Sinusoidal endothelial COX-1-derived prostanoids modulate the hepatic vascular tone of cirrhotic rat livers

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Graupera, Mariona, Sandra March, Pablo Engel, Juan Rodés, Jaume Bosch, and Joan-Carles García-Pagán. Sinusoidal endothelial COX-1-derived prostanoids modulate the hepatic vascular tone of cirrhotic rat livers. Am J Physiol Gastrointest Liver Physiol 288: G763–G770, 2005.—Cirrhotic rat liver exhibits a hyperresponse to the α1-adrenergic agonist methoxamine (Mtx) that is associated with enhanced thromboxane A2 (TXA2) production and is abrogated by indomethacin. To further elucidate the molecular mechanisms involved in the hyperresponse to vasoconstrictors, portal perfusion pressure dose-response curves to Mtx were performed in CCl4 cirrhotic rat livers. Impaired vasorelaxation in cirrhotic livers is completely prevented by COX-1, but not by COX-2, inhibition (21), suggesting that, despite increased COX-2 expression in cirrhotic livers, COX-1 is the isoenzyme that modulates increased vascular tone.

Nearly all tissues express COX-1 under normal conditions (10). COX-2 is an inducible enzyme. However, several tissues, including the liver, also express COX-2 constitutively (2, 20, 29). There may be a segregated utilization of COX-1 and COX-2 in prostanoid biosynthesis (28, 34). COX-2 is mainly involved in the immediate prostanoid biosynthesis that occurs within minutes after stimulation with some agonists, such as α1-adrenoceptor stimulation (42), whereas COX-2 is involved predominantly in delayed prostanoid biosynthesis (41). Furthermore, it has been shown that thromboxane synthase (TXA), the terminal enzyme that metabolizes PGH2 to TXA2, preferentially utilizes COX-2-derived PGH2 when limited amounts of arachidonic acid (AA) are supplied, but can efficiently metabolize COX-1-derived PGH2 when AA supply increases (41).

The liver is composed of parenchymal cells, the hepatocytes, and nonparenchymal cells (NPCs), mainly Kupffer cells, hepatic stellate cells (HSC), and sinusoidal endothelial cells (SEC). It has been reported that, in the normal liver, SEC and Kupffer cells are the primary sources of COX-dependent eicosanoid production (43). The current study was aimed at further elucidating the role of COX-derived TXA2 production on the hyperresponse of the hepatic vascular bed to vasoconstrictors, by characterizing which COX isof orm is responsible for the increased synthesis of TXA2. In addition, we also investigated COX-1 and COX-2 protein expression in the hepatic tissue and the specific localization of COX-1 in different parenchymal and NPCs isolated from control and CCl4 cirrhotic rat livers using a double-immunofluorescence staining.

MATERIALS AND METHODS

Induction of Cirrhosis by CCl4

Male Wistar rats weighing 175–200 g underwent inhalation exposure to CCl4 as previously described (3–4, 14). Phenobarbital (0.3 g/l) was added to the drinking water (20, 22). A high yield of micronodular cirrhosis is obtained after ~12–15 wk of CCl4 inhalation. When the cirrhotic rats developed ascites, administration of CCl4 and phenobarbital was stopped, and the perfusion experiments were performed 1 wk later. Control animals received only phenobarbital. The animals were kept in environmentally controlled animal facilities at

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G763
the Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS). All experiments were performed according to the criteria of the Committee for the Care and Use of Laboratory Animals in the Hospital Clinic and IDIBAPS.

**Isolated Perfused Liver System**

A flow-controlled perfusion system was employed in this study, as described previously (22). Livers were perfused with Krebs’ buffer in a recirculating fashion with a total volume of 100 ml at a constant flow rate of 35 ml/min. An ultrasonic flow probe (model T201; Transonic Systems, Ithaca, NY) and a pressure transducer were placed on line, immediately ahead of the portal inlet cannula, to continuously monitor portal flow and perfusion pressure. Another pressure transducer was placed immediately after the thoracic vena cava outlet cannula for measurement of outflow pressure. The flow probe and the two pressure transducers were connected to a PowerLab (4SP) linked to a computer using the Chart version 4.0.1 for Windows software (AD-Instruments, Mountain View, LA). The average portal flow and inflow and outflow pressures were sampled and recorded every second and later exported to data management software to be analyzed.

The perfused rat liver preparation was allowed to stabilize for 30 min before the vasoactive substances were added. The gross appearance of the liver, stable perfusion pressure, bile production over 0.4 μl min⁻¹ g liver⁻¹, and a stable buffer pH (7.4 ± 0.1) were measured during this period (22). If any viability criteria were not satisfied, the experiment was discarded.

**Portal Perfusion Pressure Dose-Response Curve to the α₁-Adrenergic Agonist Mtx**

A portal perfusion pressure-response curve to Mtx was performed by adding increasing doses of Mtx (10⁻⁶, 10⁻⁵, 10⁻⁴, 5 × 10⁻⁴ mol/l) to the reservoir every 5 min in different groups of perfused cirrhotic rat livers preincubated with vehicle (n = 6), the selective COX-1 inhibitor SC-560 (5 μM, n = 6), or the selective COX-2 inhibitor SC-236 (5 μM, n = 6). These doses have been shown to selectively and effectively inhibit COX-1 and COX-2 prostanoid production, respectively (1, 26, 37).

**Measurement of TXB₂**

In 10 cirrhotic livers, 2-ml samples of the perfusate were obtained before SC-560 or SC-236 administration and after the dose response to Mtx. The samples were stored at −80°C. TXB₂ (the end metabolite of TXA₂) was quantified in duplicate using a commercially available enzyme immunossay (20). TXB₂ production was expressed as percent increase over baseline after Mtx administration.

**Western Blot Analysis of COX-1 and COX-2 Protein Expression in Liver Tissue**

Protein expression for COX-1 and COX-2 was assessed in liver samples isolated from six cirrhotic rats with ascites and from seven control animals. Tissue samples were prepared in RIPA lysis buffer and samples isolated from six cirrhotic rats with ascites and from seven control animals. Tissue samples were prepared in RIPA lysis buffer containing (1 μg/ml each) leupeptin, pepstatin A, and aprotinin. Next, aliquots from each sample containing equal amounts of protein (50 μg) were run on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The efficiency of the transfer was visualized by Ponceau staining. The blots were subsequently blocked for 2 h with Tris-buffered saline containing 0.05% (vol/vol) Tween 20 and 5% (wt/vol) nonfat dry milk and probed with mouse anti-COX-1 monoclonal antibody (1:1,000) or rabbit anti-COX-2 polyclonal antibody (1:1,000) for 16 h at 4°C, followed by an incubation with goat anti-mouse (1:10,000) or donkey anti-rabbit (1:10,000) horseradish peroxidase-conjugated secondary antibodies for 2 h at 4°C. After 30 min, immunodetection was performed using the enhanced chemiluminescence blotting detection system (Pierce, Rockford, IL).

**COX-I Immunohistochemistry**

Liver tissue from control and CCl₄ cirrhotic rats was fixed in 2% paraformaldehyde for 1 h at 4°C and cryoprotected overnight in a 30% sucrose solution (wt/vol) at 4°C. Frozen liver sections (8 μm) were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100 in PBS. Liver sections were stained and evaluated for COX-1 protein expression by simple fluorescence immunohistochemistry. Samples were incubated overnight at 4°C with a mouse anti-COX-1 monoclonal antibody (1:100) or with PBS-1% BSA as a negative control. The slides were washed three times with PBS and incubated with a Cy3-labeled goat anti-mouse IgG monoclonal antibody (1:500). All antibodies were diluted in PBS with 1% BSA. Samples were analyzed by confocal microscopy using a ×40 objective (Leica TCS NT, Heidelberg, Germany).

**Lever Cell Isolation**

Parenchymal cell. Control (n = 5) and cirrhotic (n = 5) rats were anesthetized with an intraperitoneal injection of ketamine hydrochloride (100 mg/kg body wt ketalar) and the hepatocytes were isolated by in situ collagenase perfusion through the portal vein according to the method of Munthe-Kaas et al. (33) with minor modifications. Briefly, livers were perfused for 10 min at a flow rate of 20 ml/min at 37°C with Hanks’ balanced salt solution (HBSS) without calcium and magnesium containing 12.5 mmol/l HEPES (pH 7.4), 0.5 mmol/l EGTA, and 2% BSA and then 30 min at a flow rate of 5 ml/min at 37°C with 0.015% collagenase (A type), HBSS containing 12.5 mmol/l HEPES (pH 7.4), and 4 mmol/l CaCl₂. For cell isolations from cirrhotic livers, the concentration of collagenase was increased by 25%. The resultant digested liver was excised, and in vitro digestion was performed at 37°C with 0.01% collagenase (A type) HBSS containing 12.5 mmol/l HEPES (pH 7.4) and 4 mmol/l CaCl₂ for 10 min. The cells were washed through nylon filters (100 μm; Becton-Dickinson Labware, Franklin Lakes, NJ), collected in cold Krebs buffer, and centrifuged at 50 g for 3 min. The obtained pellet contains the hepatocytes, whereas the supernatant is enriched in NPCs. Then the hepatocytes were washed two times in cold Krebs buffer.

NPC. Supernatants containing NPCs (i.e., Kupffer cells, HSC, and SEC) were obtained from control and cirrhotic rat livers. Briefly, after collagenase perfusion, the NPC-enriched supernatant was centrifuged at 800 g for 10 min at 4°C, and the obtained pellet was resuspended in Dulbecco’s PBS (DPBS) and centrifuged at 800 g for 20 min through a 25–50% Percoll gradient at room temperature. The interface of the gradient containing Kupffer cells and SEC was seeded on 37°C, collagenase culture plates and incubated at 37°C for 1 h. Cell monolayers adhered to the dishes were characterized as Kupffer cells and were collected in cold PBS. The nonattached cells are enriched in SEC.

**COX-1 Expression by Flow Cytometry Analysis**

The COX-1 expression in the obtained enriched population of hepatocytes, Kupffer cells, and SEC was analyzed by double-immunofluorescence staining using Zenon mouse IgG labeling kits (IgG₁ FITC or IgG₂b, biotin). Double-immunofluorescence technique does allow the quantitative analysis of the expression of specific molecules in heterogeneous cell population without requiring the purification of individual cell populations or subpopulations (13, 31). Thus, with double-immunofluorescence staining, we are able to identify and define endothelial and Kupffer cells populations with specific markers in a heterogeneous cell suspension, and then the COX-1 protein expression was determined in the population positive for these markers.

Each cell population was identified using a specific cell marker [asialoglycoprotein (ASGRP) for hepatocytes, ED2 for Kupffer cells, and rat endothelial cell antigen (RECA)-1 for SEC]. Cells were fixed in 2% paraformaldehyde for 20 min at 4°C and permeabilized in 0.2%...
Triton X-100 in PBS. In all cases, parallel isotype control experiments were performed. Hepatocytes were stained by double immunofluorescence with a biotinylated anti-COX-1 (1:100) and a rabbit anti-ASGRP polyclonal antibody (1:100) for 30 min at 4°C. After being washed, the hepatocytes were treated with FITC-labeled donkey F(ab′)2 anti-rabbit IgG heavy and light chain [IgG(H+L)] (1:200) and with streptavidin-Cy-chrome (1:100). Hepatocyte acquisition was performed at a logarithmic scale, and the hepatocyte population was identified using anti-ASGRP antibody (7). Kupffer cells were studied by double immunofluorescence with a biotinylated anti-COX-1 (1:100) and anti-ED2-FITC (1:100). After being washed, Kupffer cells were treated with streptavidin-Cy-chrome (1:100). Kupffer cell acquisition was performed at a logarithmic scale, and the Kupffer cells population was identified using anti-ED2 antibody (15). SEC were studied by double immunofluorescence with a biotinylated anti-COX-1 (1:100) and anti-RECA-1-FITC (1:100). After being washed, SEC were treated with streptavidin-Cy-chrome (1:100). SEC acquisition was performed at a logarithmic scale, and the SEC population was identified using anti-RECA-1 antibody (16). Fluorescence was analyzed using a FACSCalibur (Becton-Dickinson Immunocytometry Systems, San Jose, CA) equipped with CellQuest software to detect FITC (FL1) and Cy-chrome (FL3) fluorescence [mean fluorescence intensity (MFI)]. At least 4,000 cells positive for each population were analyzed. MFI indicate the relative amount of protein expression, and the negative control was set at 5 MFI.

**Isolation and Culture of HSC**

HSC were isolated from control (n = 2) and cirrhotic (n = 2) rat livers. Briefly, livers were perfused for 10 min at a flow rate of 20 ml/min at 37°C with Gey’s balanced salt solution (GBSS) with 0.6% heparin and then 30 min at a flow rate of 5 ml/min at 37°C with 0.015% collagenase (A type), 0.005% DNase, and 0.16% pronase GBSS. For cell isolations from cirrhotic animals, the concentration of collagenase was increased by 25%. The resultant digested liver was excised, and in vitro digestion was performed at 37°C with 0.01% collagenase, 0.01% DNase, and 0.004% pronase GBSS. The cells were passed through nylon filters (100 μm) and centrifuged at 50 g for 3 min. The supernatant was centrifuged at 800 g for 5 min, and the pellet was then washed twice with RPMI medium. Cells were grown in Iscove’s modified Dulbecco’s medium, and experiments were performed (at 3–5 days after isolation; see Ref. 5). Cells showed a typical autofluorescence because of vitamin A-rich fat droplets and were highly viable and 90% pure. To further confirm that these cells were actually HSC, we performed oil red staining that confirmed the presence of the characteristic lipid droplets of HSC (19).

**Oil Red Staining**

Cells after isolation were plated on coverslips; after 3 days in culture, cells were fixed in 4% paraformaldehyde (for 10 min at 4°C). Subsequently, cells were washed two times with PBS and two times with isopropanol alcohol (60%) and incubated 1 h and 30 min with oil red O (Sigma). Thereafter, cells were differentiated with 60% isopropanol alcohol and mounted using an aquos medium.

**COX-1 Western Blot Analysis in HSC**

Protein expression for COX-1 in HSC isolated from two cirrhotic rats with ascites and two control rat livers was assessed by Western blot following the same methodology as for the liver tissue samples. COX-1 expression was not assessed in these cells by flow cytometry because of the lack of good markers for identifying the HSC population. Markers like glial fibrillary acidic protein or desmin, frequently used by most investigators, fail to stain a subpopulation of HSC in normal rat liver (3, 35). Moreover, desmin also stains rat liver myofibroblast (9, 27).

**Drugs and Reagents**

TXB2 enzyme immunossay kits, SC-560, mouse anti-COX-1 monoclonal antibody, and rabbit anti-COX-2 polyclonal antibody were obtained from Cayman Chemical (Ann Arbor, MI). Mtx, GBSS, DPBS, goat anti-mouse and donkey anti-rabbit horseradish peroxidase-conjugated secondary antibodies, leupeptin, pepstatin A, aprotnin, and other chemical reagents were purchased from Sigma (St. Louis, MO). Mouse anti-ED2 monoclonal antibody and mouse anti-RECA-1 monoclonal antibody were from Serotec (Oxford, UK). Cy3-labeled goat-anti-mouse IgG monoclonal antibody and FITC-labeled donkey F(ab′)2 anti-rabbit IgG(H+L) were from Jackson ImmunoResearch Laboratories. Percoll was from Amersham Biosciences (Uppsala, Sweden). Collagenase A, pronase, and DNase were from Roche Diagnostics (Mannheim, Germany). Zenon mouse IgG labeling kits (IgG1 FITC or IgG2a, biotin) were from Molecular Probes (Leiden, The Netherlands). Iscove’s modified Dulbecco’s medium and RPMI medium were from Life Technologies (Paisley, UK). Streptavidin-Cy-chrome and nylon filters (100 μm) were obtained from Becton-Dickinson Labware.

**Statistical Analysis**

Statistical analysis was performed using the StatView package of statistical programs. The unpaired Student’s t-test and ANOVA were used as adequate. All data are reported as means ± SE. Differences were considered significant at a P value < 0.05.

**RESULTS**

**Effect of COX-1 and COX-2 Inhibition on the Portal Perfusion Pressure Response to Mtx**

Preincubation with SC-560, a COX-1 selective inhibitor, significantly blunted the hyperresponse to Mtx observed in cirrhotic rat livers (Fig. 1A). In contrast, no significant changes in the response to Mtx were observed after preincubation with the selective COX-2 inhibitor SC-236 (Fig. 1B).

**TXB2 production**

SC-560 preincubation, but not SC-236, prevented the increase in TXB2 observed after Mtx (Fig. 2). Indeed, TXB2 production after preincubation with SC-236 was similar to that found in our laboratory in cirrhotic rat livers preincubated with vehicle (data not shown; see Ref. 21).

**COX-1 and COX-2 Expression**

COX-1 and COX-2 protein expression was detected by Western blot and immunohistochemistry analysis in liver tissue collected from control and cirrhotic rats. There were no significant differences in COX-1 protein expression, but COX-2 expression was consistently upregulated in cirrhotic livers (data not shown). Immunohistochemistry for COX-1 also showed a similar number of positive cells in control and cirrhotic rat livers, which were mainly located along the sinusoids (Fig. 3). The pattern of staining was compatible either with Kupffer cells, SEC, or HSC.

**COX-1 Protein Expression in Different Hepatic Cell Types**

Double immunofluorescence staining of each population of hepatocytes, Kupffer cells, and SEC using flow cytometry showed COX-1 expression in Kupffer cells and in SEC from control and cirrhotic livers but not in hepatocytes (Fig. 4). More than 70% of ED2 positive cells (Kupffer cells) expressed...
COX-1, without significant differences between control and cirrhotic rat livers (Fig. 5A). Furthermore, the intensity of expression was also similar (Fig. 5B). In contrast, COX-1 was expressed in > 50% of cirrhotic RECA-1-positive cells (SEC) vs. < 20% in RECA-1-positive cells (control SEC; \( P = 0.003 \); Fig. 5C). In addition, COX-1 expression had a greater (almost double) intensity in cirrhotic than in control RECA-1-positive cells (Fig. 5D).

HSC isolated from control and cirrhotic rat livers were highly viable and 90% pure (Fig. 6). A downregulation in COX-1 expression was observed in HSC isolated from cirrhotic rat livers compared with control livers (Fig. 7).

**DISCUSSION**

Cirrhotic livers exhibit an exaggerated response to vasoconstrictors (4, 22, 23, 38) that is one of the factors increasing hepatic vascular resistance and therefore aggravating portal hypertension. Recent experiments from our group have shown that COX-derived vasoconstrictor prostanoids modulate the response to Mtx in cirrhotic rat livers. This is based on the observation that indomethacin, a nonselective COX inhibitor, completely abolished the hyperesponse to Mtx of cirrhotic livers (20). Furthermore, the finding of increased production of TXA2 after Mtx administration, of increased expression of TXA2 synthase, and the observation that the selective TXA2 receptor blocker SQ-29548 corrects the hyperesponse to Mtx of cirrhotic rat livers and completely prevented the increased production of TXA2. These findings were not seen when the selective COX-2 inhibitor SC-236 was used. A previous study from our group showed that a COX-1-dependent increase in TXA2 was the main factor mediating the impaired vasorelaxation to ACh of cirrhotic rat livers (21). Taken together, our

Fig. 1. Portal perfusion pressure (PP) dose-response curve to methoxamine (Mtx) in cirrhotic rat livers preincubated with the selective inhibitor of cyclooxygenase (COX)-1 SC-560 (A) and the COX-2 inhibitor SC-236 (B) compared with those preincubated with vehicle. \( \Delta \), Change.

Fig. 2. Thromboxane (TX) B2 production after Mtx in perfused CCl4 cirrhotic rat livers preincubated with SC-560 or SC-236.

Fig. 3. COX-1 staining of liver sections from control (A and B) and CCl4 cirrhotic (C and D) rats. Sections were incubated with either PBS/1% BSA as negative control (A and C) or anti-COX-1 antiserum (B and D). COX-1-positive cells, in red (arrow), were widespread along the sinusoids.
current results and the results of our previous study (21) suggest that prostanoids, and more specifically TXA2, are important modulators of two of the pathophysiological mechanisms thought to play a major role in the increased vascular resistance of cirrhosis, the impaired response to endothelium-dependent vasodilators, and the hyperresponse to vasoconstrictors.

As expected from the findings of our preceding study (20), COX-1 protein expression was found to be unchanged, whereas COX-2 protein expression was upregulated in cirrhotic compared with control rat livers. This is in accordance with previously published data also showing increased expression of COX-2 in the liver of rats with portal hypertension induced by bile duct ligation (43). Unexpectedly, however, the present study confirms that, in cirrhotic livers, the synthesis of TXA2 is mainly the result of COX-1 and not COX-2 (21). Thus the physiological significance in the liver of prostaglandins derived from COX-2 remains to be determined, both in normal conditions and cirrhosis.

COX-1 immunohistochemical studies showed widespread staining for this protein along the sinusoid, suggesting that the positively labeled cells may be Kupffer cells, SEC, or HSC. This was confirmed by liver cell isolation studies where COX-1 expression was detected in Kupffer cells, SEC, and HSC. This is in accordance with previous data suggesting that Kupffer cells are major producers of prostanoids within the liver (14). However, the number of Kupffer cells that expressed COX-1 and the amount of COX-1 protein per cell were not different between control and cirrhotic livers, suggesting that Kupffer cells were not mainly responsible for the increased response to Mtx and TXA2 production observed in cirrhotic rat livers. Furthermore, it has been observed that Kupffer cells do not express α1-adrenergic receptors, so it is unlikely that they can be stimulated by Mtx (11). HSC isolated from control and cirrhotic rat livers expressed COX-1. These findings are in accordance with previous studies showing constitutive expression of COX-1 in human hepatic myofibroblasts (12). However, HSC from cirrhotic livers have a significant lower expression of COX-1 than HSC from control livers, making it unlikely that HSC are responsible for the increased production of COX-1-derived vasoconstrictive prostanoids after Mtx. In conditions of chronic liver damage, HSC undergo a process of activation from the quiescent storing phenotype to the highly proliferative myofibroblast-like phenotype (36), which results with remarkable proliferative, synthetic, and contractile properties. In this situation, HSC may still be involved in the regulation of the response to Mtx in cirrhotic rat livers, more likely as a TXA2-sensitive cells than a TXA2 source, since it has been shown that TXA2 produce a marked contraction of cultured HSC (36). Furthermore, activated HSC exhibit an overexpression of COX-2, which plays an important role in the regulation of monocyte chemoattractant protein-1, a potent chemoattractant for monocytes, lym-

Fig. 4. Two-color cytometric analysis of COX-1 expression on hepatocytes, Kupffer cells, and sinusoidal endothelial cells (SEC). Isolated hepatocytes from control (A) and cirrhotic (B) liver were stained with biotinylated COX-1 monoclonal antibody (mAb) and asyaloglycoprotein (ASGRP) antiserum, washed, and incubated with avidin streptavidin-Cy-chrome and FITC-labeled donkey anti-rabbit antibody. Isolated Kupffer cells from control (C) and cirrhotic (D) liver were stained with biotinylated COX-1 mAb and ED2-FITC, washed, and incubated with streptavidin-Cy-chrome. Isolated SEC from control (E) and cirrhotic (F) liver were stained with biotinylated COX-1 mAb and rat endothelial cell antigen (RECA)-FITC. Nos. indicate the percentage of positive cells. FL1, green fluorescence; FL3, red fluorescence.
phocytes, and mesenchymal cells (17). All these data suggest that, in activated HSC, prostaglandins are mainly derived by COX-2 and play an important role regulating leukocyte infiltration rather than vascular tone. In this situation, we can speculate that COX-2 upregulation might be playing a role in COX-1 downregulation.

A low proportion of SEC from control rat livers constitutively express COX-1. Interestingly, the number of SEC from cirrhotic rat livers that were positive for COX-1 was significantly greater (almost 3-fold) compared with control livers. In addition, the intensity of COX-1 expression in cirrhotic SEC was double that in controls. High flow rate has been shown to upregulate COX-1 expression in cultured vascular endothelial cells. Interestingly, this effect was associated with a downregulation in COX-1 expression in cocultured smooth muscle cells. This last phenomenon was dependent on prostaglandin synthesis, suggesting therefore that an endothelial COX-1-derived product may negatively regulate smooth muscle cell COX-1 (25). We can speculate that the high blood pressure and shear stress in the sinusoid may promote COX-1 overexpression on SEC. Therefore, an increase in endothelial COX-1-derived products may lead to downregulation of HSC COX-1.

Fig. 5. COX-1 expression in Kupffer cells (A and B) and SEC (C and D) by flow cytometry. The Kupffer cells were identified with mAb anti-ED2 (15), and SEC were identified with mAb anti-RECA-1 (16). ED2-positive cells were gated for the analysis of COX-1 expression on enriched Kupffer cell preparation. The %positive ED2 cells expressing COX-1 is shown in A. The mean fluorescence intensity (MFI) of the ED2-positive gated cells expressing COX-1 is shown in B. RECA-1-positive cells were gated for the analysis of COX-1 expression on enriched SEC preparation. The %positive RECA-1 cells expressing COX-1 is shown in C. The MFI of the SEC-positive gated cells expressing COX-1 is shown in D. Filled bars, control rat livers (CT); open bars, cirrhotic rat livers (CH). NS, not significant.

Fig. 6. Oil red staining of hepatic stellate cells in culture. Small lipid droplets staining with the oil red O were visualized inside the cells by phase contrast microscopy.

Fig. 7. COX-1 hepatic stellate cell (HSC) protein expression. Top: Western blot analysis of COX-1 (relative molecular mass 70 kDa) in HSC samples from two control (CT1 and CT2) and 2 cirrhotic rats with ascites (CH1 and CH2). HSC protein extracts were electrophoresed and probed with specific anti-COX-1 antibodies. Bottom: COX-1 band intensities were determined by scanning densitometry.
Recent studies have suggested that endothelial cells express α₁-adrenoceptors (39, 40). It has been shown that the activation of G protein-coupled receptors, such as those for α₁-adrenergic agonists, vasopressin, or endothelin 1, stimulates release of AA by activating phospholipase A₂, leading to the formation of its vasoactive-derived metabolites (42). We hypothesize that activation of this α₁-adrenoceptor by Mtx may promote the release of AA from membrane phospholipids, which, in a setting where COX-1 is overexpressed, may result in an enhanced production of vasoconstrictor-derived prostanooids, in particular PGH₂. The hyperresponse to vasoconstrictors is a phenomena that extends to different vasoconstrictors. Therefore, although we have not tested other vasoconstrictors, we think that the phenomena may apply to all vasoconstrictors sharing G protein-coupled receptors linked to phospholipase A₂ activation.

Previous studies from our group showed that macrophages infiltrating CCl₄ cirrhotic rat liver overexpress TXA₂ synthase (20), the enzyme responsible for the synthesis of TXA₂ from its precursor PGH₂. Thus we cannot rule out the possibility of transcellular production of TXA₂ within the vessel wall (8).

The endothelium responds to chemical and hormone signals by releasing mediators that modulate the response of vascular smooth muscle cells, platelets, and leukocytes. Endothelial dysfunction may be defined as an imbalance between the synthesis, release, or effect of these endothelial factors. Taking into account all this information, we hypothesize that the increased expression of COX-1 in SEC from cirrhotic livers may be another hitherto unrecognized factor contributing to the endothelial dysfunction observed in cirrhosis (21, 24). This concept is further supported by the fact that overexpression of COX-1 in endothelial cells is not unique to the cirrhotic livers, since it has been also observed in other vascular beds in circumstances where there is endothelial dysfunction (18, 32).

In conclusion, the present study provides evidence that the hyperresponse to Mtx of cirrhotic rat liver appears to be mainly mediated by COX-1-derived TXA₂. SEC from cirrhotic livers showed increased expression of COX-1, suggesting that these cells may be responsible for the increased availability of PGH₂ and consequently for the increased synthesis of TXA₂ after Mtx stimulation. These abnormalities represent a new mechanism contributing to portal hypertension in cirrhosis and may establish the rationale to explore and develop new therapeutic strategies for this condition.

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GRANTS

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