

# Cyclooxygenase-1 Is Involved in Endothelial Dysfunction of Mesenteric Small Arteries From Angiotensin II–Infused Mice

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**Abstract**—Angiotensin II induces endothelial dysfunction by reducing NO availability and increasing reactive oxygen species. We assessed whether cyclooxygenase (COX)-1 or COX-2 participate in the angiotensin II–induced endothelial dysfunction in murine mesenteric small arteries and examined the role of reduced nicotinamide-adenine dinucleotide phosphate–dependent reactive oxygen species production. Mice received angiotensin II (600 ng/kg per minute, SC), saline (controls), angiotensin II + apocynin (reduced nicotinamide-adenine dinucleotide phosphate oxidase inhibitor, 2.5 mg/day), or apocynin alone for 2 weeks. Endothelial function of mesenteric arteries was assessed by pressurized myograph. In controls, acetylcholine-induced relaxation was inhibited by  $N^G$ -monomethyl-L-arginine and unaffected by DFU (COX-2 inhibitor), SC-560 (COX-1 inhibitor), or ascorbic acid. In angiotensin II–infused animals, the attenuated response to acetylcholine was less sensitive to  $N^G$ -monomethyl-L-arginine, unaffected by DFU, and enhanced by SC-560 and, similarly, by SQ-29548, a thromboxane–prostanoid receptor antagonist. Moreover, response to acetylcholine was unchanged by ozagrel, a thromboxane synthase inhibitor, and normalized by ascorbic acid. Apocynin prevented the angiotensin II–induced vascular dysfunctions. In angiotensin II–infused mice, RT-PCR analysis showed a significant COX-2 downregulation, whereas COX-1 expression was upregulated. These changes were unaffected by apocynin. Modulation of COX isoform by angiotensin II was also documented by immunohistochemistry. In small mesenteric vessels, the reduced NO availability and oxidant excess, which characterize endothelial dysfunction secondary to angiotensin II, are associated with a reduced COX-2 and an increased COX-1 function and expression. Angiotensin II causes an oxidative stress–independent COX-1 overexpression, whereas angiotensin II–mediated oxidant excess production stimulates COX-1 activity to produce a contracting prostanoid endowed with agonist activity on thromboxane–prostanoid receptors. (*Hypertension*. 2007;49[part 2]:679–686.)

**Key Words:** angiotensin II ■ microcirculation, cyclooxygenase ■ endothelium ■ oxidative stress

It is widely accepted that angiotensin (Ang) II is greatly implicated in the development of endothelial dysfunction in small resistance arteries,<sup>1,2</sup> mainly through an increased generation of reactive oxygen species (ROS) via vascular reduced nicotinamide-adenine dinucleotide phosphate [NAD(P)H] oxidase activation.<sup>3–6</sup> Mesenteric arteries from Ang II–infused mice are characterized by a blunted endothelium-dependent relaxation, as a consequence of a reduced NO availability because of oxidant excess.<sup>5</sup> Recent evidence indicates that another pathway involved in endothelial dysfunction is cyclooxygenase (COX) activity. Thus, prostaglandins (PGs) have been shown to play a major role in modulating vascular tone, and COX represents a key enzyme in PG synthesis. Indeed, COX metabolizes arachidonic acid into the unstable intermediate  $PGH_2$ , which, in turn, is converted by an array of downstream enzymes to form a range of bioactive

prostanoids.<sup>7</sup> Under physiological conditions, prostacyclin ( $PGI_2$ ) is the major prostanoid identified and released by endothelial cells mediating several protective effects on the vascular wall, including relaxation and inhibition of platelet aggregation and adhesion.<sup>7</sup> The predominant opponent of  $PGI_2$  is thromboxane ( $TXA_2$ ), specifically acting on thromboxane–prostanoid (TP) receptors mainly located on smooth muscle cells where it causes vasoconstriction.<sup>8</sup> Under pathological conditions, such as inflammation or atherosclerosis, the  $PGI_2$  production decreases, and COX-derived vasoconstrictor substance release, including  $TXA_2$ , becomes predominant.<sup>9</sup> Two distinct COX isoenzymes, COX-1 and COX-2, have been described. Although COX-1 is constitutively expressed to produce physiologically relevant prostanoids, COX-2 is regarded as an inducible isoform, which can be rapidly upregulated by a number of stimuli.<sup>10,11</sup>

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A direct interaction between Ang II and the COX pathway has been described. Indeed, COX-1–derived prostanoids acting on TP receptors were shown to be involved in a murine model of Ang II–induced hypertension.<sup>12</sup> In addition, an attenuation of COX-2 expression in rat renal cortex by Ang II was observed.<sup>13,14</sup> Worth noting, in aorta from spontaneously hypertensive rats, an animal model of oxidative stress, increased ROS production was shown to activate COX-1 leading to the production of endoperoxides, which, in turn, stimulate TP receptors on vascular smooth muscle cells.<sup>8</sup> Based on these findings, ROS might be hypothesized as a mechanism whereby Ang II interacts with the COX pathway to induce endothelial dysfunction.

Therefore, the present study was designed to evaluate whether any COX isoform contributes to the Ang II–mediated endothelial dysfunction in peripheral small arteries and whether this interaction is mediated by ROS generation. To address this issue, we studied endothelium-dependent relaxation in mesenteric resistance arteries from Ang II–infused mice. Finally, the impact of NAD(P)H oxidase–derived ROS generation on interaction between Ang II and COX pathways was also investigated.

## Methods

### Animals

All of the experiments were carried out in accordance with the provisions of European Union Council Directive 86–609, recognized by the Italian Government. DBA/1N male mice (Charles River), aged 12 to 14 weeks, were used. Mice, under short anesthesia with chloral hydrate (8.5 mg SC), were implanted subcutaneously with Alzet osmotic minipumps (Alza Corp) that infused 600 ng/kg per minute of Ile<sup>5</sup>-Ang II (Peninsula) dissolved in 0.9% saline. Vehicle-infused (saline, osmotic minipumps) mice served as controls. In 2 additional groups of Ang II–infused and vehicle-infused mice, 2.5 mg/day of apocynin, an NAD(P)H oxidase inhibitor,<sup>15</sup> were given in the food. Mice were located in single cages, and the food was prepared and given daily to the animals. The dose of apocynin was chosen on the basis of previous data showing that the increase in vascular NAD(P)H oxidase activity by Ang II was prevented by apocynin.<sup>6</sup> Mice were treated for 2 weeks. Systolic blood pressure was measured by the tail-cuff method (BP recorder 58600, Ugo Basile). The average of 3 pressure readings was obtained.

### Preparation of Small Mesenteric Arteries for Reactivity Experiments

A second-order branch of the mesenteric arterial tree ( $\approx$ 2 mm in length) was dissected and mounted on 2-glass microcannulae in a pressurized myograph, as described previously.<sup>16</sup> Vessels were equilibrated for 60 minutes under constant intraluminal pressure (45 mm Hg) in warmed (37°C) and bubbled (95% air and 5% CO<sub>2</sub>) physiological salt solution, which contained (in mmol/L): NaCl 120,

NaHCO<sub>3</sub> 25, KCl 4.7, KH<sub>2</sub>PO<sub>4</sub> 1.18, MgSO<sub>4</sub> 1.18, CaCl<sub>2</sub> 2.5, EDTA 0.026, and glucose 5.5 (pH 7.4). Vessels were considered viable and used if they constricted  $>$ 70% of their resting lumen diameter in response to an extraluminal application of high-potassium (125 mmol/L of KCl) physiological salt solution containing 100  $\mu$ mol/L of noradrenaline (NA).

### Experimental Design

Endothelium-dependent and -independent relaxations were assessed by measuring the responses of mesenteric arteries retrieved from control or Ang II–infused mice (n=8 each group) to cumulative concentrations of acetylcholine ([ACh] 1 nM to 100  $\mu$ mol/L) and sodium nitroprusside (0.01 to 100  $\mu$ mol/L), respectively. All of the experiments were performed in vessels precontracted with NA (10  $\mu$ mol/L). To avoid the possibility that NA might induce a different contractility in vessels from controls, Ang II–infused, or Ang II plus apocynin-treated animals, in preliminary experiments we performed a concentration–response analysis of NA effects (from 1 nM to 100  $\mu$ mol/L) to establish the dose of NA able to elicit similar contractions among the groups (data not shown). After the titration study, the dose of 10  $\mu$ mol/L of NA, which induced similar contracting responses in all of the groups, was selected (Table).

### Influence of COX-1 and COX-2 Activity and NO Availability on Endothelium-Dependent Relaxation

The participation of COX-1 and COX-2 isoenzymes in modulation of endothelial function was assessed by construction of concentration–response curves to ACh after 30-minute preincubation of mesenteric vessels with SC-560 (1  $\mu$ mol/L; selective COX-1 inhibitor)<sup>17</sup> or DFU (1  $\mu$ mol/L; selective COX-2 inhibitor).<sup>18</sup>

To evaluate the NO availability, concentration–response curves to ACh were constructed in the presence of the NO synthase inhibitor N<sup>G</sup>-monomethyl-L-arginine ([L-NMMA] 100  $\mu$ mol/L; 30-minute preincubation). Then, to examine whether COX-1 could influence NO availability, ACh was tested in an additional group (n=4) of mesenteric vessels from Ang II–infused mice under simultaneous presence of L-NMMA and SC-560.

### Involvement of TP Receptors and Thromboxane Synthase on Endothelium-Dependent Relaxation

To ascertain the contribution of TP receptors to endothelial dysfunction elicited by Ang II, in mesenteric vessels from Ang II–infused animals (n=8) a concentration–response curve to ACh was constructed after 30-minute incubation with SQ-29548 (1  $\mu$ mol/L; TP receptor antagonist).<sup>19</sup> Then, to rule out the possibility that the COX-1–derived endoperoxide could exert any biological effect on specific receptors different from TP receptors, ACh was applied to mesenteric vessels during simultaneous incubation with SC-560 and SQ-29548. Finally, to assess the participation of COX-1–derived TXA<sub>2</sub> to endothelial dysfunction, in an additional group of mesenteric vessels from Ang II–infused animals (n=6), a response curve to ACh was constructed in the presence of ozagrel, a selective TX synthase inhibitor (1  $\mu$ mol/L; 30-minute incubation).<sup>20</sup>

### Influence of ROS on Endothelium-Dependent Relaxation

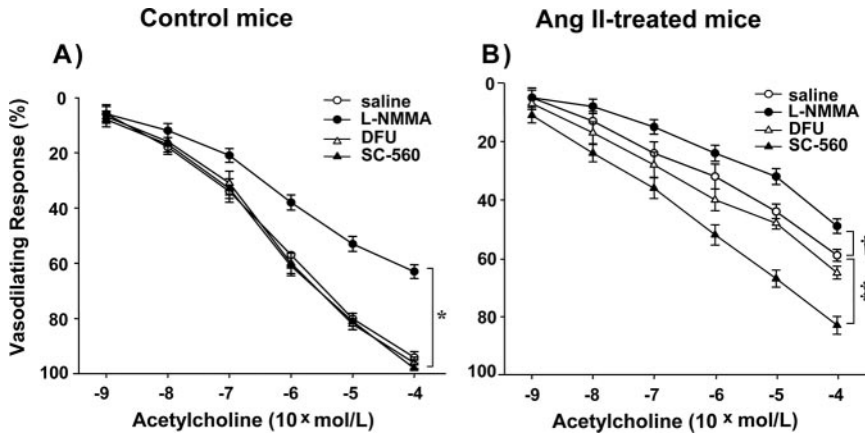
This set of experiments (n=6 for each group) was performed to confirm the major pathophysiological role of Ang II–mediated ROS

**Body Weight, Systolic Blood Pressure, and Lumen Diameters at Resting Condition and After NA Precontraction of Mesenteric Small Arteries from Ang II–Infused Mice $\pm$ Apocynin**

Parameter	Control	Ang II	Ang II+Apocynin	Apocynin
Body weight, g	25.4 $\pm$ 1.4	24.3 $\pm$ 1.0	25.8 $\pm$ 1.2	27.5 $\pm$ 0.9
SBP, mm Hg	104 $\pm$ 2	149 $\pm$ 3*	122 $\pm$ 3†‡	106 $\pm$ 2
Resting lumen diameter, $\mu$ m	213 $\pm$ 12	194 $\pm$ 9	218 $\pm$ 14	215 $\pm$ 10
Lumen diameter precontracted with NA, $\mu$ m (%)	58 $\pm$ 2 (73 $\pm$ 1)	58 $\pm$ 3 (70 $\pm$ 1)	62 $\pm$ 3 (71 $\pm$ 1)	60 $\pm$ 2 (72 $\pm$ 1)

n=8 per group; results are mean $\pm$ SEM.

\* $P$ <0.001 vs control; † $P$ <0.001 vs Ang II; ‡ $P$ <0.01 vs control.



**Figure 1.** Endothelium-dependent relaxations elicited by ACh in mesenteric resistance arteries from control (A) or Ang II-infused mice (B), without (saline) or with L-NMMA, DFU, or SC-560. Each point represents the mean of 8 experiments  $\pm$  SEM. \* $P < 0.0001$ ; † $P < 0.05$ ; ‡ $P < 0.01$ .

excess in decreasing NO availability. Thus, concentration–response curves to ACh were constructed in the absence and in the presence of the antioxidant compound ascorbic acid (10 mmol/L; 30 minutes preincubation), L-NMMA, and during simultaneous incubation with L-NMMA and ascorbic acid.

### RNA Extraction and RT-PCR

Expression of mRNA for COX-1 and COX-2 isoforms was assessed by RT-PCR, as described previously.<sup>21</sup> Specimens of mesenteric vessels were disrupted with cold glass pestles, and total RNA was isolated by TRIzol (Life Technologies) and chloroform. Total RNA (1  $\mu$ g) served as a template for cDNA synthesis in a reaction based on moloney murine leukemia virus reverse transcriptase. cDNA samples were subjected to PCR in the presence of specific primers based on the nucleotide sequences of cloned mouse COX isoforms.<sup>22</sup> PCR was carried out by a PCR-Express thermocycler (Hyaid). Amplification conditions were: 15 s at 94°C, 15 s at 55°C, and 1 minute at 72°C for 35 cycles followed by 7 minutes at 72°C. Aliquots of RNA not subjected to reverse transcription were included in PCR reactions to verify the absence of genomic DNA. The efficiency of RNA extraction, reverse transcription, and PCR was evaluated by primers for mouse  $\beta$ -actin. PCR products were separated by 1.8% agarose gel electrophoresis and stained with ethidium bromide. PCR products were then visualized by UV light and subjected to densitometric analysis by National Institutes of Health image software. The relative expression of COX-1 or COX-2 mRNA was normalized to that of  $\beta$ -actin.

### Immunostaining of COX-1 and COX-2

After collection, mesenteric specimens were immediately fixed in cold 4% paraformaldehyde diluted in PBS and paraffin embedded at 56°C. Sections were sequentially treated with the following: 1% hydrogen peroxide in methanol for 5 minutes; microwave antigen retrieval (300 W for 5 minutes, twice in 10 mmol/L of sodium

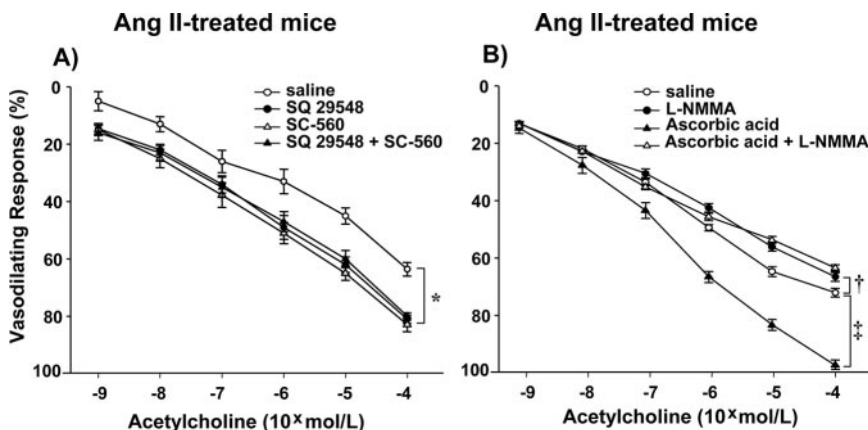
citrate; pH 6.0); normal swine serum (1:20; Sigma Chemicals); primary antibody solutions diluted in 0.1% BSA and 0.1% sodium azide in PBS (overnight at 4°C); biotinylated anti-rabbit immunoglobulins (code No. E0432, Dakopatts); peroxidase-labeled streptavidin complex (LSAB plus, Dakopatts); and, finally, 3,3'-diaminobenzidine tetra-hydrochloride (Dakopatts), as reported previously.<sup>23</sup> Specimens were counterstained with hematoxylin. COX-1 and COX-2 were detected by using rabbit anti-COX-1 (code No. 160109) and anti-COX-2 (code No. 160126) polyclonal antibodies (Cayman Chemical) diluted at 1:50 and 1:100, respectively. Negative controls were obtained by substituting the primary antibodies with preimmune rabbit serum. Endogenous peroxidases and avidin-binding activity were assayed by incubating slides with 3,3'-diaminobenzidine tetrahydrochloride alone or with streptavidin-horseradish peroxidase complex/3,3'-diaminobenzidine tetrahydrochloride, respectively.

### Drugs and Solutions

Apocynin was purchased from Fluka (Sigma-Aldrich). ACh, ascorbic acid,  $N^G$ -monomethyl-L-arginine, physiological salt solution, SC-560 (5-[4-chlorophenyl]-1-[4-methoxyphenyl]-3-trifluoromethylpirazole) and sodium nitroprusside were purchased from Sigma Chemicals. SQ-29548 was provided by Cayman Chemical and ozagrel hydrochloride (2E-3-[4-{1H-Imidazol-1-ylmethyl}phenyl]-2-propenoic acid hydrochloride) by Tocris Bioscience. DFU (5,5-dimethyl-3-[3-fluorophenyl]-4-[4-methylsulphonyl]-phenyl-2[5H]-furanone) was kindly supplied by Merck Research Laboratories. Drug solutions were made in distilled water except for SC-560, DFU, and SQ-29548 (dimethylsulphoxide). Further dilutions were prepared with distilled water.

### Data Analysis

Results are presented as mean  $\pm$  SEM and analyzed by repeated-measures ANOVA, followed by Student–Newman–Keuls test or by



**Figure 2.** Endothelium-dependent relaxations to ACh in mesenteric resistance arteries from Ang II-infused mice. A, relaxant responses without (saline) or with SQ 29548, SC-560, or both. B, relaxant responses without (saline) or with L-NMMA, ascorbic acid, or both. Each point represents the mean of 8 experiments  $\pm$  SEM. \* $P < 0.01$ ; † $P < 0.05$ ; ‡ $P < 0.0001$ .

unpaired Student's *t* test where appropriate. A value of  $P < 0.05$  was considered statistically significant. Maximal ACh-induced and sodium nitroprusside-induced responses ( $E_{\max}$ ) were calculated as the maximal percentage increments of lumen diameter. "n" indicates the number of experiments.

## Results

### Body Weight, Systolic Blood Pressure, and Lumen Diameters

Body weight, taken immediately before animal sacrifice, was similar in all of the groups (Table). At the end of treatment, systolic blood pressure was significantly higher in Ang II-infused mice compared with controls, an effect that was significantly reduced, but not normalized, by apocynin administration. Apocynin treatment alone did not affect systolic blood pressure values (Table). Baseline and NA-induced contraction levels of lumen diameters of vessels did not significantly differ among groups (Table).

### Effects of SC-560, DFU, and L-NMMA on Endothelium-Dependent Relaxation

In control mice, relaxation of resistance arteries evoked by ACh was not modified by exposure to SC-560 or DFU (Figure 1A), whereas it was significantly blunted by L-NMMA (inhibition:  $-34 \pm 1$ ; Figure 1A). In Ang II-infused animals, relaxation to ACh was attenuated ( $P < 0.001$ ) in comparison with controls and significantly improved, but not normalized, by SC-560 ( $P < 0.05$  versus control; Figure 1B). Vascular response to ACh was unaffected by DFU (Figure 1B). In these vessels, the inhibitory effect exerted by L-NMMA on relaxation to ACh (inhibition:  $-11 \pm 1\%$ ), was significantly ( $P < 0.001$ ) lower compared with controls (Figure 1B). Under the same conditions, SC-560 application failed to enhance the inhibitory effect of L-NMMA on endothelium-dependent relaxation (ACh alone,  $62 \pm 1\%$ ; ACh plus L-NMMA,  $51 \pm 1\%$ ; inhibition,  $-11 \pm 2\%$ ; ACh plus SC-560,  $83 \pm 2\%$ ; ACh plus L-NMMA and SC-560,  $71 \pm 1\%$ ; inhibition:  $-11 \pm 1\%$ ).

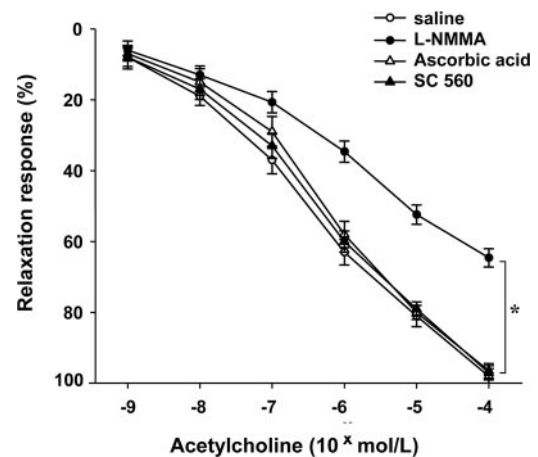
### Involvement of TP Receptors and Thromboxane Synthase on Endothelium-Dependent Relaxation

In this series of experiments in mesenteric arteries from Ang II-infused animals, the attenuated relaxation to ACh was confirmed ( $P < 0.001$  versus controls; Figure 2A). The response to ACh was enhanced by SQ-29548 reaching almost identical values to those obtained with COX-1 blockade. When SC-560 and SQ-29548 were simultaneously applied to these vessels, no further increase in response to ACh was obtained (Figure 2A). In additional mesenteric vessels from Ang II-infused mice ( $n=6$ ), the relaxation to ACh ( $E_{\max}$ :  $64 \pm 1\%$ ) was not affected by incubation with the selective TX synthase inhibitor ozagrel ( $E_{\max}$ :  $62 \pm 1\%$ ).

### Effect of Ascorbic Acid on Responses to Acetylcholine

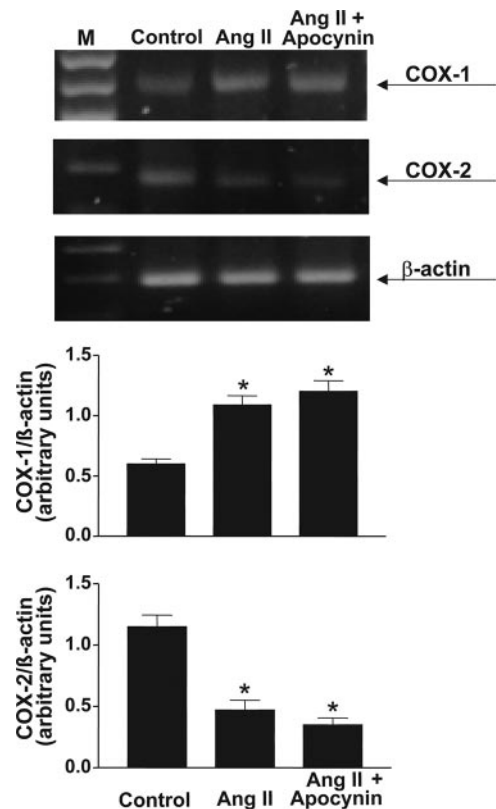
In control mice, relaxation to ACh ( $E_{\max}$ :  $97 \pm 1\%$ ) was not modified by ascorbic acid ( $E_{\max}$ :  $98 \pm 1\%$ ). By contrast, the antioxidant drug normalized the endothelium-dependent relaxation in vessels from Ang II-infused animals (Figure 2B).

### Ang II and apocynin-treated mice



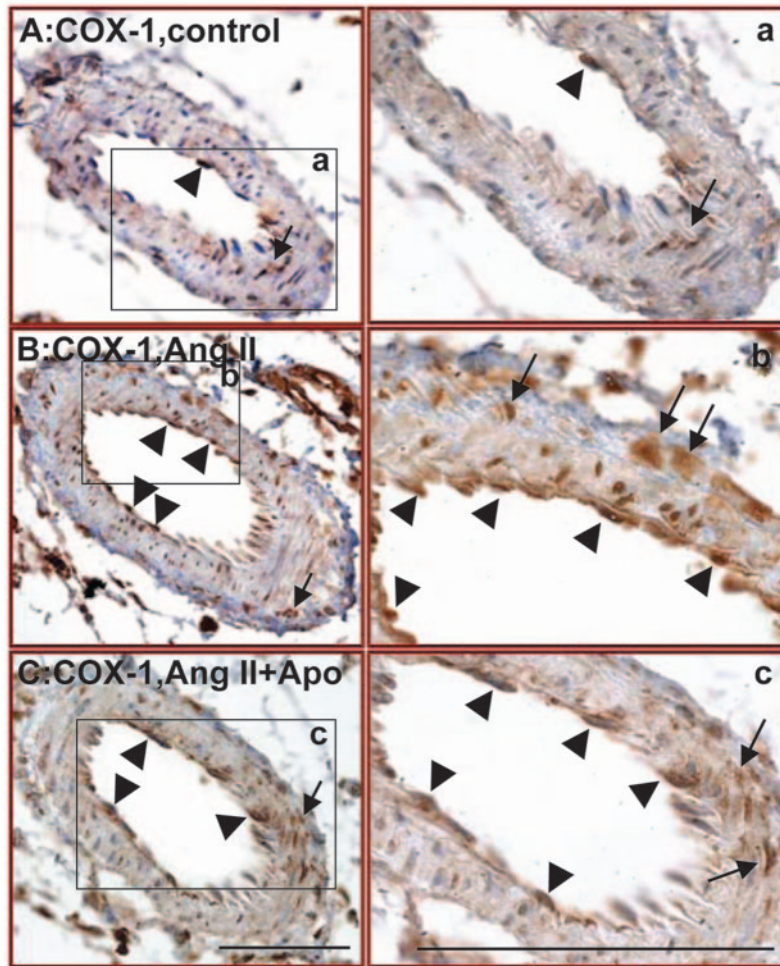
**Figure 3.** Endothelium-dependent relaxations to ACh in mesenteric resistance arteries from Ang II + apocynin-treated mice, without (saline) or with L-NMMA, ascorbic acid, or SC-560. Each point represents the mean of 6 experiments  $\pm$  SEM. \* $P < 0.0001$ .

Moreover, ascorbic acid restored the inhibitory effect of L-NMMA on ACh-induced relaxation (ACh plus L-NMMA, inhibition:  $-13 \pm 1\%$ ; ACh plus L-NMMA and ascorbic acid, inhibition:  $-35 \pm 1\%$ ; Figure 2B).



**Figure 4.** Top, representative agarose gel showing RT-PCR products for COX-1, COX-2, and  $\beta$ -actin in mesenteric vessels from control, Ang II, and Ang II + apocynin-treated mice. Bottom, column graphs referring to densitometric analysis of COX isoform cDNA bands normalized to the expression of  $\beta$ -actin. M indicates size markers. Each column represents the mean of 5 experiments  $\pm$  SEM. \* $P < 0.05$  vs controls.

## Immunostaining for COX-1 in mesenteric vessels from controls and Ang II $\pm$ apocynin-treated mice



**Figure 5.** Immunostaining for COX-1 in small mesenteric arteries from control (A), Ang II-infused (B), and Ang II+apocynin-treated (C) mice. Right panels (a, b, and c) display higher magnification of boxed areas in A, B, and C micrographs, respectively. Specific COX-1 immunostaining is detected in endothelium (arrowheads) and muscle cells (arrows). Basal COX-1 expression of endothelial and muscle cells in control mice (A) is enhanced after Ang II or Ang II+apocynin treatment (B and C). Scale bars=100  $\mu$ m.

### Role of NAD(P)H Oxidase Inhibition on Endothelium-Dependent Relaxation

The Ang II-induced vascular alterations were completely prevented by apocynin. Thus, in vessels from Ang II- and apocynin-treated animals, relaxation to ACh was preserved and unmodified by DFU. Moreover, the dilating response to ACh was no longer affected by SC-560 or by ascorbic acid (Figure 3). In addition, the inhibitory effect of L-NMMA on response to ACh was increased, resulting similar to that seen in controls (inhibition:  $-33 \pm 1\%$ ; Figure 3).

Vessels from mice treated with apocynin alone were similar to controls. Indeed, relaxation to ACh ( $E_{\max}$ :  $97 \pm 1\%$ ) was unchanged by DFU ( $95 \pm 1\%$ ), SC-560 ( $97 \pm 1\%$ ), or ascorbic acid ( $E_{\max}$ :  $96 \pm 1\%$ ) and significantly attenuated by L-NMMA ( $E_{\max}$ :  $63 \pm 2\%$ ; inhibition:  $-34 \pm 2\%$ ; *P* not significant versus control).

Endothelium-independent relaxation by sodium nitroprusside was similar in all of the groups ( $E_{\max}$ , controls:  $97 \pm 1\%$ ; Ang II:  $97 \pm 1\%$ ; Ang II plus apocynin:  $97 \pm 1\%$ ; apocynin alone:  $97 \pm 1\%$ ) and not modified by SC-560 or DFU (data not shown).

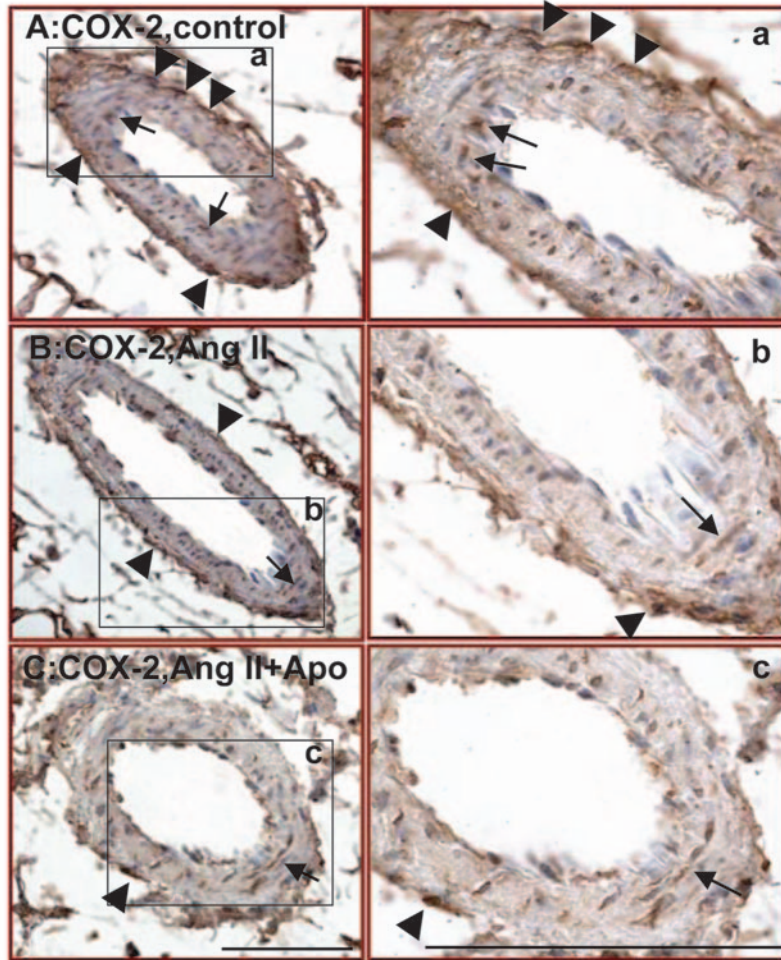
### RT-PCR Analysis of COX-1 and COX-2 Isoforms Expression

RT-PCR analysis showed a basal expression of mRNA encoding COX-1 and COX-2 in mesenteric control vessels. Ang II infusion was associated with a significant induction of COX-1, which was unmodified by simultaneous treatment with apocynin (Figure 4). In contrast, the expression of COX-2 was significantly downregulated in mesenteric vessels from Ang II-infused animals. This effect was not modified by apocynin (Figure 4).

### Immunohistochemical Analysis of COX-1 and COX-2 Isoforms

Control mesenteric arteries showed a faint COX-1 staining at the level of endothelial and smooth muscle cells (Figure 5A). In the presence of Ang II, COX-1 immunostaining was enhanced in endothelial cells and muscle layer both in cytoplasm and nuclei (Figure 5B). This picture was not modified by treatment with apocynin (Figure 5C). On the other hand, basal expression of COX-2, mainly detected within muscle cells and tunica adventitia (Figure 6A), was reduced in vessels from mice treated with Ang II both in the absence (Figure 6B) and in the presence of apocynin (Figure 6C).

### Immunostaining for COX-2 in mesenteric vessels from controls and Ang II $\pm$ apocynin-treated mice



**Figure 6.** Immunostaining for COX-2 in small mesenteric arteries from control (A), Ang II-infused (B), and Ang II+apocynin-treated (C) mice. Right panels (a, b and c) display higher magnification of boxed areas in A, B, and C micrographs, respectively. COX-2 is constitutively expressed in muscle (arrows) and adventitial (arrowheads) layers of small mesenteric arteries in control mice (A) and reduced either in Ang II-treated (B) or Ang II+apocynin-treated (C) mice. Scale bars=100  $\mu$ m.

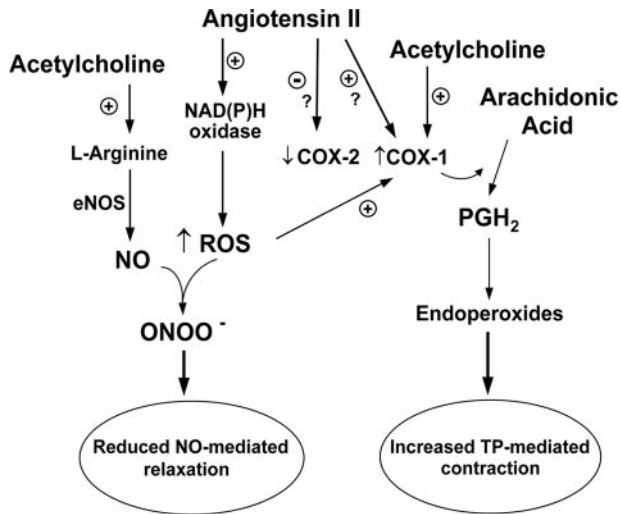
### Discussion

In the present study, we observed that, at the level of mesenteric small arteries, chronic Ang II infusion induces endothelial dysfunction because of a reduced NO availability secondary to an increased ROS production. Because all of these alterations were prevented by apocynin administration, our findings strengthen the concept that an excess of ROS, produced via vascular NAD(P)H oxidase activation, is a major determinant of Ang II-induced vascular functional changes.<sup>3-6</sup>

The first major novel finding of our study consists of the demonstration of an interaction between Ang II and COX isoforms. In vessels from Ang II-infused mice, SC-560, but not DFU, improved the relaxant response to ACH without any effect on sodium nitroprusside, indicating that COX-1, but not COX-2, contributes to the pathogenesis of Ang II-mediated endothelial dysfunction. In keeping with our functional results, RT-PCR detected a downregulation of COX-2 and a simultaneous induction of COX-1 expression in vessels from Ang II-infused animals. Furthermore, immunohistochemical analysis revealed a reduced COX-2 and a strong enhanced COX-1 staining in vessels from Ang II-infused animals as compared with controls. These findings

provide the first simultaneous demonstration of COX-2 downregulated together with COX-1 overexpressed localization within murine small resistance vasculature. Unfortunately, the present experimental design does not allow us to distinguish whether COX-1 overexpression depends on an attenuating effect of Ang II on COX-2 or whether it occurs as a direct effect of Ang II. Although previous reports documented a COX-1/COX-2 compensatory phenomenon in other districts,<sup>24-26</sup> the possibility that COX-1 upregulation is a consequence of a compensatory mechanism is in contrast with the evidence that COX-1 and COX-2 are constitutive and inducible isoforms, respectively. Further studies are needed to propose COX-1/COX-2 interaction in mesenteric small arteries. On the other hand, it was observed recently that prostanoids from the COX-1 or COX-2 pathway do not mediate or modulate pressor responses to Ang II in rat pulmonary or systemic vascular districts.<sup>27</sup> When considering that this is an acute study, this finding reinforces the possibility that Ang II effects on COX refer to the constitutive isoform, because they need time to be detected.

Our results agree with and extend previous evidence indicating a contribution of COX-1 to Ang II-mediated cardiovascular damage. Indeed, it was observed that the acute



**Figure 7.** Schematic diagram showing the hypothetical involvement of COX isoforms in Ang II-induced endothelial dysfunction. Ang II increases the generation of ROS via vascular NAD(P)H oxidase activation. ROS are able to react with NO, leading to a reduced NO availability and hyperproduction of peroxynitrites ( $\text{ONOO}^-$ ). Ang II might also directly downregulate COX-2 or upregulate COX-1. COX-1 is also activated by ACh. Under such conditions, ROS stimulate COX-1 to transform arachidonic acid into endoperoxides, which, in turn, activate the contracting TP receptors.

pressor effect of Ang II was abolished in COX-1-deficient mice.<sup>28</sup> More recently, Francois et al<sup>12</sup> showed a significant abrogation of hypertension in COX-1 knockout mice chronically treated with Ang II. Our study is, however, the first to indicate a participation of COX-1 isoenzyme in the development of functional alterations of resistance arteries induced by Ang II. Of note, COX-1 blockade improved only in part the Ang II-induced endothelial dysfunction, indicating a residual direct and specific COX-1-independent effect of Ang II on the vascular wall that has been well documented.<sup>1</sup>

An important question raised by our findings deals with the mechanism(s) whereby Ang II modulates COX isoform expression at the level of mesenteric small arteries. To address this issue we used apocynin, a selective NAD(P)H oxidase inhibitor able to reverse Ang II-induced vascular structural alterations and endothelial dysfunction, as demonstrated previously.<sup>6</sup> In our study, apocynin, while normalizing endothelial function and restoring NO availability, as documented by the restored inhibitory effect of L-NMMA on ACh-induced relaxation, failed to modify the COX-2 downregulation and COX-1 upregulation, as observed by RT-PCR and immunohistochemistry. Overall, these results allow us to propose that the oxidant excess, produced by NAD(P)H oxidase activation secondary to Ang II infusion, accounts also for COX-1 stimulation, but not overexpression, to transform arachidonic acid into endoperoxides, which then diffuse to activate responsive TP receptors located on vascular smooth muscle (Figure 7). Under these conditions, the roles of ACh are to induce an endothelium-dependent relaxation, of which the mediators include residual NO and endothelium-derived hyperpolarizing factor(s),<sup>29,30</sup> and to stimulate COX-1.<sup>31</sup> Although in healthy conditions COX-1 activity leads to vasodilating  $\text{PGI}_2$ , in pathological conditions, including hy-

perension, this isoform is, at least in part, switched to contracting prostanoids. Thus, both the enhanced COX-1 expression and its activation by oxidant excess are required for the reduced endothelium-dependent relaxation secondary to Ang II infusion. A reduction in the release of NO availability augments the extent of the endothelial dysfunction. This possibility totally agrees with and extends previous reports in aorta from the spontaneously hypertensive rat, a genetic animal model of hypertension and oxidative stress, where the activation of COX-1-derived endoperoxides by ROS was documented.<sup>31</sup>

The second major novel finding of our study deals with the COX-1-derived factor that might be implicated in the Ang II-mediated vascular alterations. The possibility that COX-1 could be itself a source of oxidative stress or produce any factor able to directly interact with NO was ruled out by the observation that COX-1 blockade did not increase the blunted inhibitory effect of L-NMMA on endothelial relaxation in Ang II-infused animals. To assess the involvement of TP receptors, we used the selective TP receptor antagonist SQ-29548, whereas the TX synthase inhibitor ozagrel allowed us to evaluate the role of  $\text{TXA}_2$ . In vessels from Ang II-infused mice, the blunted endothelium-dependent relaxation was improved by exposure to the TP receptor antagonist to similar values obtained by COX-1 blockade. In contrast, the relaxing response to ACh was not affected by TX synthase inhibition. Taken together, although excluding any participation of  $\text{TXA}_2$ , these data indicate that, on stimulation by Ang II, COX-1 mediates the production of a prostanoid acting specifically as a TP receptor agonist. We cannot exclude the possibility that  $\text{PGH}_2$ , per se, could interact with TP receptors, as was documented previously in another animal model of oxidative stress.<sup>32</sup> Thus, it has been documented that peroxynitrite anion hyperproduction, secondary to the reaction between NO and ROS, leads to attenuation of endothelial  $\text{PGI}_2$  synthase.<sup>33</sup> As a consequence, the unmetabolized substrate  $\text{PGH}_2$  would accumulate and trigger the TP receptors, thus causing vasoconstriction.

The hypothetical involvement of a putative vasoconstrictor prostanoid acting on receptor(s) other than TP receptors was ruled out by our finding that, under simultaneous COX-1 and TP receptor blockade, no further improvement of endothelium-dependent relaxation could be obtained. In line with our results, Yang et al<sup>8</sup> observed an ROS-induced production of COX-1-derived endoperoxides in aorta from spontaneously hypertensive rats. In addition, a recent study on TP knockout mice demonstrated a major involvement of COX-1-derived prostanoid acting on TP receptors in Ang II-induced hypertension and cardiac hypertrophy.<sup>12</sup> Taken together, these data extend previous findings, obtained in spontaneously hypertensive rats, indicating that oxidative stress, independent from its source, is able to stimulate COX-1 to produce a contracting endoperoxide different from  $\text{TXA}_2$  but acting on TP receptors.<sup>31</sup>

In conclusion, the present study indicates that, in murine mesenteric small arteries, endothelial dysfunction secondary to Ang II infusion is partly dependent on the COX-1 pathway. It is suggested that Ang II is associated with COX-1 overexpression and COX-2 downregulation, whereas Ang II-mediated ROS production stimulates COX-1 activity to produce a

contracting prostanoid, acting as an agonist on TP receptors. This mechanism might significantly contribute to the atherosclerotic damage mediated by Ang II.

### Perspectives

Using an in vivo model, we demonstrated a major role for the COX-1 derivative TP receptor pathway, simultaneously with COX-2 downregulation, in Ang II-induced endothelial dysfunction in murine small arteries. The current results can have important clinical implications. The demonstration that a COX-1-derivative prostanoid is greatly involved in Ang II-induced vascular alterations may represent a possible explanation for the increased cardiovascular risk in patients treated with selective COX-2 inhibitors.<sup>34</sup> In addition, the COX-1-mediated pathway might be a possible adjunctive therapeutic target to reverse endothelial dysfunction and, hopefully, to prevent cardiovascular disease, especially in those forms of human hypertension where a markedly activated renin-Ang-aldosterone system occurs.

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### Disclosures

None.

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## Cyclooxygenase-1 Is Involved in Endothelial Dysfunction of Mesenteric Small Arteries From Angiotensin II–Infused Mice

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