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Endothelium-dependent blunted membrane potential responses to ATP-sensitive K^+ channel modulators in aortae from rats with cirrhosis

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Background/Aims: In vivo **studies have shown that arterial vasodilation induced by synthetic openers of ATP-sensitive K^+ (K_{ATP}) channels is decreased in rats with cirrhosis. Since vasodilation induced by these substances is mediated by membrane potential hyperpolarization in arterial smooth muscle cells, membrane potential hyperpolarization in response to K_{ATP} channel openers may be altered in cirrhotic smooth muscle cells. The aim of the present study was to investigate the effects of K_{ATP} channel modulators (i.e. openers and blockers of these channels) on the membrane potential in smooth muscle cells in isolated aortae from cirrhotic and normal rats. The influence of endothelin-1 production by endothelial cells on smooth muscle cells membrane potential responses to K_{ATP} channel modulators was also studied.**

Methods: **Cells were impaled *in situ* (in intact and endothelium-denuded aortae) with a microelectrode that was used to measure membrane potentials. K_{ATP} channel openers were diazoxide or cromakalim; blockers were glibenclamide or tolbutamide. Bosentan (a mixed endothelin receptor antagonist) and exogenous endothelin-1 were also used. Preproendothelin-1 mRNA was assayed in aortae by RNase protection assay. Aortic wall endothelin-1 concentration was measured by double antibody radioimmunoassay technique.**

Results: **As expected, in smooth muscle cells in intact normal aortae, K_{ATP} channel openers induced membrane potential hyperpolarization and K_{ATP} channel blockers membrane potential depolarization. In smooth muscle cells in intact cirrhotic aortae, K_{ATP} channel openers and blockers did not significantly change the membrane potential. Endothelium removal or exposure of intact aortae to bosentan restored normal membrane potential responses to K_{ATP} channel modulators in cirrhotic smooth muscle cells and did not alter the effects of these substances in normal smooth muscle cells. In endothelium-denuded aortae, exposure to exogenous endothelin-1 suppressed membrane potential responses to K_{ATP} channel modulators. In intact aortae, the abundance of preproendothelin-1 mRNA and endothelin-1 did not significantly differ between normal and cirrhotic rats.**

Conclusions: **K_{ATP} channel opener-induced membrane hyperpolarization and K_{ATP} channel blocker-elicited membrane depolarization are blunted in smooth muscle cells in intact cirrhotic aortae. This blunting is due to the activation of the endothelin-1 pathway in the aortic wall, downstream to the endothelial production of endothelin-1.**

Key words: **Endothelin-1; K_{ATP} channel blockers; K_{ATP} channel openers; Membrane potential depolarization; Membrane potential hyperpolarization; Portal hypertension.**

In vivo STUDIES have shown that arterial vasodilation induced by synthetic openers of adenosine triphosphate (ATP)-sensitive K^+ (K_{ATP}) channels is decreased in rats with cirrhosis (1,2). In normal arterial smooth muscle cells (SMC), K_{ATP} channel openers are known to evoke vasodilation by inducing membrane potential hyperpolarization, in which the membrane potential becomes more negative than the resting value (3–5). Thus, a cirrhosis-induced decrease in vasorelaxant re-

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sponses to K_{ATP} channel openers suggests that the membrane potential hyperpolarization caused by these substances may be reduced in cirrhotic SMC. If membrane potential responses to K_{ATP} channel openers are altered in cirrhotic SMC, an alteration of the effects of K_{ATP} channel blockers on the resting membrane potential may also occur. The present study was performed in SMC in isolated aortae to compare membrane potential responses to K_{ATP} channel openers (diazoxide and cromakalim) (6,7) in cirrhotic rats with membrane potential responses to these substances in normal rats. The present study also compared membrane potential responses to K_{ATP} channel blockers (glibenclamide and tolbutamide) (6,7) in cirrhotic rats to membrane potential responses to these substances in normal rats. Since the vascular endothelium produces substances (such as endothelin-1) (8) which exert a paracrine modulation of smooth muscle K_{ATP} channel activity (9), the influence of endothelin-1 production by endothelial cells on membrane potential responses to K_{ATP} channel modulators (i.e., openers and blockers of these channels) was also studied.

Materials and Methods

Animals

Male Sprague-Dawley rats (Charles River Laboratoires, Saint-Aubin-Lès-Elbeuf, France) were divided into two groups. One group included normal rats (n=48). A second group (n=49) had secondary biliary cirrhosis with portal hypertension as a result of bile duct ligation, as previously described (10,11). Under ether anesthesia, the common bile duct was exposed by median laparotomy and 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 two ligatures and the abdominal incision closed. Studies were performed 4–5 weeks following bile duct ligation in rats weighing from 210 to 340 g. This delay is necessary for the development of secondary biliary cirrhosis (12).

Cirrhosis was confirmed by macroscopic examination of the liver and the presence of ascites. Studies were performed in normal rats weighing from 290 to 380 g. All rats were allowed free access to food and water up to 14 to 16 h before the study, when food was withdrawn. Protocols performed in this laboratory were approved by the French Agricultural Office in conformity with European legislation for research involving animals.

Histology and immunochemistry

Studies were performed to assess the morphological integrity of the endothelium in freshly dissected "intact aortae" and to check the efficacy of endothelium removal in "endothelium-free" aortae. Rats were killed by stunning. Specimens were fixed in 10% neutral formalin for 24 h, embedded in paraffin and serially sectioned at 5 μM. Histology was performed on hematoxylin-eosin stained sections. Immunohistochemistry was performed using the three-step immunoperoxidase method described by Mason & Summons (13). A rabbit polyclonal antibody to human von Willebrand factor (Dako, Glostrup, Denmark) was used at a 1:50 dilution. This antibody specifically stains endothelial cells and cross-reacts with various species, including the rat.

Electrophysiological studies

Tissue preparation. After animals were killed, the thoracic aorta was removed and placed in a Petri dish containing an oxygenated physiological saline solution (PSS) at 36°C, then cleansed of surrounding fatty tissue and cut into 5-mm rings (n=2–4 per aorta). PSS contained (mM) NaCl, 120; D-glucose, 11; $NaHCO_3$, 24; KCl, 4.7; $MgSO_4$, 2.4; KH_2PO_4, 1.2; $CaCl_2$, 1.8 (pH 7.4 with NaOH).

Microelectrode preparation. Microelectrodes used for electrophysiological 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 50–100 MΩ. Microelectrodes were filled with 600 mM KCl.

Membrane potential measurements. Electrophysiological studies were performed in a Faraday cage, immediately after the arteries were removed from the animal. An arterial ring was placed in a continuous-flow chamber (200 μl) percolated by gravity with solutions (flow rate: 17 μl/s). Under microscopic control, SMC in the arterial ring were impaled with a microelectrode on the adventitial side. Impalement was performed using a micromanipulator (Narishige WR91, Narishige Scientific Instrument Laboratory, Tokyo, Japan). The microelectrode was used to measure the membrane potential which was recorded on an electrometer (Axoclamp 2A, Axon Instruments, Foster City, CA, USA). Potential was displayed on a dual-input oscilloscope (Gould 1425, Gould Instruments Ltd., Hainault, United Kingdom) and was continuously recorded throughout the experiment using a pen-recorder (BD 41, Kipp and Zonen, Delft, The Netherlands). Criteria for successful impalement were as follows (14): (i) an abrupt drop in voltage as the electrode entered the cell, (ii) a stable membrane potential for at least 2 min, (iii) at the end of impalement, a sharp return to 0 mV, with resistance of the microelectrode tip unchanged, (iv) a tip potential$<$ 2 mV. Experiments were performed at 36°C.

Four sets of electrophysiological studies were performed in isolated aortae.

Study 1. Three subsets of experiments were performed to investigate the effects of K_{ATP} channel modulators in SMC in intact aortae. In the first subset, membrane potentials were measured prior to and during exposure to the K_{ATP} channel openers diazoxide (100 μM, Sigma Chemical Co, St. Louis, MO, USA) or cromakalim (10 μM, Sigma Chemical Co). In a second subset, membrane potentials were measured prior to and during exposure to the K_{ATP} channel blockers glibenclamide (10 μM, Sigma Chemical Co) or tolbutamide (500 μM, Sigma Chemical Co). In the last subset, membrane potentials were measured prior to and during exposure to cromakalim, in glibenclamide (10 μM)-pretreated aortae.

Study 2. Two subsets of experiments were performed to investigate the effects of K_{ATP} channel modulators in SMC in endothelium-denuded aortae. In the first subset, membrane potentials were measured prior to and during exposure to the K_{ATP} channel openers diazoxide or cromakalim. In a second subset, membrane potentials were measured prior to and during exposure to the K_{ATP} channel blockers glibenclamide or tolbutamide.

Study 3. Two subsets of experiments were performed to investigate the influence of endothelin-1 on membrane potential responses to K_{ATP} channel modulators in aortae. In the first subset, membrane potential responses to diazoxide and glibenclamide were measured in intact or endothelium-denuded cirrhotic aortae pretreated with the mixed endothelin receptor antagonist, bosentan (10 μM, Hoffmann-La Roche, Switzerland) (15). The second subset of experiments was performed in endothelium-denuded aortae from cirrhotic and normal rats. Membrane potential responses to diazoxide and glibenclamide were measured in arteries pretreated with exogenous endothelin-1 (20 nM, Sigma Chemical Co).

Study 4. This study investigated the influence of the endothelium on the resting membrane potential and was divided into 2 parts. The first part consisted in the analysis of certain results obtained in Study 1–3, i.e., resting potentials measured in intact aortae (in Study 1 and Study 3) were compared to resting potentials measured in endothelium-denuded aortae (in Study 2 and Study 3). In the second part of Study 4, two additional experiments were performed to investigate the potential role of endogenous (i.e., endothelial) endothelin-1 (8) in

the regulation of the resting membrane potential in cirrhotic SMC. First, membrane potential responses to bosentan were measured in SMC in intact or endothelium-denuded aortae from normal and cirrhotic rats. Second, membrane potential responses to exogenous endothelin-1 were measured in SMC in intact and endothelium-denuded aortae from normal and cirrhotic rats.

RNA isolation and preproendothelin-1 mRNA detection

Immediately after isolation, aortae were snap-frozen in liquid nitrogen and stored at −80°C until analysis.

RNA isolation. Prior to RNA preparation, aortae were reduced to a fine powder in liquid nitrogen and homogenized with a glass-glass homogenizer in a denaturing solution containing 4M guanidinium isothiocynate (GuSCN), 25 mM sodium citrate, pH 7, 0.05% sarcosyl, 0.1 M2-mercaptoethanol. Total RNA was extracted according to the technique of Chomczynski & Sacchi (16). The concentration of RNA was determined by spectrophotometry, and the integrity of the samples was documented by visualization of 18S and 28 s ribosomal bands after electrophoresis through an 0.8% agarose gel stained with ethydium bromide.

Preproendothelin-1 mRNA assay. Preproendothelin-1 mRNA was detected in aortae by an RNase protection assay. A 464-bp *Sac*I/*Taq*I fragment of the rat preproendothelin-1 cDNA (17) was ligated into a *Sac*I/*Taq*I digested pBluescriptII (SK^+) vector. In order to generate a radiolabeled cRNA probe, the vector was linearized with *Pvu*II/*Sty*I and transcription of a 260-nt RNA was performed using T7RNA polymerase (Promega, Charbonnières, France) and 400Ci/mmol α-^{32}P-UTP (Amersham, Pharmacia, Les Ullis, France) (18). The radiolabeled transcripts were separated from free labeled UTP by Nuctrap Push Columns (Stratagen, Ozyme, Montigny-Le-Bretonneux, France), dried under a vacuum and resuspended in 5 M GuSCN, 0.1 M EDTA, pH 7.0 (19). Solution hybridization was carried out in 50 ml buffered formamide (40 mM Pipes, pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% (vol/vol) formamide) containing 2 μl of radiolabeled riboprobe (1.10^5 cpm) and 30 μg of total RNA in 5 M GuSCN, 0.1 M EDTA, pH 7.0. The preparation was denatured at 95°C for 10 min, and hybridized at 55°C for 12 h. Following hybridization, RNase A (40 μl/ml) and RNases T1 (625 U/ml) (both from Boehringer Mannheim, Meylan, France) were added in 1 mM EDTA, 300 mM NaCl, 30 mM Tris-HCl, pH 7.4, for 45 min at room temperature, to digest unbound label and unprotected mRNA. Samples were then incubated at 37°C for 30 min after addition of 60 μl of a solution of proteinase K (1.7 mg/ml) (Appligene, Illkirch, France) containing 3.3% SDS. The protected hybrids were purified by phenol-chloroform extraction, ethanol precipitation and separated by electrophoresis through a 5% polyacrylamide/urea sequencing gel. Dried gel was applied to x-ray film for 12 h. Bands corresponding to the protected labeled fragments were quantified by scanning densitometry. Samples were all cohybridized with a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) riboprobe as an internal control for the total mRNA present in an individual extract. Total rat lung RNA and yeast tRNA served as positive and negative controls, respectively.

Extraction and assay of endothelin-1

Aortic wall endothelin concentration was determined as previously described (20). Briefly, snap-frozen vessels were pulverized and homogenized in 2 ml of a chloroform-ethanol 2:1 solution with 0.1% trifluoroacetic acid and 1 mM N-ethylmaleamide. Sterile water (0.8 ml) was added to each tube, which was then centrifuged at 4°C, 5000 rpm for 15 min. The aqueous phase was collected, diluted 1:9 in acetic acid 4% and passed through activated Sep-Pak C_{18} 500 mg cartridges (Waters Corporation, Milford, USA). The product of elution (2 ml 86% ethanol/4% acetic acid) was dried overnight in a speed vac centrifuge system. Endothelin-1 was quantified by double antibody radioimmunoassay technique. The results were corrected based on the recovery of ET extracted. Endothelin-1 was obtained from Sigma (St. Louis, MO, USA), endothelin-1 antibodies were from Peninsula (St. Helens, UK), [^{125}I]-endothelin-1 was obtained from Amersham International (Buckinghamshire, UK).

Statistical analysis

Values are means±SEM. Paired and unpaired Student's *t*-tests were performed where appropriate. Simple regression analysis was used. A *p*-value <0.05 was considered significant.

Results

Histology and immunochemistry

In intact aortae from normal and cirrhotic rats, endothelial cells were present in hematoxylin-eosin stained sections and in sections stained with the anti-von Willebrand factor. In endothelium-denuded aortae from normal and cirrhotic rats, no endothelial cells were visualized in hematoxylin-eosin stained sections or in sections stained with the anti-von Willebrand factor.

Electrophysiological studies

Study 1. Membrane potential responses to K_{ATP} channel modulators in intact aortae

K_{ATP} channel openers. In normal SMC, membrane potentials were significantly less negative under baseline conditions than during exposure to diazoxide (n=6) or cromakalim (n=6) (Table 1). Change in membrane potential (i.e., membrane potential hyperpolarization) was −3.8±0.7 mV and −10.3±1.1 mV with diazoxide and cromakalim, respectively.

In cirrhotic SMC, membrane potentials were not significantly changed during application of diazoxide (n=5) or cromakalim (n=10) (Table 1). Membrane potential hyperpolarization was −0.0±0.9 mV and −0.4±0.3 mV with diazoxide and cromakalim, respectively.

In SMC in glibenclamide-pretreated normal aortae (n=6), membrane potentials were not significantly different prior to and during exposure to cromakalim

TABLE 1

Membrane potential responses to K_{ATP} channel modulators in smooth muscle cells in intact aortae from normal and cirrhotic rats

	Membrane potential (mv)		
K_{ATP} channel openers	Baseline	Diazoxide (100 μM)	Cromakalim (10 μM)
Normal	−40.8±1.5	−44.7±1.1[a]	
	−43.5±2.6		−53.8±3.3[a]
Cirrhosis	−20.6±1.3[b]	−20.6±1.3	
	−21.3±1.2[b]		−21.0±1.4
K_{ATP} channel blockers	Baseline	Glibenclamide (10 μM)	Tolbutamide (500 μM)
Normal	−42.0±2.8	−35.0±2.7[a]	
	−40.5±2.4		−36.7±2.4[a]
Cirrhosis	−23.0±2.2[b]	−21.9±2.0	
	−20.4±2.1[b]		−19.0±2.4

Values are mean±SEM. [a] $p<0.05$ vs. baseline. [b] $p<0.05$ vs. corresponding normal.

(−30.3±1.7 mV vs −29.7±1.8 mV, respectively). In SMC in glibenclamide-pretreated cirrhotic aortae (n=8), membrane potentials did not significantly differ prior to and during exposure to cromakalim (−22.4±2.5 mV vs −22.1±2.5 mV, respectively).

K_{ATP} channel blockers. In normal SMC, membrane potentials were significantly more negative under baseline conditions than during exposure to glibenclamide (n=7) or tolbutamide (n=6) (Table 1). Changes in membrane potential (i.e., membrane potential depolarization) were 6.5±0.8 mV and 3.2±0.8 mV with glibenclamide and tolbutamide, respectively.

In cirrhotic SMC, membrane potentials were not significantly changed during exposure to glibenclamide (n=7) or tolbutamide (n=5) (Table 1). Membrane potential depolarization was 0.7±0.6 mV and 1.2±0.7 mV with glibenclamide and tolbutamide, respectively.

Study 2. Membrane potential responses to K_{ATP} channel modulators in endothelium-denuded aortae. In SMC in endothelium-denuded normal aortae, significant changes in the resting potential occurred during exposure to diazoxide [from −43.5±2.1 mV to −48.8±2.5 mV (n=4)] or glibenclamide [from −44.8±2.6 mV to −38.4±1.7 mV (n=5)]. Changes in membrane potential due to K_{ATP} channel modulators did not significantly differ between intact and endothelium-denuded aortae.

In SMC in endothelium-denuded cirrhotic aortae, significant changes in the resting potential occurred during exposure to diazoxide [from −29.0±1.2 mV to −32.8±1.7 mV (n=4)] or glibenclamide [from −27.0±1.1 mV to −20.9±2.0 mV (n=5)]. Changes in membrane potential due to K_{ATP} channel modulators

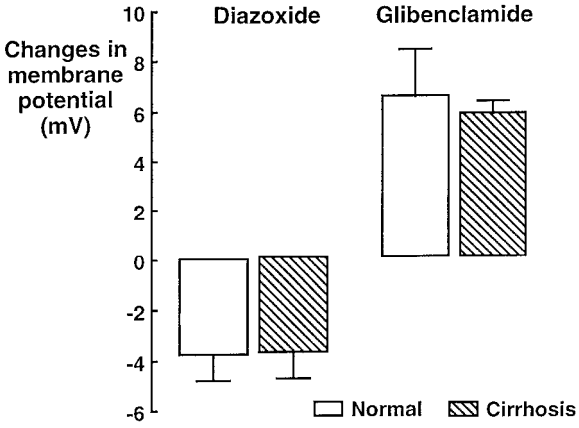

Fig. 2. Membrane potential responses to the K_{ATP} channel opener diazoxide (100 μM) and glibenclamide (10 μM) in SMC (SMC) in normal and cirrhotic aortae pretreated with bosentan (10 μM, a mixed endothelin receptor antagonist). Diazoxide-induced membrane hyperpolarization did not significantly differ between normal and cirrhotic SMC (−3.8±1.0 mV and −3.7±0.9 mV, respectively). Glibenclamide-induced membrane depolarization did not significantly differ between normal and cirrhotic SMC (6.5±1.3 mV and 5.8±0.5 mV, respectively).

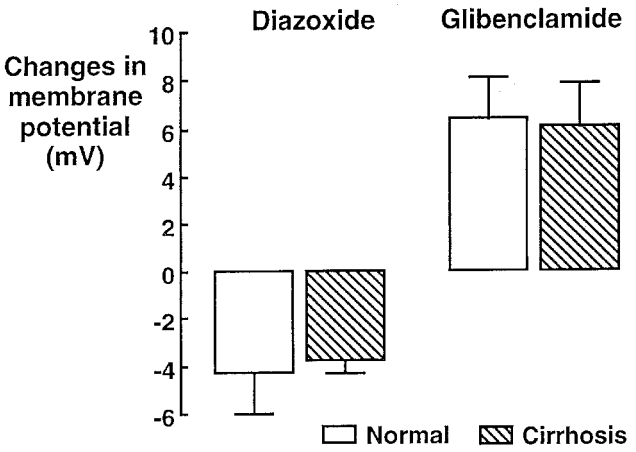

Fig. 1. Membrane potential responses to the K_{ATP} channel opener diazoxide (100 μM) and glibenclamide (10 μM) in SMC (SMC) in endothelium-denuded aortae from normal and cirrhotic rats. Diazoxide-induced membrane hyperpolarization did not significantly differ between normal and cirrhotic SMC (−4.3±1.7 mV and −3.8±0.5 mV, respectively). Glibenclamide-induced membrane depolarization did not significantly differ between normal and cirrhotic SMC (6.4±1.7 mV and 6.1±1.8 mV, respectively).

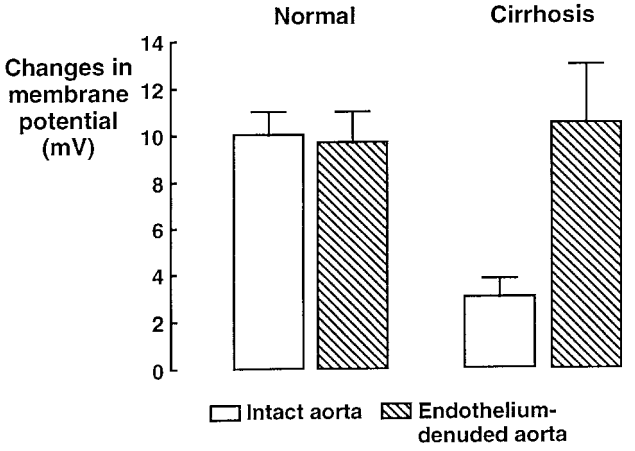

Fig. 3. Membrane potential responses to exogenous endothelin-1 (20 nM) in SMC (SMC) in intact and endothelium-denuded aortae from normal and cirrhotic rats. In intact aortae, endothelin-1-induced membrane depolarization was significantly higher in normal than in cirrhotic SMC (10.0±1.0 mV vs 3.0±0.8 mV). In endothelium-denuded aortae, endothelin-1-induced membrane depolarization did not significantly differ between normal and cirrhotic SMC.

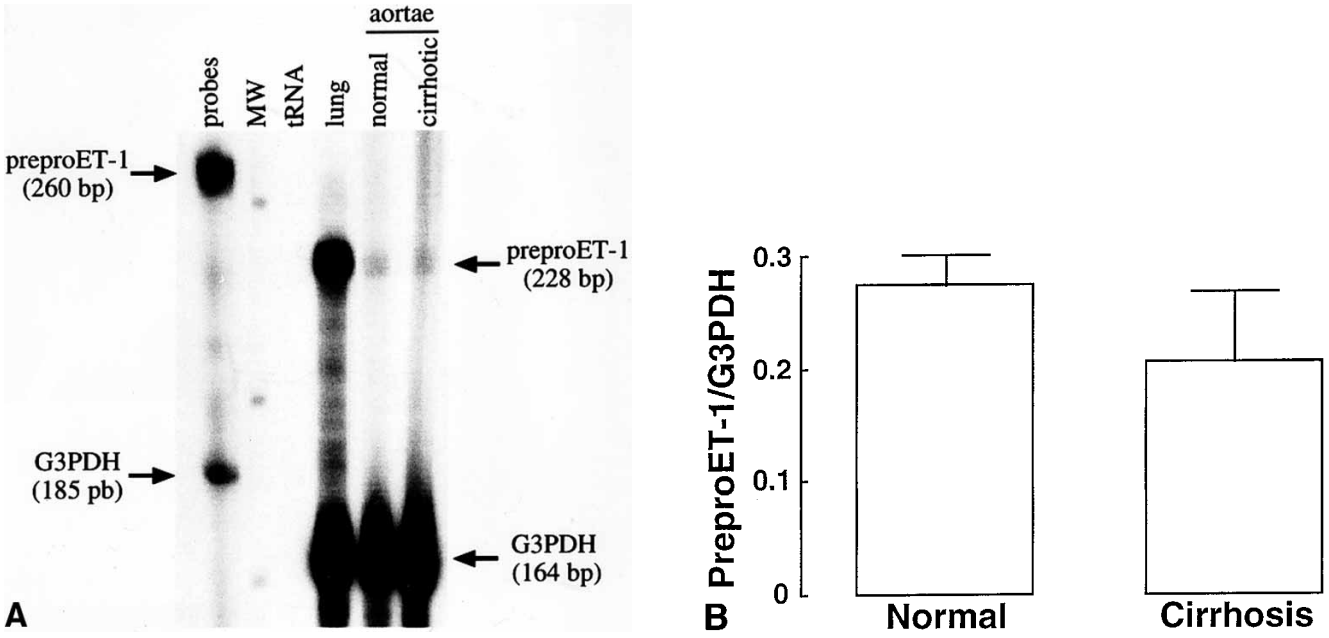

Fig. 4A. Representative autoradiogram of preproendothelin-1 mRNA in intact aortae from normal and cirrhotic rats. Thirty micrograms of total RNA of aortae were subjected to the RNAse protection assay as described in Materials and Methods. Glyceraldehyde-3-phosphate dehydrogenase H (G3PDH) mRNA, which was used as an internal standard, was detected in the same samples by co-hybridization. Yeast tRNA and rat lung mRNA served as negative and positive controls, respectively. B. Specific preproendothelin-1 (prepro-ET-1) mRNA bands quantified by scanner densitometry and normalized to glyceraldehyde-3-phosphate dehydrogenase H (G3PDH) mRNA. Results are the means±SEM of 4 experiments.

were significantly lower in intact than in endothelium-denuded aortae.

In endothelium-denuded aortae, changes in membrane potential due to K_{ATP} channel modulators did not significantly differ between normal and cirrhotic SMC (Fig. 1).

Study 3. Effects of bosentan and exogenous endothelin-1 on membrane potential responses to K_{ATP} channel modulators. In SMC in normal intact aortae pretreated with bosentan, significant changes in the resting potential occurred during exposure to diazoxide [from −44.5±1.9 mV to −48.4±2.2 mV (n=4)] or glibenclamide [from −44.3±2.4 mV to −38.5±1.8 mV (n=5)]. Changes in membrane potential due to K_{ATP} channel modulators did not significantly differ in the absence or presence of bosentan.

In SMC in cirrhotic intact aortae pretreated with bosentan, significant changes in the resting potential occurred during exposure to diazoxide [from −34.5±2.5 mV to −38.3±1.8 mV (n=4)] or glibenclamide [from −31.2±1.2 mV to −25.4±1.1 mV (n=5). Changes in membrane potential due to K_{ATP} channel modulators were significantly lower in the absence than in the presence of bosentan. In aortae pretreated with bosentan, changes in membrane potential due to K_{ATP} channel modulators were not significantly different between normal and cirrhotic SMC (Fig. 2).

In endothelium-denuded normal aortae pretreated with exogenous endothelin-1, the resting potential was −26.7 ±1.4 mV and −26.4±2.0 mV (n=4), prior to and during exposure to diazoxide (p>0.05). In endothelium-denuded cirrhotic aortae pretreated with exogenous endothelin-1, the resting potential was −17.6±1.8 mV and −17.5±1.5 mV (n=4), prior to and during exposure to diazoxide (p>0.05).

In endothelium-denuded normal aortae pretreated with exogenous endothelin-1, the resting potential was −28.0 ±1.5 mV and −26.4±2.2 mV, prior to and during exposure to glibenclamide (p>0.05). In endothelium-denuded cirrhotic aortae pretreated with exogenous endothelin-1, the resting potential was −17.3±1.7 mV and −17.3±1.5 mV, prior to and during exposure to glibenclamide (p>0.05).

Study 4. Effect of endothelium removal, bosentan and exogenous endothelin-1 on the resting membrane potential. Under baseline conditions, i.e., in intact aortae bathed in PSS, the membrane potentials were significantly less negative in cirrhotic than in normal SMC [−22.7±0.7 mV (n=35) vs −44.9±0.8 mV (n=40), respectively].

In SMC in endothelium-denuded aortae, membrane potentials were significantly less negative in cirrhotic rats than in normal rats [−30.2±1.0 mV, (n=36) vs −43.2±1.6, (n=28), respectively]. However, in cirrhotic rats, membrane potentials were significantly less negative in intact aortae than in endothelium-denuded aortae.

In intact aortae from normal rats, the resting potential was −42.8±2.8 mV and −45.3±2.8 mV (n=6), prior to and during exposure to bosentan, respectively (p>0.05). In SMC in intact aortae from cirrhotic rats, bosentan induced a significant change in the resting potential, from −19.8±2.6 mV to −26.6±2.8 mV (n=6). In endothelium-denuded normal aortae, the resting potential was −41.6±2.2 mV and −44.0±2.8 mV, prior to and during exposure to bosentan (p>0.05). In endothelium-denuded cirrhotic aortae, the resting potential was −29.2±1.7 mV and −31.8±2.1 mV, prior to and during exposure to bosentan (p>0.05).

In SMC in intact aortae, endothelin-1 induced a significant membrane potential response in normal rats [from −41.7±2.2 mV to −25.3±3.1 mV (n=3)] and in cirrhotic rats [from −22.0±1.1 mV to −19.0±0.9 mV (n=4)]. Endothelin-1-induced membrane potential depolarization was significantly higher in normal than in cirrhotic aortae (Fig. 3).

In SMC in endothelium-denuded aortae, endothelin-1 induced a significant membrane potential response in normal rats [from −43.7±1.8 mV to −34.0±2.1 mV (n=3)] and in cirrhotic rats [from −33.0±1.5 mV to −22.5±3.8 mV (n=4)]. Endothelin-1-induced membrane potential depolarization did not significantly differ between normal and cirrhotic aortae (Fig. 3).

Quantification of endothelin-1 mRNA in intact aortae

Preproendothelin-1 mRNA was identified in normal and cirrhotic aortae (Fig. 4A). The abundance of preproendothelin-1 mRNA did not significantly differ between normal and cirrhotic aortae (Fig. 4B).

Quantification of endothelin-1 in intact aortae

Aortic wall endothelin-1 concentration did not significantly differ between normal and cirrhotic aortae (2.06±0.24 pg/100 mg dried weight vs 3.65±1.12 pg/100 mg dried weight).

Discussion

This study measured membrane potential responses to K_{ATP} channel modulators (i.e. channel openers and blockers) in SMC in intact and endothelium-denuded aortae from cirrhotic and normal rats.

K_{ATP} channel openers diazoxide and cromakalim induced the expected marked membrane potential hyperpolarization in SMC in normal arteries (3–5), while these substances elicited no significant effect in cirrhotic SMC. On the other hand, K_{ATP} channel blockers, i.e. sulfonylureas glibenclamide and tolbutamide, elicited the expected membrane potential depolarization in normal SMC (3–5), while they had no significant effect on the membrane potential in cirrhotic SMC. Thus, the present study shows that membrane potential responses to K_{ATP} channel modulators are blunted in SMC in intact cirrhotic aortae.

In this study, normal membrane potential responses to K_{ATP} channel modulators were restored in SMC in endothelium-denuded cirrhotic aortae. Moreover, in SMC in intact cirrhotic aortae, the mixed endothelin receptor antagonist bosentan (15) restored normal membrane potential responses to K_{ATP} channel modulators. In addition, the membrane potential responses to K_{ATP} channel modulators were suppressed in endothelium-denuded cirrhotic and normal aortae exposed to exogenous endothelin-1. These findings indicate that blunting of membrane potential responses to K_{ATP} channel modulators in SMC in intact arteries is dependent on the integrity of the endothelium and mediated by the action of endothelin-1.

There are several possible explanations for the endothelin-1-induced blunted membrane potential responses to K_{ATP} channel modulators in cirrhotic SMC. On one hand, endothelin-1 may decrease the affinity between K_{ATP} channel modulators and the channel. On the other hand, in this study endogenous endothelin-1 caused some membrane potential depolarization in cirrhotic SMC under baseline conditions (see below). Since endothelin-1 has been shown to inhibit K_{ATP} channels in normal SMC (9), this mechanism may explain the induction of membrane potential depolarization and the blunting of membrane potential responses to K_{ATP} channel modulators in cirrhotic SMC by endothelin-1.

Another finding in the present study was the role of endothelin-1 in the altered resting membrane potential in cirrhotic SMC. As expected (21), in intact aortae, membrane potentials were significantly more depolarized at rest in cirrhotic than in normal SMC. Endothelium removal led to membrane potential repolarization in SMC from cirrhotic rats. Moreover, in SMC in intact aortae from cirrhotic rats, bosentan partially repolarized membrane potentials to a degree which was similar to that elicited by endothelium removal. In addition, bosentan-evoked membrane potential repolarization was suppressed in SMC in endothelium-denuded aortae from cirrhotic rats. Finally, exposure to exogenous endothelin-1 induced some membrane potential depolarization in SMC in endothelium-denuded

aortae from normal and cirrhotic rats. Together these findings indicate that in cirrhotic SMC, baseline membrane depolarization was partly due to a paracrine action of endothelin-1 produced by endothelial cells. On the other hand, this study shows that, in normal rats, endothelium removal or exposure of intact aortae to bosentan did not significantly change the resting potential. This finding suggests that endothelin-1 does not play a major role in the regulation of the resting potential in normal SMC.

It should be emphasized that, in this study, endothelium removal or exposure of intact arteries to bosentan, induced only a partial membrane repolarization in arterial SMC from cirrhotic rats. In other words, endothelium-independent mechanisms also play a role in cirrhosis-induced membrane SMC depolarization.

This study shows that the endothelin-1 pathway is activated in aortic walls from cirrhotic rats. This activation may be due to increased endothelin-1 production by endothelial cells. However, the abundance of preproendothelin-1 mRNA and endothelin-1 did not significantly differ between cirrhotic and normal aortae. Therefore, mechanisms downstream to the endothelial production of endothelin-1 seem to explain the activation of the endothelin-1 pathway in aortic walls from cirrhotic rats. An upregulation of smooth muscle endothelin receptors has been shown in arteries from portal hypertensive rats (22).

There are three isoforms of endothelins, i.e., endothelin-1, -2 and -3 (23). Endothelin-2 and -3 bind to endothelin receptors which are blocked by bosentan (15). Since the present study did not measure the expression of endothelin-2 or -3, one can speculate that one of these endothelins is overproduced by the endothelium of cirrhotic aortae and causes SMC membrane depolarization. However, this hypothesis is very unlikely because endothelial cells do not produce endothelin-2 or -3 (24). In other words, endothelin-1 is the only endothelin isopeptide produced in endothelial cells (24).

Under *in vivo* conditions, SMC in cirrhotic arteries are exposed to elevated levels of endogenous neurohumoral substances such as norepinephrine, angiotensin II and vasopressin (25). All these neurohumoral substances are known to induce membrane potential depolarization in normal SMC (4). Thus, under *in vivo* conditions, norepinephrine, angiotensin II and vasopressin may induce membrane potential depolarization in cirrhotic SMC. However, it should be kept in mind that the present study was performed under *in vitro* conditions and that under these conditions, the above-mentioned neurohumoral substances are not present. Thus, it is unlikely that norepinephrine, angiotensin II or vasopressin contributes to cirrhosis-induced membrane potential depolarization measured in the present study in cirrhotic SMC.

Nitric oxide has been shown to induce membrane potential hyperpolarization in arterial SMC from normal animals (26). Since there is a sustained endothelial NO overproduction in freshly isolated aortae from cirrhotic rats (27,28), an endothelium-dependent membrane potential hyperpolarization may be expected to occur in cirrhotic SMC. In fact, the present study showed a membrane depolarization, and not hyperpolarization, in resting cirrhotic SMC. Moreover, endothelium removal did not induce a membrane depolarization but a certain degree of membrane potential hyperpolarization in cirrhotic SMC. Taken together, these findings suggest that, in cirrhosis, as yet unidentified mechanisms inhibit NO-induced membrane potential hyperpolarization.

Vascular hyporeactivity to the vasodilator action of K_{ATP} channel openers has been shown in cirrhotic rats (1,2). Membrane potential hyperpolarization is the mechanism by which K_{ATP} channel openers induce vasorelaxation in normal arteries (3–5). Since the present study shows a decrease in K_{ATP} channel opener-elicited membrane potential hyperpolarization, this decrease probably contributes to the cirrhosis-induced hyporeactivity to these substances.

Vascular hyporeactivity to the vasoconstrictor action of exogenous endothelin-1 occurs in cirrhotic rats (29). Membrane potential depolarization is a mechanism by which exogenous endothelin-1 elicits a contraction in normal arteries (25). Since the present study shows a decrease in membrane potential depolarization caused by exogenous endothelin-1, this decrease probably contributes to the cirrhosis-induced hyporeactivity to this substance.

In conclusion, K_{ATP} channel opener-induced membrane potential hyperpolarization and K_{ATP} channel blocker-elicited membrane potential depolarization are blunted in SMC in intact cirrhotic aortae. This blunting is due to the activation of the endothelin-1 pathway in the aortic wall, downstream to the endothelial production of endothelin-1.

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