Regulation of hepatic eNOS by caveolin and calmodulin after bile duct ligation in rats

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Received 21 August 2000; accepted in final form 18 December 2000

Shah, Vijay, Sheng Cao, Helen Hendrickson, Janet Yao, and Zvonimir S. Katusic. Regulation of hepatic eNOS by caveolin and calmodulin after bile duct ligation in rats. Am J Physiol Gastrointest Liver Physiol 280: G1209–G1216, 2001.—In carbon tetrachloride-induced liver cirrhosis, diminution of hepatic endothelial nitric oxide synthase (eNOS) activity may contribute to impaired hepatic vasodilation and portal hypertension. The mechanisms responsible for these events remain unknown; however, a role for the NOS-associated proteins caveolin and calmodulin has been postulated. The purpose of this study is to characterize the expression and cellular localization of the NOS inhibitory protein caveolin-1 in normal rat liver and to then examine the role of caveolin in conjunction with calmodulin in regulation of NOS activity in cholestatic portal hypertension. In normal liver, caveolin protein is expressed preferentially in nonparenchymal cells compared with hepatocytes as assessed by Western blot analysis of isolated cell preparations. Additionally, within the nonparenchymal cell populations, caveolin expression is detected within both liver endothelial cells and hepatic stellate cells. Next, studies were performed 4 wk after bile duct ligation (BDL), a model of portal hypertension characterized by prominent cholestasis, as evidenced by a significant increase in serum cholesterol in BDL animals. After BDL, caveolin protein levels from detergent-soluble liver lysates are significantly increased as assessed by Western blot analysis. Immunoperoxidase staining demonstrates that this increase is most prominent within sinusoids and venules. Additionally, caveolin-1 upregulation is associated with a significant reduction in NOS catalytic activity in BDL liver lysates, an event that is corrected with provision of excess calmodulin, a protein that competitively binds eNOS from caveolin. We conclude that, in cholestatic portal hypertension, caveolin may negatively regulate NOS activity in a manner that is reversible by excess calmodulin.

endothelial nitric oxide synthase; portal hypertension; protein interactions; cholesterol

CAVEOLIN-1 IS A 22-kDa integral membrane protein implicated in regional signal transduction pathways (33). For example, caveolin has been demonstrated to bind with endothelial nitric oxide synthase (eNOS) and thereby directly inhibit nitric oxide (NO) production (5). Conversely, the calcium regulatory protein calmodulin competitively dissociates eNOS from caveolin, thereby reversing the inhibitory effects of caveolin on NOS activity (6, 7, 11, 21, 22). In addition to such distinct cell signaling functions, caveolin is also a cholesterol-binding protein and provides a functional scaffold for lipid-rich plasmalemmal vesicles termed caveolae (8, 19, 33). Prominent increases in hepatic caveolin protein levels have been detected in independent experimental models of liver disease, suggesting specific pathophysiological sequela for caveolin upregulation (10, 32). For example, an increase in caveolin expression is detected in a carbon tetrachloride (CCL4)-induced model of experimental liver cirrhosis (32). In this model, the increase in caveolin protein level has been implicated in the development of impaired liver endothelial cell (LEC) NOS activity and ensuing vasconstriction and portal hypertension observed in this model (32).

The cellular distribution of caveolin protein in normal liver and the mechanisms that regulate caveolin protein expression are unclear. Although most studies note that caveolin protein is expressed at only low levels under normal conditions in liver (23, 32), the cellular distribution within normal liver is debated. For example, some studies suggest the presence of caveolin protein in normal LEC (10, 32) while other studies report dissimilar results describing an enrichment in hepatocyte populations (25). Additionally, mechanisms of regulation of caveolin expression and signaling function remain unclear. A recent analysis of the caveolin gene promoter suggests a direct link between cholesterol influx and caveolin gene expression in cultured cells (3). In liver, this concept is supported by the detection of an increase in caveolin protein levels in a murine model of Niemann-Pick disease type C, which is characterized by prominent lipid accumulation in the liver (10). These studies raise the possibility that alterations in lipid metabolism may play a role in regulation of caveolin-1 expression and ensuing impairment of eNOS function in portal hypertensive liver.

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Therefore, the goals of this study were to 1) examine the cellular and subcellular localization of caveolin in normal liver, 2) analyze caveolin expression in an animal model of portal hypertension associated with hyperlipidemia, and 3) determine whether caveolin-mediated inhibition of NOS activity is reversible by calmodulin in portal hypertensive liver.

METHODS

Isolation and culture of liver cells. All tissue culture reagents, including MEM, FBS, penicillin, and streptomycin, were obtained from GIBCO. Rat hepatocytes and nonparenchymal cells (NPC) were isolated from normal Fisher rats as previously described (30, 31). In brief, the liver was digested with a collagenase perfusion. The digest was filtered, and the hepatocytes were separated by centrifugation at 400 rpm. The supernatant was centrifuged at 400 rpm a second time to remove remaining hepatocytes and then centrifuged at 1,600 rpm, resulting in an NPC pellet. In some experiments, LEC and, alternatively, hepatic stellate cells (HSC), were further separated from the NPC fraction using centrifugal elutriation and density gradient centrifugation, respectively (17, 18, 31). For HSC isolation, after centrifugation to obtain the NPC fraction, NPC were resuspended in a Krebs buffer that was layered on an Accudenz gradient consisting of a layer of 15.6% and a layer of 8.2%. Gradients were centrifuged at 20,000 rpm for 25 min at 20°C. The band above the 8.2% Accudenz was retrieved, and cells were resuspended in culture media and plated on plasticware. HSC cell purity was confirmed by 1) immunohistochemical expression of smooth muscle α-actin after several days in culture, 2) lack of expression of eNOS by Western blot analysis of HSC lysates, and 3) characteristic phase-contrast morphology. LEC were purified by centrifugal elutriation as previously described using a Beckman JE-6B elutriator and rotor (31). LEC purity was confirmed by the ability of >95% of cells to take up Di-I-acetylated low-density lipoprotein (31). Additionally, we have previously found that fenestrae and sieve plates are abundantly detected in these cells up to 20 h after culture by transmission electron microscopy (31). Freshly isolated cells were plated on human placental collagen (Sigma, St. Louis, MO)-coated 100-mm dishes. Animals and bile duct ligation. Bile duct ligation (BDL) was performed as previously described (34). In brief, male Fisher rats weighing 225–275 g were anesthetized. Laparotomy was performed under sterile technique, and the common bile duct was isolated and tied in two locations. The duct was ligated between the sutures, and animals were closed. In sham animals, surgery was identical, except no ties were placed and the duct was not ligated. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin from sham and BDL animals were assayed commercially through the Mayo Clinical Laboratory, and isolation from normal rat liver. First, freshly iso-

RESULTS

Caveolin-1 protein is detected in LEC. To examine the relative abundance of caveolin expression in the major liver cell types, we performed cell fractionation and isolation from normal rat liver. First, freshly iso-
lated hepatocyte and NPC fractions were separated, and cell lysates were prepared for Western blot analysis. As depicted in Fig. 1A, in normal liver, caveolin protein is enriched in NPC lysate (lanes 3 and 4 depict 100 μg of protein lysate from two representative NPC lysates) compared with hepatocyte lysate (lanes 1 and 2 depict 100 μg of protein lysate from two representative hepatocyte lysates). To further examine caveolin expression in NPC cell types, we purified and cultured HSC and LEC, which constitute the majority of NPC. As seen in Fig. 1B, caveolin protein is detected in both of these cell populations (lane 1 depicts 50 μg protein from a representative HSC lysate, whereas lanes 2 and 3 depict 50 μg of protein from two representative LEC lysates). To further establish the specificity of caveolin expression in LEC and to determine the subcellular localization of the protein, we performed immunofluorescence microscopy in LEC using a caveolin pAb. As shown in Fig. 2B, the caveolin-1 immunofluorescence signal was detected in a predominantly perinuclear distribution in LEC with additional vesicular staining also observed. Note the lack of caveolin immunofluorescence in the plasma membrane where specialized vesicles termed caveolae reside. When nonimmune serum was substituted for caveolin pAb, no significant immunofluorescence signal was detected (Fig. 2A). These studies indicate that, in liver, caveolin is detected in LEC and is distributed predominantly in a perinuclear pattern.

Increased serum cholesterol after BDL. An increase in caveolin expression in cultured cells has been linked to hyperlipidemia (3, 5, 13). To examine this paradigm in an experimental model of portal hypertension, we performed studies in the BDL rat, a model characterized by prominent cholestasis and liver injury, as evidenced by elevated bilirubin and ALT and AST levels (Table 1). We first examined cholesterol levels from serum samples of sham animals and animals after BDL. As shown in Fig. 3, serum cholesterol levels, as assessed by a spectrophotometric assay, were significantly increased in BDL animals compared with sham animals (P < 0.05, sham vs. BDL; n = 6), suggesting significant intrahepatic cholesterol retention.

Hepatic caveolin-1 protein levels are increased after BDL. To determine whether cholestasis and ensuing hypercholesterolemia are associated with elevated caveolin expression in LEC, we examined hepatic caveolin protein levels from sham and BDL animals. As shown in Fig. 4, caveolin protein levels were significantly increased in BDL animals compared with sham animals (P < 0.05, sham vs. BDL; n = 6), suggesting increased caveolin expression in LEC after BDL.

Fig. 1. Caveolin-1 protein levels are enriched in liver endothelial cells (LEC). A: parenchymal and nonparenchymal fractions of liver cells were isolated. Equal amounts of protein lysates were prepared for gel electrophoresis and Western blot analysis. As seen in the representative blot, caveolin protein levels in two representative hepatocyte lysates (HEP) are low compared with two representative nonparenchymal cell (NPC) lysates (n = 3). B: LEC and hepatic stellate cells (HSC) were purified and cultured from NPC populations as described in METHODS and prepared for gel electrophoresis and Western blot analysis. Caveolin protein is detected in both cell populations (n = 3).

Fig. 2. Subcellular localization of caveolin-1 in LEC. LEC were isolated and cultured on glass coverslips for several hours. Cells were then fixed and prepared for immunofluorescence analysis using a caveolin pAb. A: no immunofluorescence was detected in cells when nonimmune serum was substituted for caveolin pAb. B: caveolin immunofluorescence signal is detected in a predominantly perinuclear distribution in LEC (large arrow) with minimal signal detected in plasma membrane regions of the cell where caveolae reside (small arrow).
caveolin-1 protein levels in the liver, detergent-soluble liver lysates were prepared from sham and BDL animals 4 wk after BDL. As shown in Fig. 4A, top, caveolin protein levels were increased significantly in BDL animals compared with sham animals, while the depicted Ponceau stain of the membrane demonstrated no increase in the levels of several highly expressed liver proteins (Fig. 4A, bottom). Additionally, these findings were paralleled by an increase in caveolin bound to eNOS in BDL liver lysates compared with sham animals (Fig. 4B). In Fig. 4C, a densitometric analysis depicts the significant increase in hepatic caveolin protein levels after BDL (P < 0.05, sham vs. BDL; n = 6). To determine the cellular location of this increase in caveolin expression after BDL, we next performed immunoperoxidase staining using a caveolin pAb. As shown in Fig. 5A, in sham animals only low levels of caveolin are detected in liver, predominantly in a sinusoidal pattern and consistent with the cell fractionation studies depicted in Fig. 1. After BDL, immunoperoxidase staining was more intense and was detected within hepatic vascular endothelium, including the central vein (Fig. 5B) and sinusoids. These studies indicate that caveolin protein levels are increased in hepatic vasculature after BDL, similar to that previously observed in the CCl4-induced model of liver cirrhosis (32).

**Diminished NOS activity in liver after BDL and correction with excess calmodulin.** To determine whether enhanced caveolin expression, which is detected after BDL, is associated with diminution of NOS catalysis, NOS activity assays were performed by measuring the conversion of radiolabeled L-arginine to L-citrulline in detergent-soluble lysates from liver in rats after BDL and in sham rats. As seen in Fig. 6, hepatic NOS activity was significantly lower in BDL animals compared with sham animals at 0.1 μM calmodulin, which is the standard concentration of calmodulin used in this assay (P = 0.05; n = 6; see Ref. 32). Because the eNOS-caveolin interaction is disrupted by excess calmodulin (6, 7, 11, 21, 22), we next sought to determine whether the provision of a 10-fold excess in calmodulin in the NOS assay could reverse the deficiency in NOS activity detected in BDL liver lysates. Although an increase in the concentration of calmodulin from 0.1 to 1.0 μM does not affect NOS activity in sham liver, the excess calmodulin significantly increases NOS activity in BDL lysates, thereby correcting the deficient NOS activity detected in BDL liver (P ≤ 0.05; n = 6). These studies indicate that hepatic eNOS catalytic activity is reduced after BDL and that the deficiency can be corrected by the provision of excess calmodulin in this in vitro assay.

**DISCUSSION**

Caveolin-1 has been implicated as a negative regulator of eNOS activity (4, 21, 22). The findings in this study demonstrate that caveolin expression in liver vasculature is increased after BDL, a model of portal hypertension characterized by prominent cholestasis. Additionally, these studies demonstrate that the deficiency in NOS catalytic activity detected in this model can be corrected by excess calmodulin, a protein that disrupts the eNOS-caveolin protein interaction (6, 7, 11, 21, 22). Furthermore, these studies suggest an association in the liver between these events and hypercholesterolemia.

Caveolin-1 protein levels in normal liver are low, and prior studies examining the cellular localization of caveolin in liver by immunohistochemistry have detected predominantly a low-intensity sinusoidal pattern of staining (10, 25, 32). However, this pattern of expression has led to varied interpretations. While some groups interpret this pattern as indicative of expression within sinusoidal cells (10, 32), others have interpreted this histochemical distribution to indicate that caveolin expression is exclusive to the basolateral hepatocyte membrane domain (25). In support of the latter viewpoint, investigators have isolated luminal endothelial cell plasma membranes (presumably containing the cellular pool of caveolae) from rat liver and have been unable to detect caveolin protein (23). In this study, we address this issue by first separating sinusoidal and parenchymal cell fractions from the liver and directly examining caveolin protein levels in each fraction. In our analysis, caveolin protein levels are clearly in abundance within freshly isolated sinusoidal NPC compared with freshly isolated hepatocytes. Additionally, upon further analysis, caveolin protein is detected within cultured LEC and cultured HSC within the NPC fraction of liver cells. Although these studies may appear to be inconsistent with the afore-

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Table 1. **Biochemical determinations**

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<th>Sham</th>
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<tr>
<td>Total Bilirubin</td>
<td>0.2 ± 0.1</td>
<td>15.1 ± 3.0*</td>
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<tr>
<td>AST</td>
<td>124.2 ± 16.4</td>
<td>474.7 ± 51.0*</td>
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<tr>
<td>ALT</td>
<td>56.0 ± 3.8</td>
<td>204.2 ± 21.0*</td>
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Values are means ± SE; n = 4 animals in each group. BDL, bile duct ligation; AST, aspartate aminotransferase; ALT, alanine aminotransferase. *P < 0.05, sham vs. BDL.
mentioned study demonstrating a lack of caveolin protein within enriched plasma membrane fractions of LEC, the discrepancies in detection of caveolin in LEC may be clarified by our immunofluorescence microscopy analysis which demonstrates that caveolin in LEC is expressed predominantly in a perinuclear distribution rather than in plasmalemmal caveolae, thereby accounting for the lack of detection of caveolin protein within LEC plasma membranes. Enrichment of caveolin within a perinuclear distribution in cells rather than in plasma membrane caveolae is observed in specific cell types under physiological and pathopharmacological conditions (1, 19, 33). For example, in Madin-Darby canine kidney (MDCK) cells, a significant component of caveolin protein is detected within perinuclear Golgi membranes rather than plasmalemmal caveolae (19). Additionally, a recent detailed confocal microscopic analysis of intact cardiac endocardium clearly demonstrates the prominent Golgi localization of caveolin in endothelial cells in vivo (1). Furthermore, pharmacological disruption of the structural integrity of caveolae with cholesterol oxidase also precipitates a recycling of caveolin to Golgi membranes in a perinuclear distribution, thereby supporting the concept that caveolin can reside independently from plasmalemmal caveolae (8, 33). This Golgi membrane pool of caveolin has been postulated to bind and transport cholesterol between the Golgi and the plasma membrane caveolae, within lipid "rafts" or vesicles, although more diverse functions are also likely (8). The physiological basis for caveolin enrichment within Golgi of LEC is unclear, but, interestingly, the caveolin-regulated protein eNOS, although enriched within caveolae within a variety of endothelial cell types (9),

**Fig. 4.** Caveolin-1 protein levels are increased in liver lysates after BDL. Caveolin protein levels and endothelial nitric oxide synthase (eNOS) immunoprecipitates (IP) were examined in detergent-soluble liver lysates 4 wk after BDL and compared with sham animals. **A:** representative Western blot depicting an increase in caveolin protein levels in liver lysates after BDL compared with sham (top) while no increase is detected in the expression of other prominent liver proteins as assessed by Ponceau stain of transferred proteins (bottom). **B:** increase in caveolin protein levels in BDL liver lysates is paralleled by an increase in caveolin bound to eNOS as assessed by coimmunoprecipitation analysis. **C:** densitometric analysis demonstrating a significant increase in hepatic caveolin protein levels in BDL animals compared with sham. *P < 0.05 (n = 6).

**Fig. 5.** Caveolin-1 expression is increased in hepatic vasculature after BDL. Sections of liver tissue were obtained from sham and BDL animals and fixed in formalin for immunohistochemical staining for caveolin. **A:** in sham liver, only low level of immunostaining for caveolin is detected, predominantly within sinusoids. **B:** in BDL animals, immunostaining for caveolin is more prominent and detected in sinusoids and veins. Note the intense staining in a central vein (arrow).
increases in hepatic caveolin expression occur in CCl4-
For example, it has recently been demonstrated that
development of experimental portal hypertension (32).
low, enhanced expression has been implicated in the
caveolin that disrupts the eNOS-caveolin protein inter-
activates other proteins, including the putative eNOS-
activation by 10.220.32.247 on September 8, 2017 http://ajpgi.physiology.org/ Downloaded from
caveolin-1 promoter region essential for the cholester-
also targets predominantly to Golgi membranes in
LEC (31). Thus, despite the dearth of both caveolin and
eNOS within LEC plasma membranes, inhibitory in-
teractions of caveolin with eNOS likely occur within
Golgi membranes and/or Golgi-associated caveolin-
coated vesicles.

Although caveolin protein levels in normal liver are
low, enhanced expression has been implicated in the
development of experimental portal hypertension (32). For
example, it has recently been demonstrated that
increases in hepatic caveolin expression occur in CCl4-
induced cirrhosis (32). Increases in caveolin expression
were associated with enhanced caveolin binding with
eNOS, diminished NO production, and impaired he-
aptic vasodilatory responses in this model (32). Addition-
ally, preliminary studies suggest a similar para-
digm in human cirrhosis (4). In the present study,
hepatic NOS activity is also diminished in the BDL
model of portal hypertension in conjunction with
enhanced caveolin expression. A causative role for caveo-
lin in the process of NOS inhibition is supported by the
demonstration that addition of excess calmodulin, a
protein that disrupts the eNOS-caveolin protein inter-
action, corrects the deficient NOS activity in BDL liver
lysates detected in the cell-free NOS assay system.
However, excess calmodulin may also potentiate de-
ficient NOS activity in BDL liver lysates through alter-
native mechanisms, as calmodulin also binds and
activates other proteins, including the putative eNOS-
activating protein, Hsp 90 (11).

The factors within the liver that upregulate caveolin
protein are unclear; however, studies in cultured cells
suggest a direct link between cellular cholesterol levels
and enhanced caveolin expression and signaling. For
example, Feron et al. (5) detected an increase in caveo-
lin protein levels in bovine aortic endothelial cells
incubated in cholesterol-enriched serum with an asso-
ciated enhancement of binding of caveolin with eNOS
and diminished NO production. Similar findings dem-
onstrating cholesterol-dependent regulation of caveo-
lin have also been observed in MDCK cells (13). In
further support of this concept, Bist et al. (3) have
recently identified a response element within the
caveolin-1 promoter region essential for the cholester-
ol-dependent regulation of the caveolin-1 gene. In the
present studies, the increase in caveolin protein levels
detected in the BDL liver also occurs in the context of
marked increases in serum cholesterol, demonstrating
an association between hypercholesterolemia and in-
creased caveolin expression and signaling in hepatic
vasculature. We speculate that serum cholesterol may
be a causative factor for caveolin expression and sig-
signaling in our studies based on in vitro cellular and
molecular studies by others (3, 5, 13); however, serum
cholesterol levels are not prominently increased in all
models of liver cirrhosis and portal hypertension, and
thus factors other than cholesterol are also likely to
contribute to regulation of caveolin and eNOS. For
example, there are other factors coexisting between the
BDL and CCL4 models of liver injury, including alter-
ations in cytokine expression and shear stress pat-
terns, both of which may alter caveolin and NO ex-
pression and function, resulting in similar hemodynamic
disturbances in both models (15, 24, 33).

The role of a diminution of NOS activity in the
pathogenesis of intrahepatic vasoconstriction and por-
tal hypertension remains unsettled. While some inves-
tigators detect impaired hepatic eNOS activity and
NO-dependent responses in CCL4-induced cirrhotic
rats (12, 28, 32), this observation has not been univer-
sal throughout other models of intrahepatic vasocon-
striction and portal hypertension (20). In the present
study, we do detect a diminution of NOS activity in
liver lysates in the BDL rat, consistent with prior
studies in the CCL4-treated rat model of portal hyper-
tension (32). Interestingly, despite the prominent in-
trahepatic vasoconstriction observed in this model, an
increase in hepatic NOS activity has also been ob-
erved after BDL (20). These temporal variations in
NOS activity are likely related to induction of inducible
NOS (iNOS). For example, iNOS mRNA levels have
been detected in nonparenchymal liver cell types dur-
ing the first 8 days after BDL surgery (27); however, in
our study, we did not detect iNOS protein levels from
whole liver homogenates 4 wk after BDL, as assessed
by Western blot analysis. Although this does not ex-
clude the presence of untranscribed iNOS mRNA tran-
scripts, the prominent decrease in NOS activity in this
study suggests that iNOS induction, in contradistinc-
tion to earlier time points, is not prominent in our
animals 4 wk after BDL. The BDL rat has also been
used to delineate the vascular component of portal hypertension as these animals develop increased intrahepatic resistance and increased portal pressure in the absence of fully developed cirrhosis at 4 wk (Fig. 5; see Ref. 14). The detection of reversibly diminished NOS activity preceding the onset of overt cirrhosis suggests that these regulatory events may contribute to the development of the vascular component of portal hypertension.

Recent experimental evidence supports the concept that the vascular component of portal hypertension may occur through flow and pressure regulation within the sinusoids, with LEC-derived NO acting abnormally on activated, contractile stellate cells (26). On the basis of this concept, reduced NO production from LEC may result in intrasinusoidal and intrahepatic vasoconstriction, perhaps through diminished NO-dependent stellate cell relaxation (12, 28, 32). However, important hepatic resistance changes also occur in extrahepatic sites (2, 16). Thus delineation of the relative roles of these locales in flow regulation and further elucidation of the cellular mechanisms of NOS regulation in liver are likely to advance the understanding of portal hypertension.

This work was supported by National Institutes of Health Grants DK-02529 (V. Shah) and HL-53524 (Z. S. Katsis), the Northern Heartland Affiliate of the American Heart Association (V. Shah), and the Mayo Clinic Foundation.

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