Endothelial COX-1 and -2 differentially affect reactivity of MVB in portal hypertensive rats

M. A. POTENZA,1 O. A. BOTRUGNO,1 M. A. DE SALVIA,1 G. LERRO,1 C. NACCI,1
F. L. MARASCIULO,1 R. ANDRIANTSITOHAINA,2 AND D. MITOLO-CHIEPPA1
1Section of Pharmacology, Department of Pharmacology and Human Physiology, Medical
School, University of Bari, 70124 Bari, Italy; and 2Faculté de Pharmacie, Laboratoire de
Pharmacologie et Physicochimie des Interactions Cellulaires et Moléculaires, Unité Mixte de
Recherche Centre National de la Recherche Scientifique, 67401 Illkirch Cedex, France

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Potenza, M. A., O. A. Botrugno, M. A. De Salvia, G. Lerro, C. Nacci, F. L. Marasciulo, R. Andriantsitohaina, and D. Mitolo-Chieppa. Endothelial COX-1 and -2 differentially affect reactivity of MVB in portal hypertensive rats. Am J Physiol Gastrointest Liver Physiol 283: G587–G594, 2002. First published April 24, 2002; 10.1152/ajpgi.00391.2001.—Expression of constitutive and inducible cyclooxygenase (COX-1 and COX-2, respectively) and the role of prostanoids were investigated in the aorta and mesenteric vascular bed (MVB) from the portal vein-ligated (PVL) as a model of portal hypertension. Functional experiments were carried out in MVB from PVL and sham-operated rats in the absence or presence of the nonselective COX inhibitor indomethacin or the selective inhibitors of COX-1 (SC-560) or COX-2 (NS-398). Western blots of COX-1 and COX-2 proteins were evaluated in aorta and MVB, and PGI2 production by enzyme immunoassay of 6-keto-PGF1α was evaluated in the aorta. In the presence of functional endothelium, decreased contraction to norepinephrine (NE) and increased vasodilatation to ACh were observed in MVB from PVL. Exposure of MVB to indomethacin, SC-560, or NS-398 reversed the hyporeactivity to NE and the increased endothelial vasodilatation to ACh in PVL, with NS-398 being more potent than the other two inhibitors. Uregulation of COX-1 and COX-2 expressions was detected in aorta and MVB from PVL portal hypertensive rats, and increased production of 6-keto-PGF1α was observed in aorta from portal hypertensive rats. These results suggest that generation of endothelial vasodilator prostanoids, from COX-1 and COX-2 isofoms, accounts for the increased mesenteric blood flow in portal hypertension.

vasodilator prostanoids; vascular hyporeactivity; resistance vessels; cyclooxygenase; mesenteric vascular bed; portal vein-ligated

HIGH CARDIAC OUTPUT AND LOW peripheral resistance are the hemodynamic features associated with portal hypertension. An increased vasodilatation is one of the causes of this phenomenon rather than primary heart failure, but the biological mechanisms involved in this low peripheral resistance are still not clearly understood. Several indirect evidences have suggested that the hyporesponsiveness to endogenous vasoconstrictors and the pathogenesis of peripheral vasodilatation observed in portal hypertension may involve excessive release of vasodilator factors such as nitric oxide (NO; see Refs. 4 and 13) and prostacyclin (PGI2; see Ref. 12).

With respect to NO, its involvement has been characterized by the partial reversal of portal hypertension-induced hyporeactivity to vasoconstrictors with the use of NO synthase inhibitor (10, 16, 17). Because the blockade of NO synthase activity was not able to completely reverse the hyperdynamic circulation during portal hypertension (9, 29), the implication of vasodilators other than NO has been advanced. In this regard an increased level of 6-keto-PGF1α, a stable metabolite of PGI2, has been found in portal hypertensive animals (21, 24) and patients with chronic liver disease (3, 6, 19). Also, administration of the cyclooxygenase (COX) inhibitor indomethacin may attenuate the hyperdynamic circulation (3, 15, 19, 23) and the vascular hyporesponsiveness to vasoconstrictors (22, 30) associated with this pathological dysfunction (2).

However, the enzyme isoforms involved in the production of NO or PGI2 and the cellular origin of these vasodilator substances in the vessel wall are still not fully known. Both NO and PGI2 release could be physiologically stimulated by shear stress provoked by blood flow in the circulation through endothelial NO synthase and constitutive COX (COX-1) enzymes, respectively (7). In inflammatory diseases with enhanced cytokine production, large amounts of vasodilatory factors can be released and can elicit peripheral resistance lowering (25). These factors include not only NO from inducible NO synthase but also vasodilatory prostaglandins from inducible COX (COX-2).

In the present study, we hypothesized that, besides NO, both COX-1 and COX-2 might be involved in the increased vasodilatation observed in portal vein-ligated (PVL) operated rats as a model of portal hypertension. The study was carried out in the mesenteric...
vascular bed (MVB), which contains small arteries that participate actively in the regulation of peripheral resistance and thus blood pressure. First, the role of both COX-1 and COX-2 in the hyporeactivity to vasoconstrictor agonist and in regulating endothelium-dependent vasodilatation was assessed in MVB from PVL rats. Second, whether COX-1 and COX-2 expressions and the associated PGI2 release are actually enhanced in the vascular wall were also investigated during this pathology.

MATERIALS AND METHODS

Animals. The studies were performed on male Sprague-Dawley rats (Harlan) weighing 250–300 g. This investigation conforms to the authorization for the use of laboratory animals given by the Italian government (Ministry of Health). In all experiments, anesthesia was induced with pentobarbital sodium salt (35 mg/kg body wt ip).

Induction of portal hypertension. Portal hypertension was induced by portal vein constriction, as previously described (20). Briefly, the portal vein was isolated, and a calibrated constriction was performed using a single ligature of 3-0 silk around the portal vein and a 20-gauge blunt-tipped needle. The needle was then removed, leaving a calibrated stenosis of the portal vein. In the control group, the same operation was performed with the exception that, after isolating the portal vein, no ligature was placed. After the operation, the animals were housed in plastic cages and allowed free access to food until they were used. All experiments were performed 18–21 days after surgery, when hyperdynamic circulation accompanying portal hypertension is fully established (20).

In vitro perfusion system. The animals were heparinized (200 IU ip) and then decapitated and exsanguinated. The MVB was prepared and perfused as described by McGregor (11). The MVB was perfused via the superior mesenteric artery with a modified Krebs-Henseleit solution of the composition (in mM): 113 NaCl, 4.8 KCl, 1.2 MgSO4, 1.2 NaH2PO4, 1.2 CaCl2, 25 NaHCO3, and 5.5 glucose and was mounted in a perfusion system. Flow rate was adjusted at 5 ml/min with modified Krebs-Henseleit solution maintained at 37°C and continuously gassed with 95% O2-5% CO2 (pH 7.4) by means of a peristaltic pump (101-Ismatec). The preparation was also superfused with modified Krebs-Henseleit solution at the rate of 0.5 ml/min to prevent drying. Drug solutions were infused for 30 s directly in the perfusate.
proximal to the arterial cannula, using another peristaltic pump. After an equilibration period of 30–40 min, changes in perfusion pressure were measured using a pressure transducer (Isotec) and recorded continuously on a polygraph (type 3310; Graph-te-Watanabe). The perfusion pressure remained constant during the equilibration period, and it was taken as the basal value to assess the changes induced by different agents. In some experiments, the endothelial layer was removed by intraluminal perfusion with 0.5% 3-[3-cholamidopropyl]dimethylammonio]-1 propane sulfonate in Krebs-Henseleit for 25 s followed by repeated washing with Krebs-Henseleit. The presence of functional endothelium was assessed by the ability of ACh (1 μM) to induce >50% relaxation of vessels precontracted with norepinephrine (NE). The absence of a relaxation response to ACh was taken as evidence that the MVB was functionally denuded of the endothelium.

**Experimental procedure.** For contraction experiments, concentration-response curves to NE were constructed by infusion of MVB with or without functional endothelium by increasing the concentration of the agonist (0.02–200 μM) in a noncumulative manner. MVB was perfused for 30 s by the agonist to reach a steady-state response. For relaxation experiments, MVB with or without functional endothelium were contracted at 80% of their maximal contraction with NE. The concentration of NE was adjusted for each preparation to obtain the same level of precontraction in MVB from either control or PVL rats in the absence or presence of pharmacological inhibitors. When the contraction reached a plateau, the addition of an increasing concentration of ACh (1 nM to 5 μM) was performed to construct concentration-response curves to the vasodilator agent.

To investigate the involvement of COX, concentration-response curves to NE and to ACh were repeated after 30 min of preincubation of MVB with the nonselective COX inhibitor indomethacin (10 μM), the selective COX-1 inhibitor 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole (SC-560; 10 nM), or the selective COX-2 inhibitor N-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide (NS-398; 10 μM).

**Western blotting with anti-COX-1 and anti-COX-2 antibodies.** Thoracic aortic or MVB preparations were homogenized, and ~50 μg total protein from supernatant fractions were loaded in 10% SDS-polyacrylamide gels to separate COX-1 and COX-2 proteins, respectively. After electrophoresis, proteins were transferred to a nitrocellulose membrane. Immunostaining of COX-1 and COX-2 was achieved using specific monoclonal mouse anti-COX-1 (Cayman Chemical) and anti-COX-2 (Transduction Laboratories) antibodies and reacted with peroxidase-conjugated antimouse antibody (Transduction Laboratories). The housekeeping COX-1 positive control was obtained from ram seminal vesicles (Cayman Chemical), whereas COX-2 positive control was obtained from mouse macrophage lysate (Transduction Laboratories). The blots were detected using an enhanced chemiluminescence assay (Amersham) and were evaluated by densitometry.

**Prostanoid production.** To determine the production of 6-keto-PGF1α, (a stable product of PGI2), thoracic aorta with endothelium were removed from each rat, cleaned of connective tissue, and then placed in a petri dish that contained 1 ml modified Krebs-Henseleit solution for 20 min at 37°C in a 95% O2-5% CO2 incubator. At the end of this period, NE (0.3 μM) was added for 10 min followed by ACh (1 μM) for 2 min. Next, the medium was collected, and 6-keto-PGF1α, was measured by enzyme immunoassay (EIA) kits according to the manufacturer’s protocol (Cayman Chemical). The concentration of 6-keto-PGF1α, is expressed as picogram per milligram tissue wet weight.

**Drugs.** NE, ACh, and indomethacin were purchased from Sigma-Aldrich (Milan, Italy). NS-398 was obtained from Alexis Biochemical (Firenze, Italy). SC-560 and the 6-keto-PGF1α, EIA kit were from Cayman Chemical (Sfi Bio, Massy, France).

Stock solutions of each drug were prepared in distilled water except for SC-560, which was dissolved in dimethyl sulfoxide, and indomethacin, which was dissolved in distilled water containing 4% NaHCO3 and sonicated before use. Final dilutions for all drugs were prepared in modified Krebs-Henseleit solution immediately before use.

**Statistical analysis.** Contractile responses to NE were expressed as the change in the increase in perfusion pressure (mmHg). Sensitivities to NE are expressed as pD2 values, where pD2 is the negative log EC50 (the molar concentration of NE that produces 50% of the maximal response, calculated in each MVB preparation separately by logit-log regression).

### Table 1. Emax and pD2 values for norepinephrine-induced contraction in MVB from sham-operated and PVL rats in control conditions and after preincubation with Indo (10 μM) or NS-398 (10 μM)

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<tr>
<td>Indo</td>
<td>9</td>
<td>226.2 ± 12.1</td>
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<td>NS-398</td>
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<td>231.1 ± 10.6</td>
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Results are means ± SE for 7 or 9 experiments (n). For control conditions, experiments were done with (+E) or without (−E) endothelium. PVL, portal vein-ligated; Emax, maximal effect; Indo, indomethacin; MVB, mesenteric vascular bed. *P < 0.05 vs. sham control (+E) values. †P < 0.05 vs. PVL control (+E) values. §P < 0.05 vs. NS-398 sham values.

### Table 2. Emax and pD2 values for norepinephrine-induced contraction in MVB from sham-operated and PVL rats in control conditions and after preincubation with SC-560 (10 nM)

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<td>SC-560</td>
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<td>238.2 ± 10.1</td>
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Results are shown as means ± SE. For control conditions, experiments were done with or without endothelium. *P < 0.01 vs. sham control (+E) values. †P < 0.05 vs. sham control (+E) values.
Figs. 1 and 2. The corresponding maximal effect and sure reached after each addition of NE are shown in curves describing the mean plateau increase in pres-

After addition of each concentration of NE, perfusion pressure rapidly increased to a stable plateau level in MVB from sham or PVL rats. The concentration-effect curves describing the mean plateau increase in pressure reached after each addition of NE are shown in Figs. 1 and 2. The corresponding maximal effect and pD2 values are given in Tables 1 and 2. In MVB with functional endothelium, the NE-induced increase in perfusion pressure was significantly decreased in PVL compared with sham rats (Fig. 1). This decrease resulted in a reduced maximal effect without any change in pD2 values of the agonist. By contrast, after removal of functional endothelium, no difference in the increases in pressure produced by NE was observed between MVB from sham or PVL rats (Tables 1 and 2). Thus the following experiments were performed on MVB with functional endothelium only. Neither the nonselective COX inhibitor indomethacin (10 \mu M) nor the selective COX-2 inhibitor NS-398 (10 \mu M) had a significant effect on contractile responses to NE in MVB from sham rats (Table 1). However, in the vascular preparation from PVL rats, both indomethacin and NS-398 enhanced markedly the increase in pressure produced by NE (Fig. 2, A and B).

Because indomethacin is a well-known dual inhibitor of COX-1 and COX-2, we carried out another set of experiments using SC-560 (10 nM), a selective COX-1 inhibitor, to better clarify the role of the COX-1 isomorph in the hyporeactivity to NE observed in PVL rats. In this set of experiments, SC-560, like indomethacin, was not able to significantly increase the perfusion pressure produced by NE in MVB from sham rats, but it markedly enhanced the contractile response to NE in vascular preparations from PVL rats (Table 2 and Fig. 2C).

Furthermore, the potentiating effect evoked by NS-398 was significantly greater than that of the other two inhibitors. Thus indomethacin and SC-560 reversed the hyporeactivity to NE in PVL rats toward the control value only, whereas the response to NE in the presence of NS-398 in PVL rats was significantly higher than that obtained in MVB from sham rats.

Endothelium-dependent relaxation. ACh produced a concentration-dependent relaxation in the MVB preparations with endothelium from both sham and PVL rats precontracted with NE, but it failed to produce relaxation in endothelium-denuded MVB (data not shown). Concentration-response curves to ACh showed a greater relaxation in PVL than in sham MVB preparations, with a significant difference in the maximal effect (Fig. 3 and Table 3).

As shown in Fig. 4, A-C, indomethacin, NS-398, and SC-560 did not significantly modify the relaxation response to ACh in MVB from sham rats. Interestingly, the experiments carried out with SC-560 or indomethacin showed that the two inhibitors were able to significantly inhibit the endothelium-dependent vasodilation in response to ACh in MVB from PVL rats (Fig. 4, D and F, and Table 3). On the other hand, as for contraction experiments, NS-398, the selective COX-2 inhibitor, was more effective in inhibiting the ACh response than the selective and nonselective COX-1 inhibitors (Fig. 4E and Table 3).

Table 3. pD2 values and maximal effects of ACh (%relaxation) in MVB precontracted with norepinephrine from sham-operated and PVL rats in control conditions and after preincubation with Indo (10 \mu M), NS-398 (10 \mu M), or SC-560 (1 nM)

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<td>Indo</td>
<td>42.6 ± 0.5</td>
<td>41.9 ± 0.6</td>
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<tr>
<td>NS-398</td>
<td>42.6 ± 0.5</td>
<td>41.9 ± 0.5</td>
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<tr>
<td>SC-560</td>
<td>42.6 ± 0.5</td>
<td>41.9 ± 0.5</td>
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Values are means ± SE. MVB were precontracted with norepinephrine before addition of ACh. *P < 0.01 vs. control sham values. †P < 0.01 and ‡P < 0.001 vs. PVL control values.
Western blot analysis of COX-1 and COX-2. The 70-kDa protein of the COX-1 isoform was expressed in aorta and in MVB from sham and PVL rats. This expression was increased in aorta and in MVB from PVL compared with sham rats (Figs. 5A and 6A). The increase in expression of COX-1 was 1.3-fold in aorta and 1.4-fold in MVB. The 70-kDa protein of the COX-2 isoform was weakly expressed in aorta and MVB from sham rats, whereas its expression was significantly enhanced by twofold in aorta and 1.7-fold in MVB from PVL rats (Figs. 5B and 6B).

6-Keto-PGF$_{1\alpha}$ production. Assay of 6-keto-PGF$_{1\alpha}$, the stable product of PGI$_2$, showed increased production in the aorta with intact endothelium from PVL rats after stimulation with NE and ACh ($P < 0.01$; Fig. 7).

**DISCUSSION**

The present study highlights the implication of endothelial vasodilatory products from both COX-1 and COX-2 in the hyporeactivity to NE and the increased endothelium-dependent relaxation to ACh of MVB in PVL rats. These functional changes are associated with enhanced expression of COX-1 and COX-2, both in the aorta and MVB from the same animals. Finally, an increased production of 6-keto-PGF$_{1\alpha}$ is also found in the aorta from PVL rats.

A differential effect of indomethacin, SC-560, or NS-398 suggests that COX-2 generates predominantly vasodilatory products, whereas COX-1 releases both vasodilatory and vasoconstrictor products. Finally, these results shed new light on the involvement of vasodilatory products from COX-2 in counteracting increased
hypereactivity to NE and endothelial dysfunction in MVB from PVL rats.

The role of NO in the impaired responsiveness to vasoconstrictor agents in portal hypertension and in cirrhotic patients is already well documented (8, 18). Indeed, NO contributes to splanchnic hyperemia and increased vasodilatation in portal hypertensive syndrome (10, 16, 17, 25).

In the present study, we report that, besides NO, COX products also participate in the changes of vascular reactivity observed in vessels from PVL rats. Indeed, the nonselective or selective COX inhibitors, indomethacin, SC-560, and NS-398, prevented both the decreased contractility to NE and the vasodilatory response to ACh in MVB from hypertensive rats. It is possible that the vasoactive products from COX implicated might be produced from an extra endothelial source like the medial or the adventitial layer, but the perfusion pressure to NE in MVB preparations without endothelium was not different between sham-operated and PVL rats. In addition, neither indomethacin, SC-560, nor NS-398 was able to increase the perfusion pressure to NE in endothelium-denuded MVB preparations from PVL rats. Moreover, MVB from PVL displayed increased endothelium-dependent relaxation to ACh and indomethacin, and SC-560 and NS-398 were ineffective in altering the ACh-induced response in MVB from sham-operated rats. Taken together, it is most likely that the source of COX metabolites that contribute to the vascular changes observed in MVB from PVL rats is mainly from the endothelial layer of the vascular wall. These data may explain the fact that acute or chronic blockade of NO synthase activity fails to completely reverse the hyperdynamic circulation in
portal hypertension. Furthermore, they are consistent with the reports that vasodilatory prostanooids such as PGI₂ can be considered one of the vasodilators involved in the development or progression of the hyperdynamic circulation in this pathology (19, 21).

The use of indomethacin, a nonselective COX inhibitor, SC-560, a reported selective COX-1 inhibitor, or the selective COX-2 inhibitor NS-398 suggests that vasoactive products from both COX-1 and COX-2 isoforms are involved in the hyporeactivity to vasoconstrictor agents and also in the increased endothelium-induced vasodilation in MVB from PVL rats. These data were reinforced by enhanced COX-1 and COX-2 expressions and increased production of PGI₂ upon stimulation with ACh in the aorta from the same animals whose MVB preparations were used for functional assays. Furthermore, upregulation of COX-1 and COX-2 expressions was also found in the mesenteric vasculature of rats with portal hypertension syndrome. Taken together, the present data provide the first evidence for a correlation between functional data mentioning increased participation of both COX-1 and COX-2 vasoactive products and upregulation of these enzymes in the vascular wall, in association with enhanced PGI₂ release in PVL rats. Upregulation of COX-1 and an increase of its metabolites might be the consequence of increased shear stress provoked by splanchnic blood flow during portal hypertension (7). COX-2 can be induced in inflammatory diseases like tumor necrosis factor-α (TNF-α). In fact, TNF-α plays a key role in promoting the systemic and splanchnic hyperdynamic circulatory state that complicates portal hypertension. This cytokine stimulates the synthesis of NO and PGI₂ and could be the common trigger for the production of the two endothelium-derived vasodilators in PVL rats (14). Thus it might be possible that increased damaging stresses such as laminar stress and proinflam-
matory agents (1, 14) can be present in portal hypertension. Nevertheless, products from both COX-1 and COX-2 enzymes are involved in regulating vascular reactivity in PVL rats.

One of the most important findings was the differential effect of COX-1 and COX-2 inhibitors in regulating the hyporeactivity to NE and the enhanced response to ACh in MVB from portal hypertensive animals. Indeed, both indomethacin and SC-560 reversed the reduced responsiveness to NE in MVB from PVL rats toward that of control only, whereas the contractile response to NE in MVB from PVL rats obtained in the presence of the selective COX-2 inhibitor NS-398 was greater than that from sham-operated rats. In addition, the inhibitory effect of NS-398 on ACh-induced vasodilation was greater than the inhibition produced by either indomethacin or SC-560 in MVB from PVL rats. Many hypotheses can be advanced to explain the differential involvement of COX-1 and COX-2 in regulating vascular reactivity. First, activation of COX-2 can release a greater amount of vasodilatory products such as PGI₂ than the activation of COX-1. Second, both isoforms might release vasoconstrictor and vasodilator products, but the balance of vasoactive products released through COX-2 is greatly shifted toward vasodilator products. Finally, it is known that both COX products and NO contribute to the hyperdynamic circulation in portal hypertension (5, 28), and the differential effect of the selective COX-1 inhibitor SC-560 or indomethacin, compared with the COX-2 inhibitor NS-398, might result from a complex interaction between NO and COX, especially for COX-2. In fact, after the induction of the later by cytokines in vascular cells, COX-2 can be activated by NO and can release a large amount of PGI₂. COX-1 may be less sensitive to the stimulation by NO. We cannot distinguish among these possibilities. Nevertheless, blockade of the COX-2 pathway is more effective in inhibiting the hyporeactivity to NE and the increased vasodilation to ACh in MVB from portal hypertension in the present study.

In MVB from PVL rats, blockade of the COX-2 pathway with NS-398 unmasked enhanced responses to NE and reduced endothelial vasodilation to ACh. The mechanisms of vascular hypereactivity to vasoconstrictor agents are unknown, but a similar observation has been reported, for example, in human hepatic conduit artery from chronic liver injury (27). A similar increased response to vasoconstrictor agonists has been reported in omental arteries from human septic shock. In these arteries, overproduction of both NO and vasodilator products from COX are also observed (26). Hypereactivity may be a general mechanism in response to inflammatory injuries with regard to portal hypertension. Therefore, the role of vasodilatory products from COX (i.e., PGI₂ in the present study) is apparently to counteract endogenous vasoconstrictor agonists. The balance between the two processes can be shifted toward hyporeactivity, when the release of PGI₂ overcomes the compensatory mechanisms of the
vessels, as in the MVb from portal hypertensive rats in the present work.

In conclusion, the present study provides evidence for the involvement of both COX-1 and COX-2 metabolites from endothelial origin in the hyporeactivity to NE and the enhanced vasodilation to ACh in MVb from portal hypertensive animals. These functional changes were associated with an increased release of PGL2 in the aorta and upregulation of both COX-1 and COX-2 in the two vascular preparations examined. The results also shed new light on the role of COX-2 vasodilatory metabolites in counteracting hypereactivity of MVb to endogenous vasoconstrictor agonists in MVb from PVL rats. Thus generation of endothelial vasodilator prostanooids, in a major part from COX-2, accounts for the increased mesenteric blood flow in portal hypertension.

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