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# Elevated expression of caveolin-1 at protein and mRNA level in human cirrhotic liver: relation with nitric oxide

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***Background.*** Caveolin, the principal structural protein of caveolae, binds with endothelial nitric oxide synthase (eNOS) leading to enzyme inhibition. This study examined the expression of caveolin and eNOS at the protein and mRNA levels in patients with hepatocellular carcinoma and hepatitis C-related cirrhosis, and in control noncirrhotic liver specimens obtained from patients with metastatic liver carcinoma. ***Methods.*** Anti-eNOS, anti-caveoin-1, and anti-calmodulin antibodies were used for Western blotting. For in situ hybridization (ISH), human eNOS and caveolin-1 peptide nucleic acid probes were used with a catalyzed signal amplification system. ***Results.*** Western blotting showed marked overexpression of caveolin-1 protein in cirrhotic liver, while caveolin-1 was almost undetectable in control liver tissue. Endothelial NOS was expressed at a slightly higher level in cirrhotic liver than in control liver tissue. Calmodulin was expressed abundantly in control liver tissue and at a low level in cirrhotic liver tissue. By ISH, eNOS mRNA was localized on portal vein and hepatic lining cells, and caveolin-1 mRNA was almost undetectable in normal liver tissue. In cirrhotic liver tissue, caveolin-1 mRNA was overexpressed on hepatic sinusoidal lining cells, while eNOS mRNA expression was similar to that in normal liver. ***Conclusions.*** Enhanced caveolin-1 expression may be associated with a significant reduction in NO catalytic activity in cirrhosis.

**Key words:** caveolin-1, endothelial nitric oxide synthase, in situ hybridization, sinusoidal endothelial cell

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## Introduction

Caveolae are small invaginations of the cell surface and are thought to play a role in important physiological functions, such as cell surface signaling, endocytosis, and intracellular cholesterol transport.[1] Unique membrane proteins, designated caveolin-1, -2, and -3, have been shown to be the major constituents of caveolae.[2–4] Animal studies have revealed that caveolin-1 and -2 are present abundantly in vascular endothelial cells, adipocytes, smooth muscle cells, and fibroblasts.[5] Caveolin-1, the first discovered marker protein for caveolae and the most extensively characterized, is implicated in regional signal transduction pathways.[1] Caveolin has been demonstrated to bind with endothelial nitric oxide synthase (eNOS) and thereby directly inhibit nitric oxide (NO) production.[6,7] The inhibitory effect of caveolin on eNOS activity is completely reversed by $Ca^{2+}$-calmodulin.[7] In experimental animals, sinusoidal endothelial cells (SEC) isolated from livers of rats subjected to carbon tetrachloride treatment or bile duct ligation exhibited decreased eNOS-specific activity, whereas eNOS protein and mRNA expression was unchanged.[8] Moreover, impaired eNOS activity associated with increased caveolin-1 protein caused vasoconstriction and portal hypertension observed in a carbon tetrachloride-induced model of experimental cirrhosis.[9]

While most of the data on eNOS and caveolin-1 have been obtained using rat liver,[9,10] there is relatively little information on the situation in intact human liver. We previously studied the immunohistochemical and immunoelectron microscopic localization of eNOS and caveolin-1 in normal human and cirrhotic liver tissues, and found increased immunoreactivity of caveolin-1 on SEC in cirrhotic liver.[11] In the present study, we examined the expression of eNOS, caveolin-1, and calmodulin proteins by Western blotting, and we investigated the mRNA expression of eNOS and caveolin-1 by in

situ hybridization (ISH) in cirrhotic and noncirrhotic human liver tissues.

## Patients, materials, and methods

### *Patients and materials*

As controls, wedge biopsy specimens from noncirrhotic portions of the liver were obtained from five patients (four men and 1 woman; aged from 52 to 68 years, with a mean age of 63.3 years) who underwent hepatectomy for metastatic liver carcinoma (four with colonic carcinoma and one with gastric carcinoma). Cirrhotic liver specimens were obtained from macroscopically cirrhotic portions surgically resected from five patients (all men; aged from 58 to 67 years, with a mean age of 62.8 years) who had hepatocellular carcinoma combined with hepatitis C-related cirrhosis. The study was approved by the local ethics committee.

### *Western blotting*

Western blotting was conducted using fresh control and cirrhotic liver tissues. Briefly, liver tissues were homogenized in 10 volumes of homogenization buffer (20 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 0.1 mM phenyl methane sulfonyl fluoride [PMSF], 20 mM pepstatin A, and 20 mM leupeptin), using a polytron homogenizer at setting 7 for 90 s. The homogenates were centrifuged at 100 000 *g* for 45 min. The membranes were washed three times, resuspended in 10 volumes of homogenization buffer, homogenized using a Teflon/glass homogenizer, and centrifuged. The membrane proteins thus obtained were used for immunoblotting. Proteins (20 mg/ml) were separated on sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS/PAGE; 7.5% gel for eNOS, 12.5% gel for calmodulin, and 15% for caveolin-1) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The blots were blocked with 5% (w/v) dried milk in phosphate-buffered saline (PBS) for 30 min, incubated with anti-eNOS (×500), anti-caveolin-1 (×1000), or anti-calmodulin (×500) antibodies in 0.1% Tween 29 in PBS, and then processed by the Vectastain ABC system (Vector Laboratories, Burlingame, CA, USA). The immunoreactive bands were developed with diaminobenzidine solution containing 0.01% $H_2O_2$.

### *In situ hybridization technique*

Messenger RNA of eNOS and caveolin-1 was detected in formalin-fixed, paraffin-embedded sections by ISH, using peptide nucleic acid (PNA) probes and the catalyzed signal amplification (CSA) technique.[12] Four-μm-thick liver sections were adhered to silanated, RNAse-free glass slides (prepared by heating in an oven at 60°C for 30 min). The sections were dewaxed in xylene (twice for 15 min each), followed by a graded ethanol series, rehydrated in RNAse-free distilled water, and incubated for 30 min in Target Retrieval Buffer (Dako, Glostrup, Denmark) preheated and maintained at 95°C. The slides were cooled at room temperature for 20 min and then digested with 20 μg/ml proteinase K (Dako) at room temperature for 30 min. The slides were rinsed in distilled water and rapidly air-dried. The air-dried sections were covered with approximately 15 ml of hybridization solution containing 10% (w/v) dextran sulfate, 10 mM NaCl, 30% (v/v) formamide, 0.1% (w/v) sodium pyrophosphate, 0.2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) Ficoll, 5 mM $Na_2$ ethylene diamine tetraacetic acid (EDTA), 50 mM Tris-HCl, pH 7.5, and 10 μg/ml PNA probe. ENOS antisense (fluorescein isothiocyanate [FITC]-GCTTCTCGTAGGC CTTCA), eNOS sense (FITC-CGAAGAGCACCCG GAAGT),[13] caveolin-1 antisense (FITC-GGCTGATG CACTGAATCT), and caveolin-1 sense (FITC-CCGA CTACGTACTTAGA)[14] probes were used. The slides were evenly covered with the hybridization solution and incubated in a moist chamber at 43°C for 90 min. Following hybridization, the coverslips were removed, and the slides were transferred to prewarmed Tris-buffered saline (TBS) (150 mM NaCl, 10 mM Tris; pH 7.5, 1.0%) in a water bath at 49°C and washed for 30 min with gentle shaking (PNA Hybridization Kit; Dako, Tokyo, Japan). A nonisotopic, colorimetric signal amplification system (GenPoint kit, Dako) was used to visualize specific hybridization signals. Sections were incubated with an FITC-horseradish peroxidase reagent for 15 min, washed three times with Tris-buffered saline with Tween (TBST) (150 mM NaCl, 10 mM Tris; pH 7.5, 1.1% v/v Tween 20), and then immersed in a solution containing $H_2O_2$ and biotinyl tyramide for 15 min, followed by three washes with TBST. This catalyzed signal amplification step enhanced the deposition of biotin at the site of probe hybridization. The sections were then incubated in streptavidin-horseradish peroxidase for 15 min and washed three times in TBST. Colorimetric signals were localized after incubation in diaminobenzidine solution containing 0.01% $H_2O_2$, and counterstaining with hematoxylin was carried out for light microscopic examination.

## Results

We investigated the protein expression of eNOS, calmodulin, and caveolin-1 by Western blotting in normal and cirrhotic liver tissues. Samples containing 20 mg of membrane protein were subjected to electrophoresis on SDS/PAGE gel (eNOS, 7.5%; calmodulin, 12.5%; caveolin-1, 15%) and analyzed by blotting. Caveolin-1

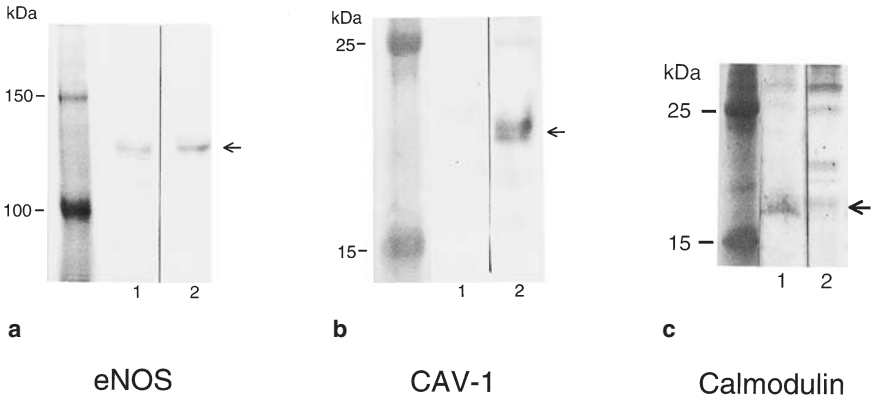


**Fig. 1a–c.** Western blot analysis of expression of **a** endothelial nitric oxide synthase (*eNOS*), **b** caveolin (*CAV*)-1, and **c** calmodulin proteins in human control and cirrhotic liver tissues. Samples containing 20 mg protein were subjected to sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS/PAGE; 7.5% gel for eNOS, 12.5% for calmodulin, 15% for CAV-1) and analyzed by blotting. *Lane 1* denotes control liver. *Lane 2* denotes cirrhotic liver. **a** eNOS immunoblots; **b** CAV-1 immunoblots; **c** calmodulin immunoblots. CAV-1 is found in abundance in cirrhotic liver, but calmodulin is abundant in control. Endothelial NOS protein level in cirrhotic liver remains almost the same as in normal liver. Positions of molecular mass markers are shown (*kDa*)

was found in abundance in cirrhotic liver and was almost undetectable in control liver tissue. Endothelial NOS expression was slightly higher in cirrhotic liver than in control liver tissue. Calmodulin was found in abundance in control liver tissue and at a low level in cirrhotic liver tissue (Fig. 1). All five cases were tested independently because fresh tissue was used in the blot. Although only one case compared with one control is presented in Fig. 1, the same tendency was observed in the other four pairs of cirrhotic and control specimens. The results were based on visual comparison and were not confirmed quantitatively.

Next, we investigated the expression of eNOS and caveolin-1 at the mRNA level by ISH, using the peptide nucleic acid probe. In control liver tissue, eNOS mRNA was localized on the portal vein and hepatic lining cells, but caveolin-1 mRNA was expressed at a low level on hepatic sinusoidal cells (Fig. 2). In cirrhotic liver tissue, caveolin-1 mRNA expression was enhanced on hepatic sinusoidal lining cells compared with control liver tissue, while eNOS mRNA remained almost the same as in control liver (Fig. 3).

Similar results were obtained in all the control noncirrhotic ($n = 5$) and cirrhotic ($n = 5$) liver tissues.

## Discussion

We previously studied the immunohistochemical and immunoelectron microscopic localization of eNOS and caveolin-1 in normal and cirrhotic human liver tissues.[11] In non-cirrhotic liver tissue, immunohistochemical and immuoelectron microscopy localized caveolin-1 at a low level on hepatic sinusoidal lining cells, and eNOS was scanty on hepatic lining cells. In cirrhotic liver tissue, however, caveolin-1 was significantly increased on SEC, and eNOS was slightly increased. In the present study, we sought to further investigate the relationship between caveolin and NO by examining the protein expression of caveolin-1, eNOS, and calmodulin by Western blotting and the mRNA expression of caveolin-1 and eNOS mRNA by ISH. Our results provide unequivocal evidence that, in cirrhotic liver, caveolin is increased, while eNOS remains more or less unchanged.

In a preliminary experiment to establish the conditions for detecting eNOS and caveolin-1 mRNA in liver tissue, we failed to obtain any signal, using RNA and DNA probes to detect mRNA both by Northern blotting and ISH. We therefore tried using peptide nucleic acid (PNA) probes for ISH. PNAs are pseudopeptides with DNA-binding capability. They are nucleotide analogs capable of binding, in a sequence-specific fashion, to DNA and RNA.[15] In PNA, the sugar phosphate backbone found in DNA/RNA is replaced by a polyamide backbone, keeping the distance between the nucleotide bases identical to that in DNA/RNA.[15] The advantage of PNA over DNA/RNA in hybridization is probably due to the uncharged RNA backbone as opposed to the charged DNA/RNA backbone, and the high conformational flexibility of the PNA molecules.[15] Although PNAs have many important potential applications in the diagnostic and therapeutic fields, initially we did not obtain satisfactory results using PNA probes and the standard ISH detection method. We next employed the CSA technique, which is based on the

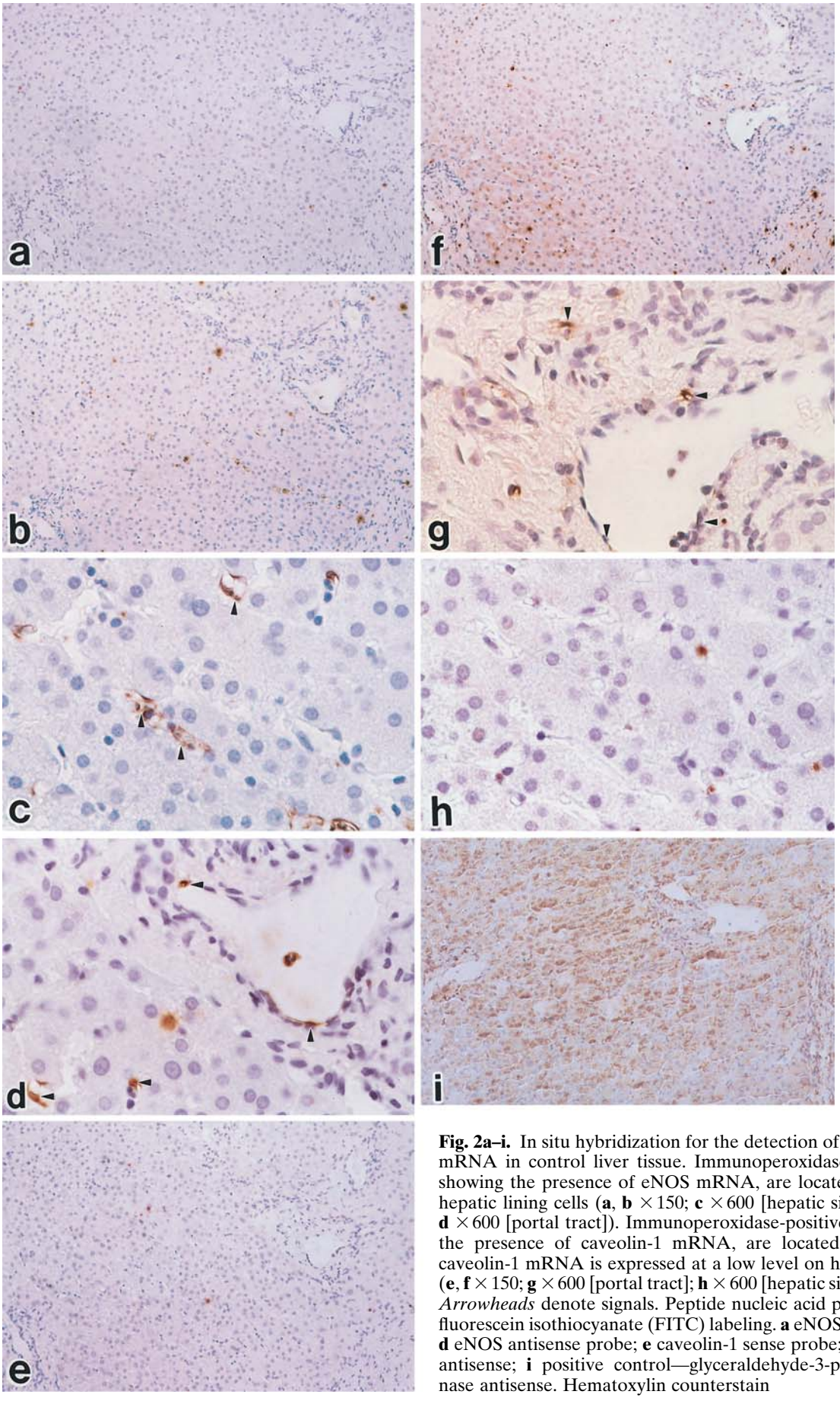


**Fig. 2a–i.** In situ hybridization for the detection of eNOS and caveolin-1 mRNA in control liver tissue. Immunoperoxidase-positive substances, showing the presence of eNOS mRNA, are located on portal vein and hepatic lining cells (**a**, **b** ×150; **c** ×600 [hepatic sinusoidal lining cells], **d** ×600 [portal tract]). Immunoperoxidase-positive substances, showing the presence of caveolin-1 mRNA, are located on portal vein, but caveolin-1 mRNA is expressed at a low level on hepatic sinusoidal cells (**e**, **f** ×150; **g** ×600 [portal tract]; **h** ×600 [hepatic sinusoidal lining cells]). *Arrowheads* denote signals. Peptide nucleic acid probes were used with fluorescein isothiocyanate (FITC) labeling. **a** eNOS sense probe; **b**, **c**, and **d** eNOS antisense probe; **e** caveolin-1 sense probe; **f**, **g**, and **h** caveolin-1 antisense; **i** positive control—glyceraldehyde-3-phosphate dehydrogenase antisense. Hematoxylin counterstain

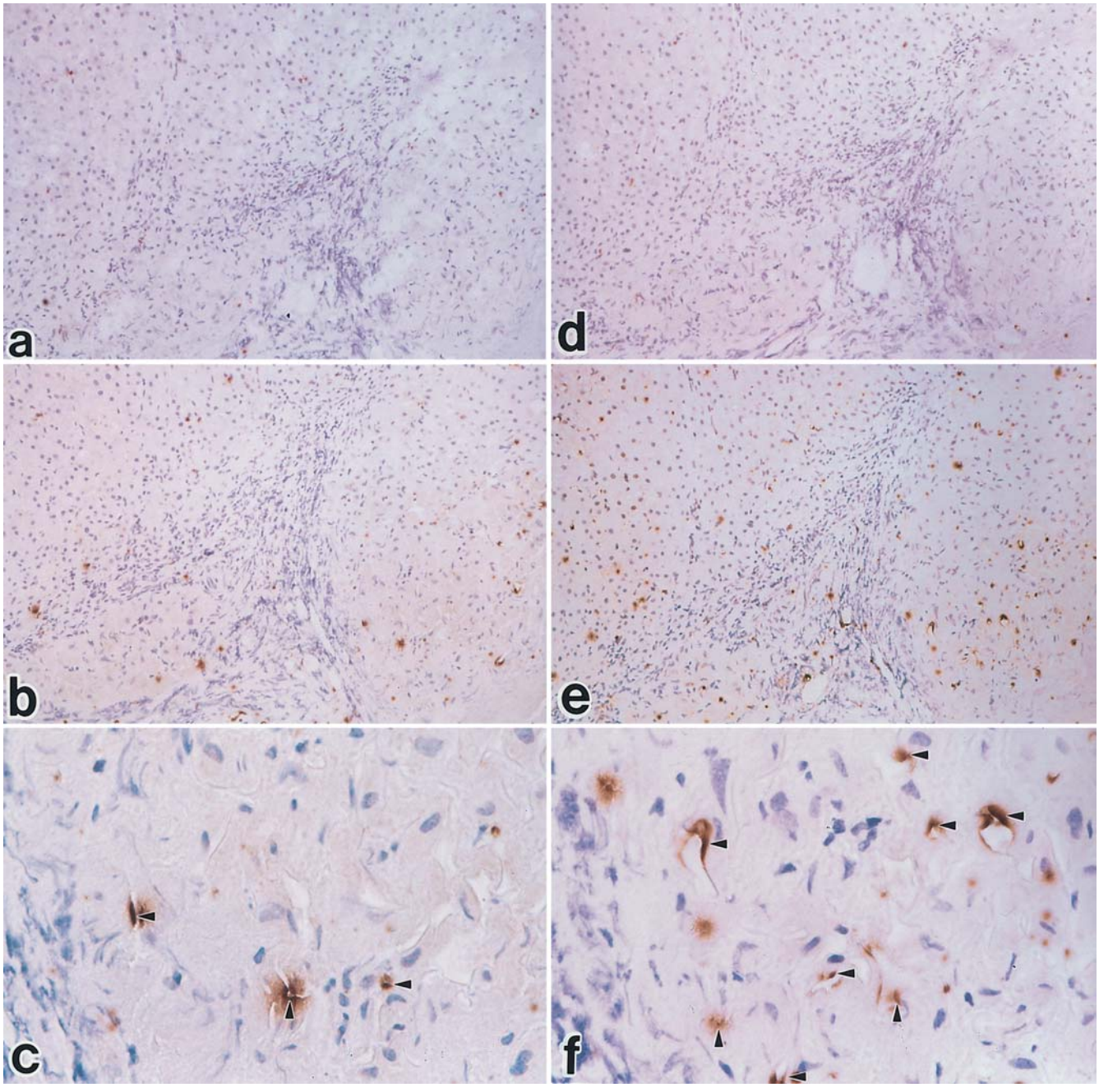


**Fig. 3a–f.** In situ hybridization for the detection of eNOS and caveolin-1 mRNA in cirrhotic liver tissue. Immunoperoxidase-positive substances, showing the presence of eNOS mRNA, are located on portal vein and hepatic lining cells (**b** ×150; **c** ×600). *Arrowheads* denote signals. A large number of sinusoidal mesenchymal cells show signals with the caveolin-1 antisense probe (**e** ×150; **f** ×600). Peptide nucleic acid probes were used with FITC labeling. **a** eNOS sense probe; **b** and **c** eNOS antisense probe; **d** caveolin-1 sense probe; **e** and **f** caveolin-1 antisense

peroxidase-catalyzed deposition of biotinyl tyramide. When oxidized by horseradish peroxidase (HRP), tyramide is converted to an intermediate that is highly reactive with electron-rich aromatic amino acids in proteinaceous specimens. The deposition of biotinyl tyramide, followed by binding with HRP-conjugated streptavidin, results in the catalyzed amplification of signals at the hybridization site of the biotinylated probes.[16,17] Using the PNA probes and the CSA technique, we succeeded in demonstrating the mRNA of eNOS and caveolin-1.

The allosteric interactions of eNOS with its regulatory proteins in the cirrhotic liver may play an important role in regulating vascular resistance.[9] It is well

established that the binding of eNOS with the ubiquitous calcium-regulatory protein calmodulin promotes NO production.[18] Moreover, caveolin-1, a putative signaling molecule, interacts with eNOS and decreases the catalytic activity of the enzyme.[7,19,20] Based on these studies, a molecular paradigm has been proposed whereby NO production is regulated through reciprocal and competitive interactions of calmodulin and caveolin with eNOS; in other words, eNOS is activated through binding with calmodulin and is inhibited through binding with caveolin.[20–22] In an experimental cirrhotic rat model, NOS activity was significantly reduced in cirrhotic rat liver, although the eNOS protein level was unaltered.[9] The protein level of caveolin-1 was markedly increased in the cirrhotic liver.[9] In the present study, we found markedly increased caveolin-1 protein and mRNA expression in cirrhotic liver specimens, as in the rat study. However, contrary to the rat study, eNOS expression appeared to be unchanged or even slightly increased in human cirrhosis. In addition, we found a decrease in calmodulin in cirrhosis. The results in this study present a general picture of reduced NO enzyme activity in cirrhosis, which may be associated with the enhanced caveolin expression.

Although eNOS has been described as constitutive, its expression may be regulated by mechanical, biological, and pharmacological factors. The factors positively influencing eNOS expression include shear stress,[21] estrogen,[22] and transforming growth factor β (TGF-β),[23] whereas the negative factors include tumor necrosis factor (TNF-α)[24] and lipopolysaccharide.[25] In cirrhosis, these factors have been reported to influence eNOS expression.[26]

The upregulated caveolin-1 in sinusoidal cells in cirrhotic liver is likely to bind with the colocalized eNOS, resulting in the inactivation of eNOS. The factors within the liver that upregulate caveolin protein are unclear. However, a response element within the caveolin-1 promoter region has been identified, which is essential for the cholesterol-dependent regulation of the caveolin-1 gene.[27] As further support of this concept, an increase in caveolin protein levels was detected in bile-duct ligated rats.[10] Because the rats also showed a marked increase in serum cholesterol, the results demonstrated an association between hypercholesterolemia and increased caveolin expression and signaling in the hepatic vasculature.[10] Our results suggested that caveolin overexpression may also be related to some pathological states in cirrhosis.

As a result of cirrhosis, SEC lose the fenestral structure and start to form a basal lamina, transforming into continuous endothelial cells, a process called capillarization.[28] The increase in caveolin-1 expression in cirrhosis may be due to morphological changes of the sinusoidal endothelial fenestrae. In another study, caveolin-1 protein expression increased in liver tissue exposed to bacterial endotoxin, and was localized primarily to vascular tissues in both the pericentral and periportal areas of the liver.[29]

Recently, an eNOS-interacting protein that binds to the carboxyl-terminal region of the eNOS oxygenase domain has been reported. This protein modulates the activity of eNOS by uncoupling it from plasma membrane caveolae and by assuring the translocation of the enzyme from caveolae to intercellular compartments.[30] Further elucidation of the cellular mechanisms of NOS regulation in liver may advance the understanding of portal hypertension.

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