phosphorylated Akt) increased in SMA isolated from 1 day after PVL surgery. In contrast, SMA isolated from 2 days and 14 days after the PVL did not show significant differences in the levels of phosphorylated Akt between sham and PVL animals (data not shown). These observations may suggest a possible link between Akt activation and eNOS phosphorylation and subsequent NO production in the early stage of portal hypertension (1 day after PVL).

An involvement of the PI3K/Akt pathway in NO overproduction in PVL was also demonstrated in our study. When SMA beds were treated with an inhibitor of the PI3K pathway, NO production was significantly reduced, an effect that was accompanied by an increase in the contractile response to methoxamine in portal hypertensive animals. Our results strongly imply a role for eNOS phosphorylation by the PI3K/Akt pathway in the excessive NO production observed in the splanchnic circulation in portal hypertensive animals.

There may be a possible involvement of other kinases that phosphorylate eNOS in our early model of portal hypertension. Recent in vitro studies have demonstrated that other kinases such as AMPK, PKA, and cGMP-activated kinases can also phosphorylate Ser1177 (1, 4). Furthermore, PKC and AMPK can phosphorylate Thr495 of eNOS (4, 11, 21). The physiological relevance of these kinases and vascular function has yet to be demonstrated. Together with a growing body of recent evidence described previously, our finding strongly suggests that eNOS phosphorylation by Akt...
may be the most plausible mechanism of early induction of NO production in the mesenteric circulation.

Akt is known as an important downstream target of PI3K. Furthermore, the PI3K/Akt pathway has been shown to directly activate eNOS by phosphorylation at serine-1177, which is induced by various cytokines and mechanical forces such as shear stress (10, 13). It has been shown that shear stress is not increased on day 1 of PVL (6). Thus, in our early model of portal hypertension, it is unclear that shear stress is involved in the early upregulation of eNOS via the PI3K/Akt pathway. This speculation is also supported by a study (16) in which NO overproduction started even at 3 h after the PVL surgery, a time point much earlier than the significant shear stress induced in the PVL model. Further studies will be needed to elucidate the factors that are involved in the initiation of eNOS phosphorylation.

Besides phosphorylation, there are other possibilities of additional control mechanisms that may upregulate eNOS activity in early portal hypertension. eNOS can directly interact with at least five proteins in vitro and in vivo: calmodulin, heat shock protein 90 (HSP90) (9, 14), dynamin-2 (26), caveolin-1 and -3 (9, 14), and the intracellular domains of G protein-coupled receptors (19). The first three proteins stimulate NO activity, whereas the latter three proteins are inhibitory. A study by Shah et al. (27) showed involvement of HSP90, an activator of eNOS, in the upregulation of eNOS catalytic activity in mesenteric beds. Interestingly, the specific HSP90 inhibitor geldanamycin partially reversed the NO-mediated hyporeactivity to vasoconstrictor, suggesting that HSP90 signaling may partially mediate excessive NO production in the mesenteric beds in portal hypertension. In addition, in vitro evidence has shown that HSP90 binding to Akt is necessary for Akt to be active (25). Collectively, these observations may raise a potential involvement of HSP90 and other protein-protein interactions in the induction of early upregulation of eNOS catalytic activity in the development of portal hypertension.

The involvement of another NOS isoform, neuronal NOS (nNOS), in the hyperdynamic circulation has been suggested in CCl4-induced cirrhotic rats with ascites (32). At present, the role of nNOS in the mesenteric arterial bed is not known.

In conclusion, upregulation of eNOS catalytic activity, not eNOS expression, is the initial event that induces NO overproduction in the splanchic circulation. eNOS phosphorylation by Akt may be a potential mechanism of the initial induction of eNOS activity and NO-mediated hyporesponsiveness to vasoconstrictor in portal hypertension. Various cytokines and growth factors have been shown to induce the PI3K/Akt pathway and subsequent phosphorylation of eNOS and also to influence protein-protein interaction with eNOS. Determining the factors that initiate phosphorylation of eNOS by the PI3K/Akt pathway is an important step for preventing the initiation of portal hypertension, which causes devastating complications of liver diseases.

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