Opposite regulation of endothelial NO synthase by HSP90 and caveolin in liver and lungs of rats with hepatopulmonary syndrome

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Submitted 26 March 2007; accepted in final form 6 August 2007

Frossard J-L, Schiffer E, Cikirikcioglu B, Bourquin J, Morel DR, Pastor CM. Opposite regulation of endothelial NO synthase by HSP90 and caveolin in liver and lungs of rats with hepatopulmonary syndrome. Am J Physiol Gastrointest Liver Physiol 293: G864–G870, 2007; doi:10.1152/ajpgi.00136.2007.—The hepatopulmonary syndrome is a complication of cirrhosis that associates an overproduction of nitric oxide (NO) in lungs and a NO defect in the liver. Because endothelial NO synthase (eNOS) is regulated by caveolin that decreases and heat shock protein 90 (HSP90) that increases NO production, we hypothesized that an opposite regulation of eNOS by caveolin and HSP90 might explain the opposite NO production in both organs. Cirrhosis was induced by a chronic bile duct ligation (CBDL) performed 15, 30, and 60 days before sample collection and pharmacological tests. eNOS, caveolin, and HSP90 expression were measured in hepatic and lung tissues. Pharmacological tests to assess NO released by shear stress and by acetylcholine were performed in livers (n = 28) and lungs (n = 28) isolated from normal and CBDL rats. In lungs from CBDL rats, indirect evidence of high NO production induced by shear stress was associated with a high binding of HSP90 and a low binding of caveolin to eNOS. Opposite results were observed in livers from CBDL rats. Our study shows an opposite posttranslational regulation of eNOS by HSP90 and caveolin in lungs and liver from rats with CBDL. Such opposite posttranslational regulation of eNOS by regulatory proteins may explain in part the pulmonary overproduction of NO and the hepatic NO defect in rats with hepatopulmonary syndrome.

shear stress; endothelial nitric oxide synthase; lung perfusion; liver perfusion

THE HEPATOPULMONARY SYNDROME (HPS) is a major pulmonary vascular complication that occurs in 4–24% of patients with chronic liver disease (8, 15, 17). The syndrome is defined by hypoxemia (Pao2 < 80 mmHg) related to pulmonary vascular dilatation and occurs in patients with cirrhosis or portal hypertension (13, 28). A decreased hypoxic vasoconstriction of pulmonary arterioles and/or an excess in vasodilators explain the pathological pulmonary vasodilatation (28). One of the mediators responsible for the vasodilatation is nitric oxide (NO). An excess in exhaled NO has been found in patients with HPS (6, 29, 30), and this NO excess disappears after liver transplantation (31).

Rats with chronic bile duct ligation (CBDL) develop HPS over time (5, 10, 32). In the liver, biliary cirrhosis develops with an increase in portal vein pressure. In lungs, hypoxemia and pulmonary vasodilatation increase with the duration of bile duct ligation (5, 10, 32). High pulmonary NO production is involved in the pathophysiology of the experimental disease because chronic pharmacological inhibition of NO production prevents the occurrence of HPS (23). NO originates from the endothelial constitutive (eNOS) and the inducible (iNOS) forms of NO synthase, the inducible isoform being localized in pulmonary intravascular macrophages (23).

eNOS expression increases over time in lungs of CBDL rats (9, 19). The increased expression begins 1–2 wk after bile duct ligation and correlates with the onset of gas-exchange abnormalities and pulmonary vasodilatation. Interestingly, endothelin overproduction originating from livers of CBDL rats contributes to the excessive NO release by acting on endothelin B receptors located on pulmonary endothelial cells (18, 21). Pulmonary endothelin B receptor expression is increased in CBDL rats, and their stimulation by endothelin increases the expression and activity of eNOS (20).

In contrast to lungs, the activity of eNOS is decreased in livers from CBDL rats, and an increased caveolin binding to eNOS explains the decreased production of hepatic endothelial NO, caveolin being a negative regulatory protein of eNOS (14, 27, 33, 34).

Considering the opposite activity of eNOS in lungs and livers from CBDL rats, we hypothesized that eNOS might be differently regulated by caveolin in liver and lungs from CBDL rats. We then determined the binding of caveolin to eNOS in both organs. However, eNOS regulation is much more complex than a simple eNOS-caveolin interaction, and the presence of heat shock protein 90 (HSP90) associated with eNOS would conversely increase eNOS activity. Consequently, we also assessed the binding of HSP90 to eNOS in liver and lungs from CBDL rats. Consequences of the dysregulation of eNOS activity by caveolin and HSP90 were measured in rat perfused liver and lungs by pharmacological tests.

MATERIALS AND METHODS

Induction of biliary cirrhosis. Cirrhosis was induced by a CBDL performed 15 (CBDL-15), 30 (CBDL-30), and 60 (CBDL-60) days before sample collection (Western blotting and immunoprecipitation) or pharmacological tests. After laparotomy under 2–3% isoflurane anesthesia, Sprague-Dawley rats (150–175 g) had a double ligation of the common bile duct with section between the two ligatures. Sham-operated rats had laparotomy and were studied 15 days later. The animal welfare committee of the University of Geneva and the veterinary office approved the protocol, and the study conformed to the veterinary office approved the protocol, and the study conformed to 18 U.S.C. Section 1734 solely to indicate this fact.

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the American Veterinary Medical Association guidelines on humane treatment of laboratory animals.

**Western blotting and immunoprecipitation.** We previously measured eNOS, caveolin, and HSP90 expression in pancreatic tissues by Western blotting and the binding of caveolin and HSP90 to eNOS by immunoprecipitation (11). Pulmonary and hepatic samples of the present study were collected from the same rats. We used the mouse monoclonal anti-eNOS from BD Biosciences-Pharmingen (Basel, Switzerland), the rabbit polyclonal anti-caveolin-1 from BD Biosciences-Pharmingen, and the rat monoclonal anti-HSP90 from Stressgen (Bioggio, Switzerland).

**Immunostaining.** We located eNOS, caveolin, and HSP90 in hepatic and pulmonary cells by immunostaining as previously described in the pancreas (11). Pulmonary and hepatic tissues were collected from the same rats. We used the primary rabbit polyclonal anti-caveolin-1, the monoclonal mouse anti-HSP90, and the rabbit polyclonal anti-eNOS.

**Pharmacological test in rat perfused livers.** We perfused livers from normal (NL, n = 14) and CBDL-30 rats (n = 14) with norepinephrine (NE) and NE + acetylcholine (ACh), as previously described (25), in the presence or the absence of the NO synthase inhibitor Nω-nitro-l-arginine methyl ester (l-NAME). Livers were perfused with a Krebs-Henseleit-bicarbonate (KHB) solution for 20 min, followed by KHB + 10^{-7} \text{ M NE}, KHB + 10^{-6} \text{ M NE}, KHB + 10^{-5} \text{ M NE}, KHB + 10^{-5} \text{ M NE} + 10^{-7} \text{ ACh}, KHB + 10^{-5} \text{ M NE} + 10^{-6} \text{ ACh}, and KHB + 10^{-5} \text{ M NE} + 10^{-5} \text{ ACh} (3 min for each perfusion). Similar experiments were performed in livers perfused with KHB and 1 mM l-NAME.

**Pharmacological tests in rat perfused lungs.** We performed equivalent experiments in perfused rat lungs by using the methodology previously described (7). After a 15-min stabilization period (baseline values), a bolus of angiotensin II (1 μg in 100 μl) was injected and the increase in pulmonary arterial pressure was recorded. Following the spontaneous return of the pressure to baseline values, the thromboxane mimetic 9,11-dideoxy-9\alpha,11\beta-epoxy methanoprostaglandin F_{2α}, (U-46619, 1 μg in 15 μl) was injected. Such bolus was able to produce a long-lasting increase in pulmonary artery pressure. After pressure stabilization, an ACh bolus (36 μg in 100 μl) was injected. The same protocol was applied in NI (n = 6) and CBDL rats (CBDL-30, n = 8) perfused with KHB solution and in NI (n = 6) and CBDL rats (CBDL-30, n = 8) perfused with KHB + 1 mM l-NAME solution. An additional group (n = 8) was perfused with KHB + geldanamycin (GE, 12 μg/ml) solution. GE binds to the ATP site of HSP90, preventing the association of the regulatory protein to eNOS. Lungs perfused with KHB + GE solution were injected with a bolus of angiotensin II (1 μg in 100 μl).

**Statistical methods.** Data are presented in graphs using a median and first and third interquartile ranges (box) and minimum and maximum (whiskers) presentation. Difference between groups was analyzed with Kruskal-Wallis tests followed by Dunn’s multiple-comparison test if the P value was <0.05.

**RESULTS**

**Expression of eNOS in livers and lungs.** eNOS expression was measured in hepatic homogenates isolated from sham-operated and CBDL rats (Fig. 1). eNOS expression did not change with the severity of the disease (P = 0.46). When eNOS protein was immunoprecipitated, similar results were obtained, and eNOS expression in immunoprecipitates was not modified by biliary cirrhosis (data not shown; P = 0.52). Despite a trend, eNOS expression did not increase significantly over time in pulmonary homogenates (Fig. 1; P = 0.20) and immunoprecipitates (data not shown; P = 0.07; n = 5).

**Expression of caveolin in livers and lungs.** In hepatic homogenates, caveolin expression significantly increased with the duration of biliary cirrhosis (Fig. 2; P = 0.01). When hepatic samples were immunoprecipitated with eNOS, the expression of caveolin also significantly increased (P = 0.003) over time. In pulmonary homogenates, caveolin expression did not change significantly (Fig. 2; P = 0.14), whereas the caveolin expression in eNOS immunoprecipitates significantly decreased (P = 0.01).

**Expression of HSP90 in livers and lungs.** In hepatic homogenates, HSP90 expression did not change with the duration of biliary cirrhosis (Fig. 3, P = 0.68). When hepatic tissues were immunoprecipitated with eNOS, the binding of HSP90 to eNOS significantly changed (P = 0.007). However, the increased binding in CBDL-15 livers and the decreased binding in CBDL-30 and CBDL-60 rats did not reach significance compared with sham-operated rats. In pulmonary homogenates, HSP90 expression did not change significantly (P = 0.39), but HSP90 expression in eNOS immunoprecipitates significantly increased with the severity of biliary cirrhosis (P = 0.002).

**Localization of eNOS, caveolin, and HSP90 in hepatic and pulmonary endothelial cells.** eNOS was localized in endothelial cells along large vessels in lungs and livers as well as in the alveolo-capillary membranes and sinusoidal lining cells (Fig. 4). Similarly to eNOS, caveolin was expressed in endothelial cells of large vessels and capillaries in lungs and livers, as well as within hepatocytes (data not shown). HSP90 was localized in alveolo-capillary membranes, hepatic endothelial cells, and pulmonary epithelial cells and cholangiocytes (data not shown). Thus the three proteins were localized in endothelial cells in both organs.
Pharmacological tests in perfused organs. In livers isolated from normal and CBDL-30 rats and perfused with KHB or KHB + L-NAME solutions, we measured the response to NE (Fig. 5A). The hepatic resistance during the perfusion of $10^{-5}$ M NE was similar in the NI-KHB group (median 100%), CBDL-KHB group (median 78%), and CBDL-L-NAME group (median 82%) but was significantly increased in the NI-L-NAME group (median 200%) ($P = 0.03$). As we previously showed (25), the perfusion of KHB + L-NAME solution did not increase the hepatic resistance.

Fig. 2. Caveolin expression in hepatic and pulmonary homogenates (A; $n = 4$ in each group) and in eNOS immunoprecipitates (B; $n = 5$ in each group) collected from sham-operated and CBDL-15, CBDL-30, and CBDL-60 rats. Caveolin expression in CBDL-15, CBDL-30, and CBDL-60 rats is expressed as % changes, taking values measured in sham-operated rats as 100%. To increase clarity, 100% value is shown with a dotted line. *$P < 0.05$ vs. sham-operated group.

Fig. 3. Heat shock protein 90 (HSP90) expression in hepatic and pulmonary homogenates (A; $n = 4$ in each group) and in eNOS immunoprecipitates (B; $n = 6$ in each group) collected from sham-operated and CBDL-15, CBDL-30, and CBDL-60 rats. HSP90 expression in CBDL-15, CBDL-30, and CBDL-60 rats is expressed as % changes, taking values measured in sham-operated rats as 100%. To increase clarity, 100% value is shown with a dotted line. *$P < 0.05$ vs. sham-operated group.
not modify hepatic resistances in the absence of NE (data not shown).

In normal precontracted livers, vasodilatation induced by ACh was small (<10%) and was not modified with increasing doses of ACh (data not shown). At low ACh concentrations, the relaxation did not change in livers from CBDL-30 rats. At $10^{-5}$ M concentration, ACh mainly acted on smooth muscle cells, as demonstrated by a small contraction (+4%).

In lungs (Fig. 5B), because NE is catabolized by endothelial enzymes, we used angiotensin II to increase mean pulmonary pressure. The increase in mean pulmonary arterial pressure during angiotensin II perfusion was similar in the Nl-KHB (median 34%), Nl-l-NAME (median 33%), and CBDL-KHB (median 80%) groups, whereas the mean pulmonary pressure significantly increased in the CBDL-l-NAME group (median 240%; Fig. 5B; $P = 0.006$).

The effect of ACh in perfused lungs was biphasic, an early relaxation being followed by a late contraction. The early relaxation observed in the NI-KHB group was significantly decreased in the CBDL-KHB group (Fig. 6A), and the late contraction, which was minimal in the NI-KHB group, significantly increased in the CBDL-KHB group (Fig. 6B). Addition of l-NAME unmasked a late contraction in the NI-l-NAME group, but the early vasodilatation remained similar in the NI-l-NAME and CBDL-l-NAME groups to the NI-KHB group. Of note, l-NAME had no effect on mean pulmonary arterial pressure in the absence of angiotensin II (data not shown).

Effect of GE in lungs from CBDL rats. In lungs from CBDL rats, the response to angiotensin II was similar in the CBDL-l-NAME and CBDL-GE groups and was significantly different in both groups to the response observed in the Nl-KHB group ($P < 0.003$; Fig. 5B). Although we did not directly measure NO during lung perfusion, we hypothesized that GE, by inhibiting the activity of HSP90, prevented endothelial NO release by shear stress.

DISCUSSION

The experimental model of CBDL is characterized by an increased pulmonary NO production that correlates with pulmonary vasodilatation and abnormalities in gas exchange (9, 19), as well as a decreased hepatic NO production that impairs hepatic vascular relaxation (14, 27, 33, 34). Because caveolin and HSP90 are two major regulatory proteins of eNOS activity that modulate NO release (12, 35), we hypothesized that an opposite regulation of eNOS by caveolin and HSP90 might explain such results. Interestingly, we found an opposite post-
The translational regulation of eNOS by HSP90 and caveolin in liver and lungs of rats with HPS. We also present indirect evidence that high NO release by shear stress in lungs from CBDL rats parallels the high binding of HSP90 and the low binding of caveolin to eNOS, whereas opposite results are observed in livers from CBDL rats. Moreover, GE, which inhibits HSP90 activity, prevents NO release in lungs from CBDL rats, demonstrating the effect of increased HSP90 binding to eNOS on pulmonary endothelial NO production.

eNOS, caveolin, and HSP90 expression in livers. In patients with advanced liver diseases, portal hypertension results from high splanchnic inflow and increased vascular hepatic resistance and is responsible for major complications. Besides remodeling of hepatic architecture, an increase in hepatic vascular tone significantly contributes to the high hepatic resistance. Impaired release of NO from the endothelium partly explains the incapacity of the intrahepatic vasculature to relax. Therefore, upregulating eNOS activity may become a new strategy to correct the increased hepatic vascular tone in patients (44) and rats (1) with cirrhosis, simvastatin enhancing Akt-dependent eNOS phosphorylation with a concomitant increase in NO release and decrease in hepatic resistance. Besides eNOS posttranslational regulation by enzyme phosphorylation, protein-protein interaction might be another important mechanism that modulates NO production.

Expression of hepatic eNOS varies widely during cirrhosis according to the species and the types of experimental models used, eNOS expression being either unchanged, increased, or decreased. eNOS is not modified by prehepatic portal hypertension (37, 38) but decreases (37, 38) or remains unchanged (34) in CCl4-induced cirrhosis in rat. In CBDL rats, we found no modification in eNOS expression with the duration of cirrhosis, in accordance with Wei et al. (40), who found similar eNOS protein expression in normal and CBDL-24 rats. The expression of eNOS was mainly visualized in sinusoidal lining cells as previously described (27, 34, 37). In normal human livers, eNOS is sparse, but the expression increases during cirrhosis (42, 43). Interestingly, the activity of eNOS is decreased in most studies (14, 27, 33, 34). One explanation might be that caveolin binds to eNOS with a concomitant decrease in enzyme activity (33).

Fig. 5. Norepinephrine (NE)-induced increase in hepatic resistances (%; A) and angiotensin II (ATII)-induced pulmonary artery pressures (%; B) over baseline values (0%). Livers were perfused with Krebs-Henseleit-bicarbonate (KHB) solution and 10^{-5} M NE, and lungs were perfused with KHB solution and ATII (1 μg in 100 μl). Normal (NI) organs perfused with KHB solution (NI-KHB), NI organs perfused with KHB + 1 mM N^o-nitro-l-arginine methyl ester solution (NI-L-NAME), organs from CBDL rats perfused with KHB solution (CBDL-KHB), and organs from CBDL rats perfused with KHB + L-NAME solution (CBDL-L-NAME), as well as lungs from CBDL rats perfused with KHB + geldanamycin solutions (CBDL-GE) are shown. CBDL took place 30 days before organ perfusion. *P < 0.05 vs. NI-KHB group, and **P < 0.05 vs. CBDL-KHB group.

Fig. 6. Early relaxation (%; A) and late contraction (%; B) of pulmonary artery after acetylcholine (ACh) injection (36 μg) in lungs perfused with KHB and precontracted with 9,11-dideoxy-9α,11α-epoxy methanoprostaglandin F_{1α} (1 μg). Baseline values are 0%. CBDL was performed 30 days before organ perfusion. *P < 0.05 vs. NI-KHB group.
partly explain the decreased NO release and concomitant increase in intrahepatic resistance (42, 43). Hepatic coimmunoprecipitation of caveolin and eNOS with decreased NO production is also described in experimental remote trauma injury (2), endotox shock (16), and chronic ethanol administration (39). We confirm such results in CBDL livers. Caveolin is detected in endothelial lining cells, as previously published (33, 42, 43), and in hepatocytes (data not shown). In contrast to Ogi et al. (24), we did not find caveolin in proliferating cholangiocytes.

eNOS regulation is much more complex than a simple eNOS-caveolin interaction, and the presence of HSP90 associated with eNOS would conversely increase eNOS activity. Upon stimulation by shear stress, the association of eNOS with HSP90 is increased, with a concomitant increase in NO production (3). In liver homogenates, HSP90 expression is not modified by biliary cirrhosis, in contrast to aorta isolated from CBDL-30 rats (36). HSP90 was ubiquitous because we localized it in endothelial lining cells, cholangiocytes, and hepatocytes.

Protein expression in lungs. Unexpectedly, pulmonary eNOS expression does not increase significantly in CBDL rats, in contrast to previous results (4, 9, 45). In these studies, pulmonary eNOS increases 2 and 5 wk after bile duct ligation and the high expression is associated with an enhanced activity of the enzyme (9, 45). HSP90 and caveolin expression have never been measured in lungs from CBDL rats, whereas caveolin is a marker of vasculogenesis in early lung development in mice (26). Caveolin is expressed in alveolar epithelial type I cells but is absent in alveolar epithelial type II cells (22).

Pharmacological tests. Lungs from CBDL rats have previously been perfused with blood solution (5). In this study, the baseline pulmonary pressure is identical in normal and CBDL rats but pulmonary pressure significantly increases over lung perfusion, with a marked edema at the end of the perfusion (5). Similarly to our results (Fig. 5), the pressive response to angiotensin II is not different between the two experimental groups. The pressive response to increasing concentrations of KCl is also similar in sham-operated and CBDL rats (4). In these studies, the responses to angiotensin or KCl were not measured in the presence of NO inhibition.

We hypothesized that a higher pressive response to angiotensin II in the presence of l-NAME compared with similar perfusion in the absence of l-NAME reflects shear stress-induced NO release by endothelial cells following cell stimulation by the vasoconstrictor. Using this indirect method, we found that NO release was higher in CBDL-30 than in normal rats. Such method has already been studied (41) in lungs perfused at constant flow rate with low-viscosity solutions (such as KHB solution). Although NO inhibition does not modify pulmonary pressure in the absence of vasoconstriction, l-NAME enhances the pulmonary vascular resistance induced by U-46619 (41). In CBDL-30 lungs, the angiotensin II-induced pulmonary artery pressure increase was higher in the presence of l-NAME than in the absence of NO inhibition. Such difference was not found in normal perfused lungs.

In contrast, NO released by ACh is much lower in the CBDL-KHB group than in the NI-KHB group, suggesting that the regulation of eNOS by caveolin and HSP90 acts differently on NO release depending on the signaling pathways that produce NO (drugs vs. shear stress). ACh-induced NO release has not previously been investigated in perfused cirrhotic lungs, but in lobar pulmonary arteries, ACh-induced NO release is similar in normal and CBDL rats (9). Thus the response of the overall pulmonary circulation to ACh in perfused lungs differs from the response of isolated pulmonary arteries.

The late ACh-induced vascular contraction was higher in the CBDL-KHB group than in the NI-KHB group because of the absence of endothelial NO released by ACh. We hypothesize that in the NI-KHB group, contraction to ACh was masked by the high endothelial NO release. In isolated lobar pulmonary arteries, the vasoconstriction to phenylephrine is lower in CBDL than in normal rats, the low response being attributed to an increased NO production (9).

In perfused lungs, the contractile response to NE was higher in the NI-l-NAME group than in the NI-KHB group, suggesting that NO is released by shear stress during the NE perfusion as we previously described (25). In contrast, the pressive response to NE was similar in the CBDL-KHB and CBDL-l-NAME groups. This result is in accordance with previous studies that found a decreased eNOS activity in biliary cirrhosis (14, 27, 33, 34). Moreover, the fact that the response to NE was higher in the NI-l-NAME group than in the CBDL-KHB group may suggest a hyporesponsiveness of smooth muscle cells in CBDL rats or the presence of vasodilators other than NO.

After NE preconstriction, the response to ACh was small as previously described (14). The response was similar while perfusing increasing doses of ACh and was not significantly different in NI-KHB and CBDL-KHB groups. The only difference between groups was a slight vasoconstriction in cirrhotic lungs perfused with l-NAME, suggesting that endothelium alteration permits ACh to act on smooth muscle cells.

In summary, we found an opposite posttranslational regulation of eNOS by HSP90 and caveolin in liver and lungs of rats with HPS. The high NO release by shear stress in lungs from CBDL rats parallels the high binding of HSP90 and the low binding of caveolin to eNOS, whereas opposite results are observed in livers from CBDL rats. Moreover, GE, the HSP90-specific inhibitor, was able to prevent the high NO production in cirrhotic lungs, demonstrating that increased HSP90 binding to eNOS is responsible for the high pulmonary endothelial NO production. In the liver, high caveolin binding and low binding of HSP90 to eNOS explain the decreased shear stress-induced NO release. Thus posttranslational regulation of eNOS by HSP90 and caveolin is an important mechanism that explains why NO production differs in lungs and liver from CBDL rats.

ACKNOWLEDGMENTS

We thank Manuel Jorge-Costa, Sylvie Roulet, and Jean-Pierre Giliberto for excellent technical assistance.

GRANTS

This work was supported by the Fonds National Suisse de la Recherche Scientifique (grant no. 3200B0-109977 to C. M. Pastor).

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