Evidence for an Endothelium-Derived Hyperpolarizing Factor in the Superior Mesenteric Artery From Rats With Cirrhosis

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In cirrhosis, in splanchnic arteries, endothelium-dependent relaxation may persist even if overactive nitric oxide synthase (NOS) and cyclooxygenase (COX) are inhibited. In normal arteries, a significant endothelium-dependent relaxation to acetylcholine persists after NOS/COX inhibition. This relaxation is caused by smooth muscle cell (SMC) membrane hyperpolarization, which is sensitive to a combination of the potassium channel blockers apamin and charybdotoxin, and is mediated by an endothelium-derived hyperpolarizing factor (EDHF). The aim of this study was to detect EDHF and evaluate its pathophysiologic role in isolated superior mesenteric arteries from cirrhotic rats. Arterial rings were obtained and exposed to N^w-nitro-L-arginine (L-NNA, a NOS inhibitor) and indomethacin (a COX inhibitor). Acetylcholine-induced membrane potential responses and concentration-response curves to the relaxant of acetylcholine were obtained with and without apamin plus charybdotoxin. Acetylcholine-induced responses were measured in certain rings from endothelium-denuded arteries. Contractions caused by the α_1-adrenoceptor agonist phenylephrine were obtained in cirrhotic and normal rings with and without apamin and charybdotoxin. Significant acetylcholine-induced, endothelium-dependent, apamin- and charybdotoxin-sensitive, SMC membrane hyperpolarization and relaxation were found. An apamin- and charybdotoxin-sensitive hyporesponsiveness to the contractile action of phenylephrine was found in cirrhotic rings. In conclusion, in cirrhotic rats, in the superior mesenteric artery exposed to NOS/COX-inhibitors, an EDHF exists that may replace NOS/COX products to induce endothelium-dependent arterial relaxation. (HEPATOLOGY 2000;32:935-941.)

In portal hypertension, in splanchnic and systemic arteries, certain mechanisms induce endothelium-dependent smooth muscle cell (SMC) relaxation and hyporeactivity to vasoconstrictors by activating endothelial nitric oxide synthase (NOS) and cyclooxygenase (COX) to produce nitric oxide (NO) and prostacyclin (PGI_2).[1-12] However, it has been shown that, in portal hypertensive rats, NOS inhibition, alone or combined with COX inhibition, did not suppress arterial SMC relaxation and hyporeactivity to vasoconstrictors.[3,4,6,8] These findings show that a NOS/COX-inhibitor–insensitive SMC relaxation may be involved in arterial alterations associated with portal hypertension. It should be kept in mind that, in normal arteries, a significant endothelium-dependent arterial SMC relaxation (caused by shear stress or acetylcholine) persists even after NOS/COX inhibition.[13-17] This NOS/COX-inhibitor–insensitive relaxation is caused by SMC membrane hyperpolarization caused by endothelium-derived hyperpolarizing factors (EDHFs).[15,17-20] In normal splanchnic arteries, a combination of the K^+ channel blockers, apamin and charybdotoxin,[21] is known to inhibit EDHF-induced SMC hyperpolarization and relaxation.[22-26] In addition, a combination of barium (a K^+ channel blocker)[27] and ouabain (an inhibitor of the sodium-potassium-adenosine triphosphatase (Na^+/K^+ ATPase)[28] is also known to inhibit EDHF-induced responses in normal mesenteric arteries.[24] In fact, it has been shown in normal small splanchnic arteries, EDHF is K^+ that effluxes through apamin- and charybdotoxin-K^+ channels located in endothelial cells.[24] The resulting increase in myoendothelial K^+ concentrations hyperpolarizes and relaxes underlying SMCs by activating barium-sensitive K^+ channels and the Na^+/K^+ ATPase (Fig. 1).[24] Taken together, the findings obtained in normal arteries suggest that EDHF may explain the earlier-mentioned NOS/COX-inhibitor–insensitive SMC relaxation in arteries from portal hypertensive animals. Thus, the first aim of the present study was to detect EDHF in splanchnic and systemic arteries from cirrhotic rats by investigating acetylcholine-induced relaxation in isolated arteries exposed to NOS/COX inhibitors. The second aim was to determine the effects of a combination of apamin and charybdotoxin, on one hand, and barium plus ouabain, on the other, on EDHF-induced relaxation in cirrhotic arteries. In addition, the effects of these different pharmacologic combinations on EDHF-mediated relaxation were compared between cirrhotic and normal arteries. Finally, the role of EDHF in cirrhosis-induced arterial hyporeactivity to vasoconstrictors was examined.

MATERIALS AND METHODS

Animals. Male Sprague Dawley rats (Charles River Laboratoires, Saint-Aubin-Lès-Elbeuf, France) were divided into 2 groups. One group included normal rats (n = 54). A second group (n = 56) had secondary biliary cirrhosis with portal hypertension as a result of bile duct ligation as previously described.[29] Under pentobarbital anesthesia, the common bile duct was exposed by median laparotomy and

Abbreviations: SMC, smooth muscle cell; NOS, nitric oxide synthase; COX, cyclooxygenase; NO, nitric oxide; PGI_2, prostacyclin; EDHF, endothelium-derived hyperpolarizing factor; Na^+/K^+ ATPase, sodium-potassium-adenosine triphosphatase; L-NNA, N^w-nitro-L-arginine.

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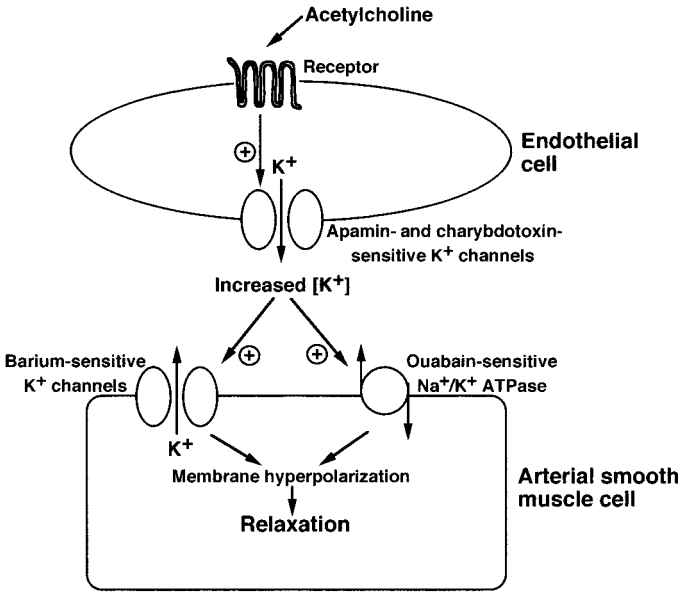

FIG. 1. K^+ is an acetylcholine-induced endothelium-derived hyperpolarizing factor in the rat small superior mesenteric artery exposed to inhibitors of NOS and COX. In the endothelial cell, binding of acetylcholine to its surface receptor leads to activation of apamin- and charybdotoxin-sensitive K^+ channels located in the plasma membrane. The resulting efflux of K^+ outside the cell increases myoendothelial K^+ concentration, which activates barium-sensitive K^+ channels and the ouabain-sensitive sodium-potassium-adenosine triphosphatase (Na^+/K^+ ATPase) located in the plasma membrane of underlying SMCs. Activation of barium-sensitive K^+ channels and Na^+/K^+ ATPase leads to plasma membrane hyperpolarization and SMC relaxation.

occluded by double ligature with a nonresorbable suture (7-0 silk). The first tie was made below the junction of the hepatic ducts, and the second was made above the entrance to the pancreatic ducts. The common bile duct was then resected between the 2 ligatures and the abdominal incision was closed. Studies were performed 4 to 5 weeks after bile duct ligation in rats weighing from 210 to 340 g. This delay is necessary for the development of secondary biliary cirrhosis.[30] Studies were performed in normal rats weighing from 300 to 380 g. All rats were allowed free access to food and water for up to 14 to 16 hours before the study, when food was withdrawn. Protocols performed in this laboratory were approved by the French Agriculture Office in accordance with European legislation for research involving animals.

Tension Measurements. Cirrhotic and normal rats were killed with an overdose of pentobarbital. The thoracic aorta and the main branch of the superior mesenteric artery were removed and placed in a petri dish containing refrigerated (4°C) modified Krebs-Henseleit salt solution containing: 118.3 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 1.17 mmol/L $MgSO_4$, 1.18 mmol/L KH_2PO_2, 25 mmol/L $NaHCO_3$, 0.026 mmol/L ethylenediaminetetraacetic acid, and 11.1 mmol/L glucose.[8,11,31] Arteries were cleaned of loose connective tissue. In most experiments, great care was taken not to injure the endothelium; in some experiments, the endothelium was removed mechanically by inserting the tip of a pair of small forceps into the lumen and rolling the tissue back and for several times on a paper towel wet with modified Krebs-Henseleit salt solution. Studies were performed in freshly isolated arteries. Arteries were cut into 3-mm rings that were then suspended horizontally between 2 stainless steel stirrups in individual organ chambers filled with 10 mL of modified Krebs-Henseleit solution. The solution was continuously bubbled with a mixture of 95% O_2/5% CO_2 and maintained at 37°C with an outer water jacket and a circulating heat pump. One of the stirrups was anchored to the organ chamber and the other was connected to a strain gauge to record isometric tension (60-2997, Harvard Apparatus, So. Natick, MA). Contractions and relaxations were recorded with a multichannel polygraph (1302-06, Gould Electronics, Balainvilliers, France) and expressed as absolute values (grams). For the superior mesenteric artery, a baseline force of 1g was applied by changing the position of the transducer. For the aorta a baseline force of 1.5g was applied. These tensions were identified in preliminary studies to provide optimum vasoactive responses. After 45 minutes of equilibration, the presence of a functional endothelium was confirmed on the basis of relaxation evoked by the endothelium-dependent dilator acetylcholine (10^{-6} mol/L) in arterial rings precontracted with phenylephrine (10^{-7} mol/L, an α_1-adrenoceptor agonist). Rings with less than 60% relaxation of phenylephrine-induced contractions were discarded. Then, the organ chambers were rinsed 3 times with warm, modified Krebs-Henseleit salt solution and the following studies were performed.

Relaxations to increasing acetylcholine concentrations (from 10^{-9} mol/L to 10^{-5} mol/L) were investigated in arteries that were preconstricted with phenylephrine (10^{-7} mol/L) and pretreated (for 30 minutes) or not with a combination of N^w-nitro-L-arginine (L-NNA, 10^{-4} mol/L, a NOS inhibitor) plus indomethacin (10^{-5} mol/L, a COX inhibitor). Preliminary results have shown that, in the superior mesenteric artery, 10^{-4} mol/L L-NNA decreased total NOS activity from 5.16 ± 0.08 to 0.09 ± 0.01 pmol/hr.mg of proteins, in cirrhotic rats ($n = 3$), and from 1.97 ± 0.28 to 0.07 ± 0.01 pmol/hr.mg of proteins, in normal rats ($n = 3$). In the aorta, 10^{-4} mol/L L-NNA decreased total NOS activity from 5.80 ± 0.54 to 0.08 ± 0.01 pmol/hr.mg of proteins, in cirrhotic rats ($n = 3$), and from 2.23 ± 0.30 to 0.06 ± 0.01 pmol/hr.mg of proteins, in normal rats ($n = 3$).

Two sets of experiments were performed in arterial rings from cirrhotic and normal rats. The first set of experiments was performed in rings from superior mesenteric arteries and aortae. In a first subset of experiments, acetylcholine-induced relaxations were measured in rings exposed or not to L-NNA plus indomethacin. In a second subset, acetylcholine-induced relaxations were measured in endothelium-denuded rings. The second set of experiments was performed in superior mesenteric arterial rings only, exposed to a combination of L-NNA and indomethacin. In a first subset of experiments, acetylcholine-induced relaxations were measured in rings exposed to apamin (10^{-7} mol/L) plus charybdotoxin (10^{-7} mol/L). In a second subset, acetylcholine-induced relaxations were measured in rings exposed to apamin plus iberiotoxin (10^{-7} mol/L, a K^+ channel blocker).[27] In a third subset, acetylcholine-induced relaxations were measured in rings exposed to barium alone (3×10^{-5} mol/L). In a last subset of experiments, acetylcholine-induced relaxations were measured in rings exposed to barium plus ouabain (10^{-3} mol/L).

Electrophysiologic Studies. As described earlier, rings were obtained in the main branch of cirrhotic superior mesenteric arteries. Rings were placed in a physiologic saline solution containing: 120 mmol/L NaCl, 11 mmol/L D-glucose, 4.7 mmol/L KCl, 2.4 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 1.8 mmol/L $CaCl_2$, 10 mmol/L HEPES (pH 7.4 with NaOH). Studies were performed in freshly isolated arteries as previously described.[28,32]

Microelectrode preparation. Microelectrodes used for electrophysiologic studies were obtained from borosilicate capillary glass (Clark GC 150F; Clark Electromedical, Pangbourne Reading, United Kingdom) pulled with a Narishige PA81 microelectrode puller (Narishige Scientific Instrument Laboratory, Tokyo, Japan). Tip diameters were about 0.5 μm and tip resistances between 50 to 100 mol/LΩ. Microelectrodes were filled with 600 mmol/L KCl.

Membrane potential measurements. Electrophysiologic studies were performed in a Faraday cage, immediately after the superior mesenteric artery had been removed from cirrhotic rats. A ring was placed in a continuous-flow chamber (200 μL) percolated by gravity with solutions (flow rate: 17 μL/s). Under microscopic control, SMCs in the superior mesenteric artery were impaled with a microelectrode. Impalement was performed by using a micromanipulator (Narishige WR91; Narishige Scientific Instrument Laboratory). The microelectrode was used to measure the membrane potential. The membrane potential was recorded on an electrometer (Axoclamp 2A; Axon Instruments, Foster City, CA). Potential was displayed on a dual-input

oscilloscope (Gould 1425, Gould Instruments Ltd., Hainault, United Kingdom) and was continuously recorded throughout the experiment by using a pen-recorder (BD 41; Kipp and Zonen, Delft, The Netherlands). Criteria for successful impalement were as follows[28,32]: (1) an abrupt drop in voltage as the electrode entered the cell, (2) a stable membrane potential for at least 2 minutes, (3) at the end of impalement, a sharp return to 0 mV, with resistance of the microelectrode tip unchanged, and (4) a tip potential less than 2 mV. Experiments were performed at 36°C.

Membrane potentials were measured before and during exposure to acetylcholine (10^{-6} mol/L) in rings pretreated for 30 minutes with a combination of L-NNA (10^{-4} mol/L) and indomethacin (10^{-5} mol/L). Membrane potential responses to acetylcholine were also measured in certain rings pretreated with L-NNA and indomethacin and exposed to a combination of apamin and charybdotoxin (10^{-7} mol/L, each). Preliminary results obtained in the superior mesenteric artery have shown that, in the absence of L-NNA and indomethacin, the SMC resting membrane potential was significantly more positive in cirrhotic (-21 ± 1 mV, n = 15) than in normal rats (-48 ± 2 mV, n = 16).[33] In other words, in the superior mesenteric artery at rest, the SMC membrane was significantly more depolarized in cirrhotic rats than in normal rats. Cirrhosis-induced SMC membrane depolarization may be caused by alteration of intracellular ionic concentrations (*e.g.*, a decrease in cytosolic K^+ concentrations).[28]

Substances. All substances were purchased from Sigma Chemical Co. (St Louis, MO).

Statistical Analysis. Values are mean ± SEM. The effects of the group (cirrhotic or normal), treatment (with or without L-NNA plus indomethacin), the type of artery (superior mesenteric artery or aorta), and the level of phenylephrine-induced preconstriction on concentration-dependent relaxation to acetylcholine were determined by multivariate ANOVA with repeated measures (SAS-STAT [SAS Institutes], procedure GLM). In the superior mesenteric artery exposed to L-NNA plus indomethacin, the effects of inhibitors (*i.e.*, apamin plus charybdotoxin; apamin plus iberiotoxin; barium alone or combined to ouabain) and the effect of the level of phenylephrine-induced preconstriction on concentration-dependent relaxation to acetylcholine were determined by multivariate ANOVA with repeated measures. In superior mesenteric arteries exposed to L-NNA and indomethacin, 2-way ANOVA was used to compare baseline phenylephrine-induced contraction with phenylephrine-induced contraction obtained in the presence of either apamin plus charybdotoxin or barium plus ouabain. A Student's t-test for paired data was used to compare membrane potentials before and during exposure to acetylcholine. A *P* value of .05 or less was considered significant.

RESULTS

Relaxation to Acetylcholine in Arteries With and Without L-NNA Plus Indomethacin. A significant concentration-dependent relaxant response to acetylcholine was found ($P = .001$) that was decreased by L-NNA plus indomethacin ($P = .001$). The L-NNA/indomethacin-induced decrease in concentration-dependent relaxation to acetylcholine did not differ between cirrhotic and normal rats (interaction term, $P = .63$). The L-NNA/indomethacin-induced decrease in concentration-dependent relaxation to acetylcholine was less marked in the superior mesenteric artery than the aorta (interaction term, $P = .004$).

None of these results were changed when the level of phenylephrine-induced preconstriction was taken into account in multivariate analysis. The concentration-dependent relaxation to acetylcholine in the superior mesenteric artery and the aorta, with and without L-NNA and indomethacin, are shown in Figure 2 (cirrhotic rats) and Figure 3 (normal rats).

Figure 4 shows representative acetylcholine-induced relaxations obtained in individual endothelium-denuded mesenteric artery from a cirrhotic rat and a normal rat. In both groups of rats, in the superior mesenteric artery (n = 3, in each group) and the aorta (n = 3, each group), the concentration-dependent relaxant response to acetylcholine was abolished by endothelium removal.

Membrane Potential Responses to Acetylcholine in SMCs in Cirrhotic Superior Mesenteric Arteries Exposed to L-NNA and Indomethacin. In 5 SMCs, the membrane potentials were significantly less negative under baseline conditions than during exposure to acetylcholine (-18 ± 2 mV and -24 ± 2 mV, respectively, $P = .001$). Endothelium removal suppressed acetylcholine-induced alterations in the membrane potential (-27 ± 1 mV and -26 ± 1 mV, before and during exposure to acetylcholine, respectively, $P = .90$).

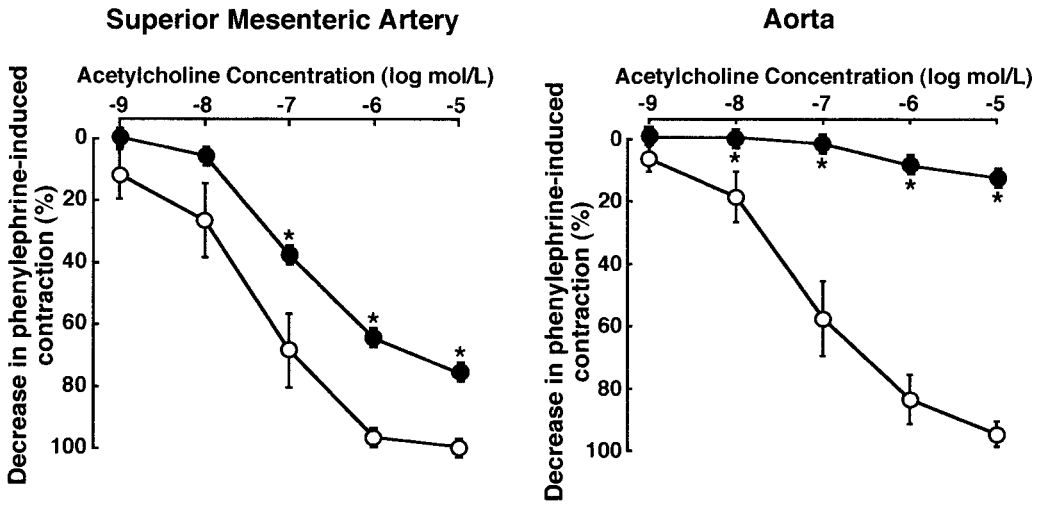

FIG. 2. Effect of inhibition of NOS and COX on concentration-dependent relaxation to acetylcholine in cirrhotic superior mesenteric arteries and aortae. Arteries were preconstricted with phenylephrine (10^{-7} mol/L). Curves were obtained in arterial rings in the absence (○) or presence (●) of a combination of L-NNA plus indomethacin. Results are expressed as the mean ± SEM of 6 to 11 experiments. A multivariate ANOVA with repeated measures was used. L-NNA plus indomethacin induced a decrease in concentration-dependent relaxation to acetylcholine in mesenteric arteries ($P = .0008$) and aortae ($P = .0001$). The L-NNA/indomethacin-induced decrease in concentration-dependent relaxation to acetylcholine was more marked in aortae than superior mesenteric arteries (interaction term, $P = .04$). *$P = .001$ versus the corresponding acetylcholine concentration in arteries without L-NNA plus indomethacin.

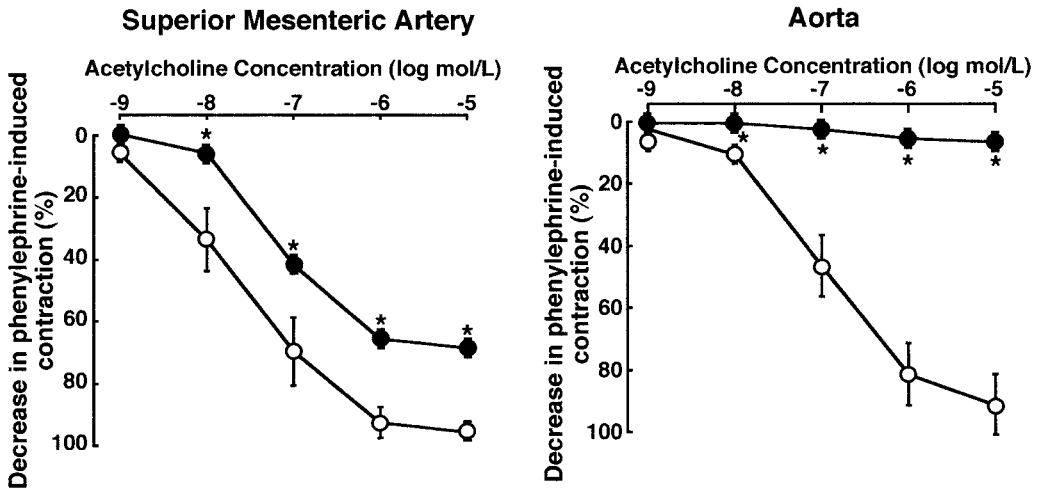

FIG. 3. Effect of inhibition of NOS and COX on concentration-dependent relaxation to acetylcholine in normal superior mesenteric arteries and aortae preconstricted with phenylephrine. Curves were obtained in arterial rings in the absence (○) or presence (●) of L-NNA and indomethacin. Results are expressed as the mean ± SEM of 6 to 8 experiments. A multivariate ANOVA with repeated measures was used. L-NNA plus indomethacin induced a decrease in concentration-dependent relaxation to acetylcholine in mesenteric arteries ($P = .002$) and aortae ($P = .0001$). The L-NNA/indomethacin-induced decrease in concentration-dependent relaxation to acetylcholine was more marked in aortae than superior mesenteric arteries (interaction term, $P = .04$). *$P = .001$ versus the corresponding acetylcholine concentration in arteries without L-NNA plus indomethacin.

In 5 SMCs, in arteries exposed to a combination of apamin and charybdotoxin, membrane potentials were not different before and during application of acetylcholine (-14 ± 2 mV and -14 ± 1 mV, respectively, $P = .70$).

Effects of Apamin Combined to Either Charybdotoxin or Iberiotoxin on Acetylcholine-Induced Relaxations in Superior Mesenteric Arteries Exposed to L-NNA and Indomethacin. The concentration-dependent relaxation to acetylcholine was lower in the pres-

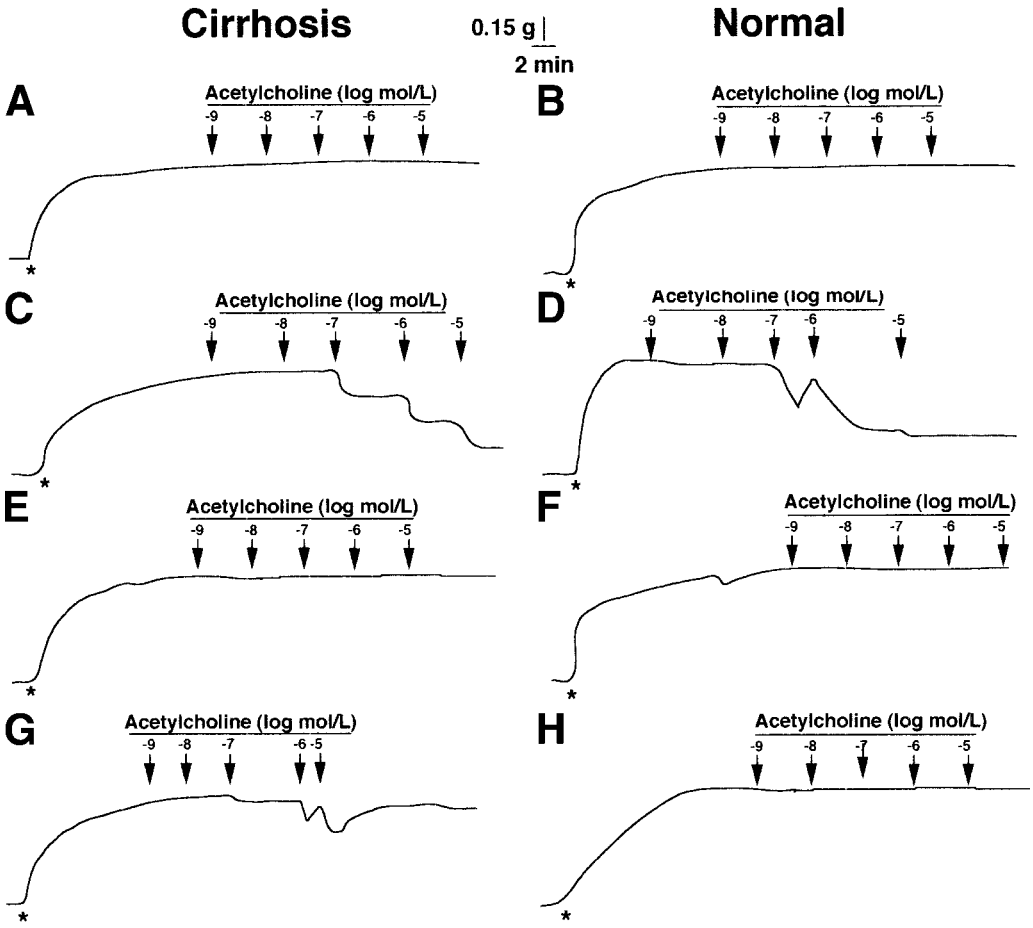

FIG. 4. Representative recordings of concentration-dependent relaxation to acetylcholine in superior mesenteric arteries. Arteries were preconstricted with phenylephrine (*). Endothelium-denuded (A, B) or intact (C, D, E, F, G, and H) arteries were used. Intact vessels were exposed to L-NNA plus indomethacin alone (C, D) or combined to either apamin plus charybdotoxin (E, F) or barium plus ouabain (G, H).

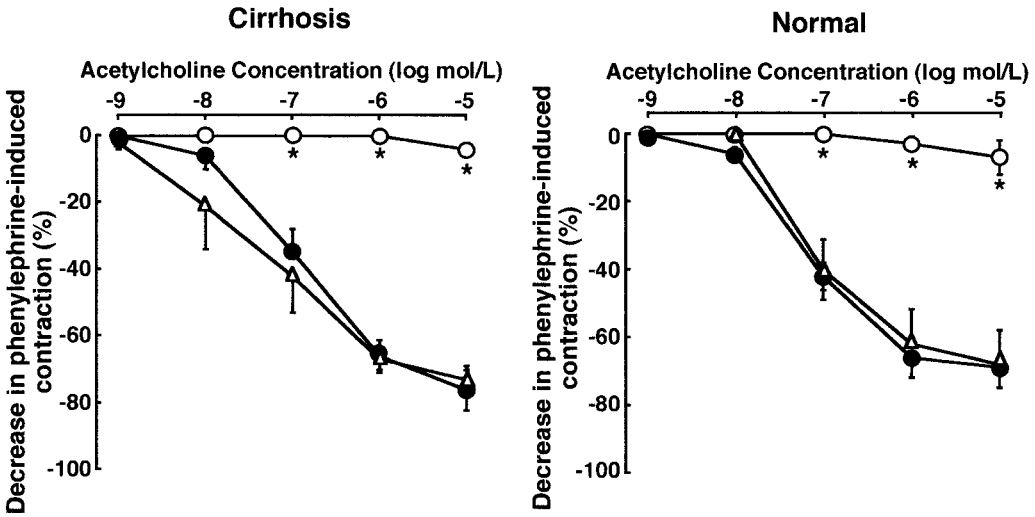

FIG. 5. Effects of apamin combined to either charybdotoxin or iberiotoxin on acetylcholine-induced, EDHF-mediated relaxation in superior mesenteric arteries from cirrhotic and normal rats. Concentration response curves to the relaxant effect of acetylcholine were all obtained in arteries exposed to L-NNA plus indomethacin. Concentration response curves were obtained under control conditions (●, n = 11 in cirrhotic rats and n = 8 in normal rats); in the presence of a combination of K^+ channel inhibitors, apamin plus charybdotoxin (10^{-7} mol/L each, ○, n = 4 in cirrhotic rats and n = 5 in normal rats); in the presence of a combination of K^+ channel inhibitors, apamin plus iberiotoxin (10^{-7} mol/L each, ▲, n = 4 in cirrhotic rats and n = 4 in normal rats). A multivariate ANOVA with repeated measures was used. Apamin and charybdotoxin decreased the concentration-dependent relaxation to acetylcholine in cirrhotic ($P = .0001$) and normal rats ($P = .0001$). The apamin/charybdotoxin-induced reduction in relaxant responses to acetylcholine was similar in both groups. Apamin plus iberiotoxin did not significantly change the concentration-dependent relaxation to acetylcholine in cirrhotic and normal rats. *$P = .0001$ versus the corresponding acetylcholine concentration in the control and apamin/iberiotoxin groups.

ence of apamin plus charybdotoxin than under control conditions ($P = .001$). The apamin/charybdotoxin-elicited decrease in acetylcholine-induced relaxation did not differ between cirrhotic and normal rats (interaction term, $P = .60$).

The concentration-dependent relaxation to acetylcholine did not differ under control conditions and in the presence of apamin plus iberiotoxin ($P = .76$). All the earlier-described results were similar when the level of phenylephrine-induced preconstriction was taken into account in multivariate analysis.

Figure 4 shows representative acetylcholine-induced relaxations under control conditions or during exposure to apamin plus charybdotoxin in cirrhotic and normal rats. Figure 5 shows the effect of each pharmacologic combination on the concentration-dependent relaxation to acetylcholine in cirrhotic and normal rats.

Effects of Barium Alone or Combined to Ouabain on Acetylcholine-Induced Relaxations in Superior Mesenteric Arteries Exposed to L-NNA and Indomethacin. The concentration-dependent relaxation to acetylcholine was lower in the presence of barium alone than under control conditions ($P = .002$). The barium-alone–elicited decrease in concentration-dependent relaxant responses to acetylcholine was similar in cirrhotic than normal rats (interaction term, $P = .48$).

The concentration-dependent relaxation to acetylcholine was lower in the presence of either barium plus ouabain than under control conditions ($P = .0001$). The barium/ouabain-elicited decrease in concentration-dependent relaxant responses to acetylcholine was less marked in cirrhotic than normal rats (interaction term, $P = .05$).

All the earlier-described results were similar when the level of phenylephrine-induced preconstriction was taken into account in multivariate analysis.

Figure 4 shows representative acetylcholine-induced relaxations in individual cirrhotic and normal arteries exposed to barium plus ouabain. Figure 6 shows the effect of barium alone and barium plus ouabain on the concentration-dependent relaxation to acetylcholine in cirrhotic and normal rats.

Reactivity to Phenylephrine in Superior Mesenteric Arteries Exposed to L-NNA and Indomethacin. Contractions caused by 10^{-7} mol/L phenylephrine were lower in cirrhotic than in normal arterial rings (0.84 ± 0.10 g (n = 11) vs. 1.40 ± 0.13 g (n = 8), respectively, $P = .004$). In the presence of apamin and charybdotoxin, phenylephrine-induced contractions did not differ between cirrhotic and normal rats (2.02 ± 0.20 g (n = 4) vs. 1.85 ± 0.57 g (n = 5), respectively, $P = .81$). In the presence of barium plus ouabain, phenylephrine-induced contractions did not differ between cirrhotic and normal rats (1.40 ± 0.11 g (n = 4) vs. 1.30 ± 0.23 g (n = 5), respectively, $P = .72$).

DISCUSSION

This study shows that in arteries without NOS/COX-inhibitors, acetylcholine induced endothelium-dependent relaxation in the superior mesenteric artery (main branch) and in the aorta from cirrhotic rats. In arteries exposed to NOS/COX-inhibitors, relaxation to acetylcholine was decreased in the superior mesenteric artery but abolished in the aorta. Thus, a NO/PGI_2-independent relaxant response to acetylcholine was present in the superior mesenteric artery but not the aorta. The present study focused on acetylcholine-induced, NOS/COX-inhibitors–insensitive relaxation in the superior mesenteric artery.

In cirrhotic mesenteric arteries exposed to NOS/COX-inhibitors, acetylcholine induced endothelium-dependent SMC membrane hyperpolarization. Thus, acetylcholine induced the release of endothelial, NO/PGI_2-independent, hyperpolarizing factors (*i.e.*, EDHFs).[15] Because SMC membrane hyperpolarization is a signal for smooth muscle relaxation,[27] EDHF

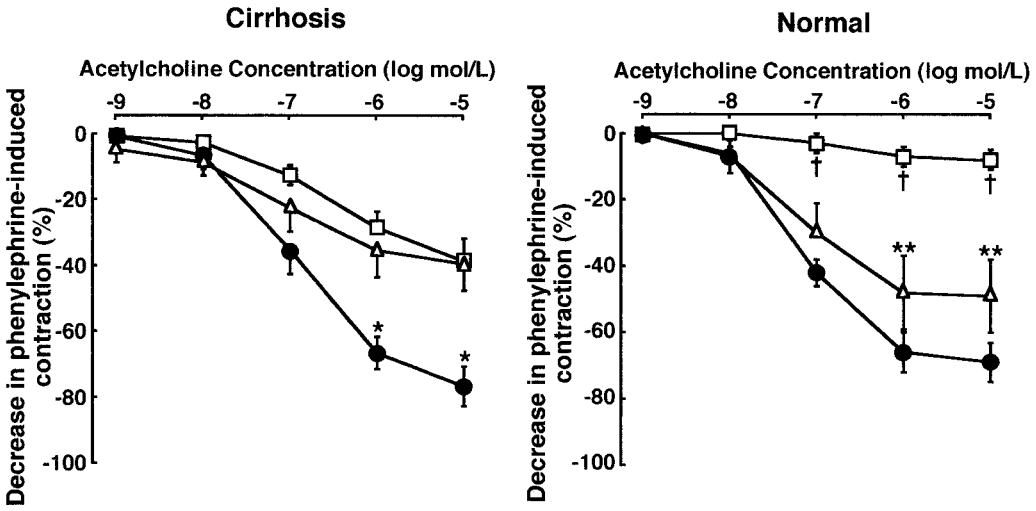

FIG. 6. Effects of barium alone or combined to ouabain on acetylcholine-induced, EDHF-mediated relaxation in superior mesenteric arteries from cirrhotic and normal rats. Concentration response curves to the relaxant effect of acetylcholine were all obtained in arteries exposed to L-NNA plus indomethacin. Concentration response curves were obtained under control conditions (●); in the presence of a K^+ channel inhibitor barium (3×10^{-5} mol/L) alone (△, n = 8 in cirrhotic rats and n = 4 in normal rats); in the presence of barium combined to the Na^+/K^+ ATPase inhibitor ouabain (10^{-3} mmol/L, □, n = 4 in cirrhotic rats and n = 5 in normal rats). A multivariate ANOVA with repeated measures was used. Barium decreased the concentration-dependent relaxation to acetylcholine in cirrhotic ($P = .004$) and normal rats ($P = .05$). The barium-alone–induced decrease in relaxant responses to acetylcholine was similar in both groups. Barium plus ouabain decreased the concentration-dependent relaxation to acetylcholine in cirrhotic ($P = .01$) and normal rats ($P = .0001$). The barium/ouabain-induced decrease in the concentration-dependent relaxation to acetylcholine was significantly less marked in cirrhotic than in normal rats (interaction term, $P = .05$). *$P = .001$ versus the corresponding acetylcholine concentration in the barium-alone and barium-plus-ouabain groups. **$P = .05$ versus the corresponding acetylcholine concentration in the control group. †$P = .0001$ versus the corresponding acetylcholine concentration in the control and barium-alone groups.

was involved in acetylcholine-induced relaxation in the present study.

In this study, overall NOS/COX-inhibitors–-insensitive relaxation to acetylcholine did not significantly differ between cirrhotic and normal rats. Thus, the degree of acetylcholine-induced, EDHF-mediated relaxation was similar in cirrhotic and normal mesenteric arteries.

In cirrhotic and normal mesenteric arteries exposed to NOS/COX-inhibitors, a combination of the K^+ channel blockers apamin and charybdotoxin suppressed acetylcholine-induced SMC membrane hyperpolarization and relaxation. Therefore, activation of apamin- and charybdotoxin-sensitive K^+ channels was involved in EDHF-induced hyperpolarization and relaxation. Moreover, our findings indicate that cirrhosis did not alter the effect of apamin plus charybdotoxin on EDHF-induced relaxation.

In this study, in cirrhotic mesenteric arteries exposed to NOS/COX-inhibitors, barium (a K^+ channel blocker) alone or combined with ouabain (Na^+/K^+ ATPase inhibitor) significantly decreased acetylcholine-induced relaxation. Thus, barium-sensitive K^+ channels and Na^+/K^+ ATPase were involved in EDHF-induced relaxation. On the other hand, barium plus ouabain suppressed (and not decreased only) acetylcholine-induced relaxation in normal mesenteric arteries. Thus, cirrhosis altered the effects of barium plus ouabain on EDHF-induced relaxation.

In small resistance mesenteric arteries, acetylcholine-induced EDHF has been shown to be K^+ that effluxes through endothelial apamin- and charybdotoxin-sensitive K^+ channels and then stimulates barium-sensitive K^+ channels and Na^+/K^+ ATPase located in the SMC plasma membrane (Fig. 1).[24] Therefore, the results of the present study suggest that K^+ may be an acetylcholine-induced EDHF in large mesenteric arteries.

In this study, an important finding in arteries exposed to NOS/COX-inhibitors was a less-marked contraction to the α_1-adrenoceptor agonist phenylephrine in cirrhotic than in normal rats. This indicates an NO/PGI_2-independent hyporeactivity to the contractile action of phenylephrine in the cirrhotic rat superior mesenteric artery. Moreover, this hyporeactivity to phenylephrine was suppressed by apamin plus charybdotoxin or barium plus ouabain. Because apamin plus charybdotoxin or barium plus ouabain inhibit EDHF-induced relaxation (see earlier), the results of the present study indicate that an EDHF-induced relaxation was the cause of NO/PGI_2-independent hyporeactivity to phenylephrine in cirrhotic arteries.

In the absence of NOS/COX-inhibitors, superior mesenteric arteries from cirrhotic rats are hyporeactive to α_1-adrenoceptor agonists. This hyporeactivity is not caused by α_1-adrenoceptor down-regulation[34,35] but by endothelial NO overproduction.[3,5,7,9,10,12,31,35,36] In this study, however, hyporeactivity to phenylephrine in superior mesenteric arteries during NO inhibition was caused by baseline EDHF-induced relaxation. Thus, EDHF replaced NO and induced arterial hyporeactivity to vasoconstrictors in cirrhotic rat superior mesenteric artery. Because NO is known to induce tonic inhibition of EDHF release in different arteries,[37] EDHF-mediated hyporeactivity to phenylephrine in cirrhotic arteries exposed to NOS inhibitors may be caused by increased EDHF release in response to inhibition of baseline NO overproduction.

Diabetes mellitus (which is common in cirrhosis) is known to be associated with decreased arterial NOS activity.[38] In portal hypertensive animals, long-term administration of a nonselective β-blocker has been shown to decrease arterial NO overproduction by inhibiting NOS expression and activity.[39] When arterial NOS activity is decreased, EDHF replaces NO to relax cirrhotic mesenteric arteries (see earlier). There-

fore, in cirrhotic patients with diabetes or in those receiving a nonselective β-blocker, EDHF may substitute for NO to decrease splanchnic vascular tone and, thus, play a role in the maintenance of portal hypertension.

In conclusion, in cirrhotic and normal rats, acetylcholine-induced, EDHF-mediated relaxation occurs in the superior mesenteric artery but not in the aorta. EDHF-induced relaxation involves the activation of apamin- and charybdotoxin-sensitive K^+ channels, barium-sensitive K^+ channels, and Na^+/K^+ ATPase. Finally, this study shows that, in cirrhotic rats, in the superior mesenteric artery exposed to NOS/COX inhibitors, EDHF may act in place of NO and PGI_2 to induce hyporeactivity to α_1-adrenoceptor agonist-mediated contraction.

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REFERENCES

1. Pizcueta MP, Piqué JM, Fernandez M, Bosch J, Rodes J, Whittle BJR, Moncada S. Modulation of the hyperdynamic circulation of cirrhotic rats by nitric oxide inhibition. Gastroenterology 1992;103:1909-1915.
2. Hou MC, Cahill PA, Zhang S, Wang YN, Hendrickson RJ, Redmond EM, Sitzmann JV. Enhanced cyclooxygenase-1 expression within the superior mesenteric artery of portal hypertensive rats: role in the hyperdynamic circulation. HEPATOLOGY 1995;22:598-606.
3. Michielsen PP, Boeckxstaens GE, Sys SU, Herman AG, Pelckmans PA. The role of increased nitric oxide in the vascular hyporeactivity to noradrenaline in long-term portal vein ligated rats. Eur J Pharmacol 1995; 273:167-174.
4. Fernandez LN, Garcia-Pagan JC, Casadeval M, Mourelle MI, Piqué JM, Bosch J, Rodés J. Acute and chronic cyclooxygenase in portal-hypertensive rats: influence on nitric oxide biosynthesis. Gastroenterology 1996; 110:1529-1535.
5. Atucha NM, Shah V, Garcia-Gardena G, Sessa WE, Groszmann RJ. Role of endothelium in the abnormal response of mesenteric vessels in rats with portal hypertension and liver cirrhosis. Gastroenterology 1996;111: 1627-1632.
6. Pilette C, Kirstetter P, Sogni P, Cailmail S, Moreau R, Lebrec D. Dose-dependent effects of a nitric oxide biosynthesis inhibitor on hyperdynamic circulation in two models of portal hypertension in conscious rats. J Gastroenterol Hepatol 1996;11:1-6.
7. Sogni P, Sabry S, Moreau R, Gadano A, Lebrec D, Dinh-Xuan AT. Hyporeactivity of mesenteric resistance arteries in portal hypertensive rats. J Hepatol 1996;24:487-490.
8. Gadano AC, Sogni P, Yang S, Cailmail S, Moreau R, Nepveux P, Couturier D, et al. Endothelial calcium-calmodulin dependent nitric oxide synthase in the in vitro vascular hyporeactivity of portal hypertensive rats. J Hepatol 1997;26:678-686.
9. Atucha NM, Ortiz MC, Fortepiani LA, Ruiz FM, Martinez C, Garcia-Estan J. Role of cyclic guanosine monophosphate and K^+ channels as mediators of the mesenteric vascular hyporesponsiveness in portal hypertensive rats. HEPATOLOGY 1998;27:900-905.
10. Hori N, Wiest R, Groszmann RJ. Enhanced release of nitric oxide in response to changes in flow and shear stress in the superior mesenteric arteries of portal hypertensive rats. HEPATOLOGY 1998;28:1467-1473.
11. Gadano AC, Sogni P, Heller J, Moreau R, Bories PN, Lebrec D. Vascular nitric oxide production during the development of two experimental models of portal hypertension. J Hepatol 1999;30:896-903.
12. Wiest R, Das S, Cadelina G, Garcia-Tsao G, Milstien S, Groszmann RJ. Bacterial translocation in cirrhotic rats stimulates eNOS-derived NO production and impairs mesenteric vascular contractility. J Clin Invest 1999; 104:1223-1233.
13. Furchgott RF, Vanhoutte PM. Endothelium-derived relaxing and contracting factors. FASEB J 1989;3:2007-2018.
14. Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991;43:109-142.
15. Cohen RA, Vanhoutte PM. Endothelium-dependent hyperpolarization. Beyond nitric oxide and cyclic GMP. Circulation 1995;92:3337-3349.
16. Rubanyi GM, Vanhoutte PM. Nature of endothelium-derived relaxing factor: are there two relaxing factors? Circ Res 1987;61:61-67.
17. Vanhoutte PM. The end of the quest? Nature 1987;327:459-460.
18. Feletou M, Vanhoutte PM. Endothelium-dependent hyperpolarization of canine coronary smooth muscle. Br J Pharmacol 1988;93:515-524.
19. Chen G, Suzuki H, Weston AH. Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. Br J Pharmacol 1988;95:1165-1174.
20. Fleming I, Bauersachs J, Busse R. Paracrine functions of the coronary vascular endothelium. Mol Cell Biochem 1996;157:137-145.
21. Nilius B, Viana F, Droogmans G. Ion channels in vascular endothelium. Annu Rev Physiol 1997;59:145-170.
22. Zygmunt PM, Högestätt ED. Role of potassium channels in endothelium-dependent relaxation resistant to nitroarginine in the rat hepatic artery. Br J Pharmacol 1996;117:1600-1606.
23. White R, Hiley CR. A comparison of EDHF-mediated and anandamide-induced relaxations in the rat isolated mesenteric artery. Br J Pharmacol 1997;122:1573-1584.
24. Edwards G, Dora KA, Gardener MJ, Garland CJ, Weston AH. K^+ is an endothelium-derived hyperpolarizing factor in rat arteries. Nature 1998; 396:269-272.
25. Doughty JM, Plane F, Langton PD. Charybdotoxin and apamin block EDHF in rat mesenteric artery if selectively applied to the endothelium. Am J Physiol 1999;276:H1107-H1112.
26. Takamura Y, Shimokawa H, Zhao H, Igarashi H, Egashira K, Takeshita A. Important role of endothelium-derived hyperpolarizing factor in shear stress-induced endothelium-dependent relaxations in the rat mesenteric artery. J Cardiovasc Pharmacol 1999;34:381-387.
27. Nelson MT, Quayle JM. Physiological roles and properties of potassium channels in arterial smooth muscle. Am J Physiol 1995;268:C799-C822.
28. Lahaye P, Tazi KA, Rona JP, Dellis O, Lebrec D, Moreau R. Effects of protein kinase C modulators on Na^+/K^+ ATPase activity and phosphorylation in aortae from rats with cirrhosis. HEPATOLOGY 1998;28:663-669.
29. Lee SS, Girod C, Braillon A, Hadengue A, Lebrec D. Hemodynamic characterization of chronic bile duct-ligated rats: effects of pentobarbital sodium. Am J Physiol 1986;251:G176-G180.
30. Kountouras J, Billing BH, Scheuer PJ. Prolonged bile duct obstruction: a new experimental model for cirrhosis in the rat. Br J Exp Pathol 1984; 65:305-311.
31. Pateron D, Oberti F, Lefilliatre P, Veal N, Tazi KA, Sogni P, Poirel O, et al. Relationship between vascular reactivity in vitro and blood flows in rats with cirrhosis. Clin Sci 1999;97:313-318.
32. Lahaye P, Fouassier L, Tazi KA, Gottardi AD, Fléjou JF, Chagneau C, Rona JP, et al. Endothelium-dependent blunted membrane potential responses to ATP-sensitive K^+ channel modulators in aortae from rats with cirrhosis. J Hepatol 1999;30:107-114.
33. Lahaye P, Rona JP, Tazi KA, Lebrec D, Moreau R. Membrane depolarization in arterial smooth muscle cells (SMC) in rats with cirrhosis. Relationships with vasodilation and endothelium [Abstract]. HEPATOLOGY 1997;26:392A.
34. Liao JF, Yu PC, Lin HC, Lee FY, Kuo JS, Yang MCM. Study on the vascular reactivity and α_1-adrenoceptors of portal hypertensive rats. Br J Pharmacol 1994;111:439-444.
35. Moreau R, Lebrec D. Endogenous factors involved in the control of arterial tone in cirrhosis. J Hepatol 1995;22:370-376.
36. Martin PY, Xu DL, Niederberger M, Weigert A, Tsai P, St John J, Schrier RW. Upregulation of endothelial constitutive NOS: a major role in the increased NO production in cirrhotic rats. Am J Physiol 1996;270:F494-F499.
37. Bauersachs J, Popp R, Hecker M, Sauer E, Fleming I, Busse R. Nitric oxide attenuates the release of endothelium-derived hyperpolarizing factor. Circulation 1996;94:3341-3347.
38. Busse R, Fleming I. Endothelial dysfunction in atherosclerosis. J Vasc Res 1996;33:181-194.
39. Pateron D, Tazi KA, Sogni P, Heller J, Chagneau C, Poirel O, Philippe M, et al. Role of nitric oxide synthase 3 (eNOS) in the systemic vasodilation of portal hypertension. Gastroenterology 2000;119:201-210.