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BASIC SCIENCE

Resistance artery mechanics and composition in angiotensin II-infused mice: effects of cyclooxygenase-1 inhibition

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Aims The aim of this study was to investigate the role of cyclooxygenase (COX)-1 on vascular alterations in structure, mechanics, and extracellular matrix (ECM) components induced by angiotensin (Ang) II in mesenteric arteries from wild-type (WT) and COX-1 knockout (*COX-1*$^{-/-}$) mice.

Methods and results Animals were infused with vehicle or Ang II (400 ng/kg/min, s.c.) ± SC-560 (COX-1 inhibitor), DFU (COX-2 inhibitor), or SQ-29548 (TP receptor antagonist). After 2 weeks, vessels were isolated and exposed to intraluminal pressures (3–140 mmHg, pressurized myograph) to determine mechanical properties. Angiotensin II-induced vascular hypertrophic remodelling in WT was reversed by SC-560 or SQ-29548, but unaffected by DFU. Angiotensin II increased vessel stiffness ($P < 0.01$), this effect being ameliorated by SC-560 or SQ-29548, but unmodified by DFU. Angiotensin II failed to modify vessel elasticity in *COX-1*$^{-/-}$ mice. In WT vessels, Ang II enhanced COX-1 immunostaining, induced collagen and fibronectin depositions and decreased elastin content ($P < 0.01$). These effects were reversed by SC-560 or SQ-29548, but unaffected by DFU. In *COX-1*$^{-/-}$ mice, Ang II did not affect ECM contents. In WT, Ang II increased COX-1 and decreased COX-2 expression, and enhanced the vascular release of 6-keto-PGF1α which was prevented by COX-1 blockade. Human coronary artery smooth muscle cells, incubated with Ang II, showed an increased expression of pro-collagen I, which was abrogated by SC-560 or SQ-29548.

Conclusion Angiotensin II-induced alterations of resistance arteries in structure, mechanics, and ECM composition were prevented by COX-1 inhibition and TP receptor antagonism, indicating that Ang II-mediated vascular damage is mediated by COX-1-derived prostanoid prostacyclin, activating TP receptors.

Keywords Angiotensin II • Microcirculation • Cyclooxygenase • Extracellular matrix • Vascular mechanics

Introduction

Angiotensin (Ang) II is greatly implicated in the development-progression of structural alterations (vascular remodelling) in small-resistance arteries.[1] Increased media:lumen ratio (M/L), which characterizes vascular remodelling, can result from a reduced outer diameter, which narrows the lumen without net growth (eutrophic remodelling), or from a thicker media encroaching on the lumen (hypertrophic remodelling).[2] Another hallmark of Ang II-induced structural abnormalities is represented by changes in the mechanical properties of arteries, with particular regard for increased stiffness.[3–5] Vascular fibrosis is critically important in determining vascular structural modifications, and it involves changes in extracellular matrix (ECM) components, including

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collagen type I and III, elastin, and fibronectin. An increase in collagen and fibronectin and a decrease in elastin contents have been shown in the media of small arteries from Ang II-infused animals.[4–6]

Cyclooxygenase (COX)-1 and its derived prostanoids are involved in the development-progression of vascular atherosclerotic damage.[7,8] Cyclooxygenase-1 is over-expressed in mesenteric small vessels of Ang II-treated mice, and its increased activity, which in turn produces contracting prostanoids(s) acting on TP receptors, represents a major pathway whereby Ang II elicits vascular functional alterations.[9] Based on this knowledge, a direct interaction between Ang II and COX-1 pathway can be hypothesized as one of the mechanisms whereby Ang II induces vascular structural changes. Accordingly, the present study was designed to evaluate whether COX-1 activation and COX-1 products acting on TP receptors contribute to changes in vascular mechanics and ECM components, which characterize the remodelling of mesenteric resistance arteries evoked by Ang II. To this aim, we employed selective COX isoforms inhibitors, chronically administered to Ang II-treated mice. Moreover, as a complementary approach, we used homozygous mice carrying a targeted disruption of *COX-1* gene. Finally, the effects of drug treatments on collagen synthesis in cultured human coronary artery smooth muscle cells (HCASMCs) were also evaluated.

Methods

All animal experiments were carried out in accordance with the provisions of the European Union Council Directive 86–609, recognized by the Italian Government.

Animal experiments

C57BL/6J male mice homozygous for *COX-1* gene disruption (*COX-1*$^{-/-}$), and littermate wild-type (WT) control animals aged 12–14 weeks, provided by Harlan Laboratories, were employed. Under anaesthesia with chloral hydrate, mice were implanted subcutaneously with osmotic minipumps (Alzet Corp., Palo Alto, CA, USA) which infused Ang II (400 ng/kg/min; Peninsula, Palo Alto, CA, USA) or saline. Three additional groups of Ang II-infused WT mice received SC-560 (COX-1 inhibitor, 5 mg/kg/day, in food), DFU (COX-2 inhibitor, 5 mg/kg/day, in food), or SQ-29548 (TP receptor antagonist, 2 mg/kg/day, i.p.). The doses of SC-560 and DFU were selected on the basis of previous reports.[10,11] SQ-29548 was administered by i.p. route owing to its very low bioavailability by oral route.[12] Mice were treated for 2 weeks. Systolic blood pressure (SBP) was measured by the tail-cuff method, as previously described.[9]

Preparation and mounting of small mesenteric arteries

A second-order branch of the mesenteric arterial tree was dissected and mounted on a pressurized myograph, as previously described.[5] Pressure–lumen diameter relationship was assessed as intravascular pressure was increased step-wise from 3 to 140 mmHg. Media thickness and lumen diameter were measured at each pressure level (see Supplementary material online, *Methods* for further details).

Vascular measurements and mechanics

Media cross-sectional area (CSA) was estimated by subtraction of internal CSA from external CSA using external plus lumen diameters.[4] The mechanical properties of mesenteric small arteries (circumferential strain, circumferential stress, and incremental elastic modulus) were calculated as previously described.[4] The growth index was calculated as $(CSA_h - CSA_n)/CSA_n$, where CSA_n and CSA_h were media CSA of normotensive and hypertensive vessels, respectively.[4]

Immunostaining of COX-1, type I collagen and fibronectin, and histochemical detection of elastin

After collection, mesenteric small arteries were immediately fixed in cold 4% paraformaldehyde and paraffin-embedded at 56°C. Eight micrometre-thick sections were immunostained by rabbit polyclonal anti-COX-1 (1:1000, Cayman), polyclonal anti-type I collagen (1:1500, Abcam), and monoclonal anti-fibronectin (1:10000, Epitomics) antibodies, as previously reported[9] (see Supplementary material online, *Methods* for further details). Histochemistry for elastic and collagen fibres was performed by the Verhoeff-van Giesson staining recommended for vascular pathology.[6] In the whole-artery section, the percentage of labelled wall area was quantified by means of an image analysis software (McBiophotonics Image J) and normalized to the total area examined.

Real-time polymerase chain reaction

Real-time polymerase chain reaction (RT-PCR) assays were employed for detection of COX-1 or COX-2 mRNA in mouse mesenteric arteries and procollagen I mRNA in HCASMCs (see Supplementary material online, *Methods* for further details).

Western blot analysis

Western blot analysis was carried out to detect COX-1 and COX-2 protein expression in mouse mesenteric arteries, and COX-1 and TP receptor protein expression in HCASMCs (see Supplementary material online, *Methods* for further details).

6-Keto-prostaglandin $F_{1\alpha}$ and 8-isoprostane assay

Concentrations of 6-keto-prostaglandin (PG) $F_{1\alpha}$ and 8-isoprostane were determined in the incubation medium of isolated mesenteric vessels by means of enzyme immunoassay commercial kits (Cayman Chemical, Ann Arbor, MI, USA), as previously described.[13] For this purpose, the incubation medium was frozen in liquid nitrogen immediately after collection, stored at −70°C until processed, and assayed in accordance with manufacturer's instructions.

Cell culture

Human coronary artery smooth muscle cells were purchased from Cascade Biologics (C-017–5C Invitrogen, Paisley UK) and maintained in a specific growth medium consisting of Basal Culture Medium 231 modified by addition of cell phenotype specific growth factors and other supplements, as recommended by the manufacturer. Twenty-four hours before the experiment, cells were plated at the density of 1×10^6cells/well in six-well culture plates in basal medium. Cells were then incubated with 0.1 μmol/L Ang II for 24 h, in the absence or in the presence of 1 h pre-incubation with SC-560 (1 μM) or SQ-29548 (10 μM). Additional cells were incubated with the TP receptor agonist U46619 (0.1 μM), alone or in the presence of SQ-29548 (10 μM). In control and Ang II-treated cells, TP receptor and COX-1 expressions were detected by western blotting. In all groups of cells, gene expression of procollagen I, a marker of collagen synthesis,[14] was detected by real-time PCR. The concentrations of Ang II and SC-560, U46619, or SQ-29548 were selected according to

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previous reports[9,14,15,17] as well as on the basis of preliminary concentration-response analysis of procollagen I gene expression (Supplementary material online, *Figure S1*).

Drugs and solutions

See Supplementary material online, *Methods*.

Statistical analysis

Results are presented as mean ± SEM. Morphological data and band optical density were analysed by two-sided one-way ANOVA followed by the Student Newman–Keuls test. Mechanical parameters were compared by repeated-measures two-sided one-way ANOVA. A value of $P < 0.05$ was considered statistically significant. Statistical analyses were performed using Prism software (GraphPad Software, Inc, version 5.0).

Results

Body weight, systolic blood pressure, and arterial morphology

Body weight was similar in all groups (*Table 1*). Systolic blood pressure was significantly increased by Ang II infusion when compared with controls, and not statistically modified by any test drug. In *COX-1*$^{-/-}$ mice, the Ang II-induced SBP increment was less evident (*Table 1*). At 45 mmHg of intraluminal pressure, lumen diameters did not differ significantly among groups (*Table 1*). In WT mice, media thickness was increased by Ang II, resulting in an increase in M/L ratio, media CSA and growth index, indicating a degree of vascular hypertrophic remodelling (*Table 1*). These changes were partly reversed by either SC-560 or SQ-29548, but not statistically modified by DFU (*Table 1*). The same patterns were observed when these parameters were studied under progressive increments of intraluminal pressure (*Figure 1A* and *B*). In *COX-1*$^{-/-}$ mice, Ang II failed to increase media thickness, M/L, or media CSA (*Table 1*). These parameters were not statistically different under intraluminal pressure increments (*Figure 1C* and *D*).

Vascular mechanics

In arteries from Ang II-infused WT mice, intraluminal pressure increments increased the media stress to a lesser degree than in control vessels (*Figure 2A*). In the SC-560 or SQ-29548 groups, the media stress returned towards control values. In contrast, this parameter was not statistically different in the DFU group (*Figure 2A*). Angiotensin II shifted to the left the stress–strain curve, indicating the presence of enhanced vessel stiffness. This effect was counteracted to a similar extent by SC-560 or SQ-29548, but it was not significantly modified by DFU (*Figure 2B*). Incremental elastic modulus plotted against increments of intraluminal pressure demonstrated a similar pattern in all experimental groups (data not shown). When examined as a function of media stress, the incremental elastic modulus was significantly greater in Ang II group, not statistically different in the DFU group, and significantly ameliorated by SC-560 or SQ-29548 (*Figure 2C*). In *COX-1*$^{-/-}$ mice, media stress values secondary to intraluminal pressure increments were similar to controls, and not affected by Ang II infusion (*Figure 2D*). Moreover, the stress–strain curve and the incremental elastic modulus as a function of media stress did not differ from controls and were not significantly modified by Ang II treatment (*Figure 2E* and *F*).

Vascular wall expression of COX-1, type I collagen, fibronectin, and elastin

The immunohistochemical amount of COX-1, constitutively expressed in endothelial and smooth muscle cells of mesenteric arteries from WT mice (*Figure 3A*), was enhanced by Ang II infusion mainly in the muscular layer (*Figure 3B*).

Mesenteric arteries from Ang II-infused WT mice displayed a marked wall thickening with a significant increment of collagen fibres, when compared with controls; type I collagen enhanced deposition was detected mainly in the outer layer of tunica media and adventitia (*Figure 4A* and *B*; *Table 2*), and such a pattern was confirmed by collagen histochemical assay (*Figure 5A* and *B*). This Ang II-induced effect was prevented to

Table 1 Physiological and vascular morphological parameters

	Wild-type mice					*COX-1*$^{-/-}$ mice	
Parameter	**Ctrl ($n = 8$)**	**Ang II ($n = 8$)**	**Ang II + SC-560 ($n = 8$)**	**Ang II + DFU ($n = 8$)**	**Ang II + SQ ($n = 8$)**	**Ctrl ($n = 8$)**	**Ang II ($n = 8$)**
Body weight, g	25.5 ± 1.6	25.2 ± 2.8	26.4 ± 2.5	25.4 ± 3.2	25.9 ± 2.9	25.8 ± 3.1	25.3 ± 2.7
SBP, mmHg	106 ± 5	169 ± 7*	157 ± 10*	163 ± 8*	160 ± 6*	104 ± 5	144 ± 6**
Lumen diameter, μm	203 ± 9	196 ± 5	202 ± 6	203 ± 4	206 ± 6	208 ± 6	197 ± 5
Media thickness, μm	11.1 ± 0.2	14.5 ± 0.3*	12.5 ± 0.2***	14.5 ± 0.2*	12.3 ± 0.4***	11.4 ± 0.3	11.7 ± 0.2
M/L (%)	5.5 ± 0.2	7.4 ± 0.2*	6.2 ± 0.3***	7.4 ± 0.1*	6.0 ± 0.3***	5.5 ± 0.1	5.9 ± 0.2
Media CSA, $10^3 \times \mu m^2$	7.4 ± 0.4	9.6 ± 0.4*	7.4 ± 0.3	9.5 ± 0.3*	8.2 ± 0.3	7.8 ± 0.3	7.7 ± 0.1
Growth index (%)	—	28.0	12.8	28.3	13.1	2.4	2.8

Ctrl, controls; SQ, SQ-29548; SBP, systolic blood pressure; M/L, media to lumen ratio; CSA, cross-sectional area.
*$P < 0.05$ vs. Ctrl.
**$P < 0.05$ vs. wild-type Ang II.

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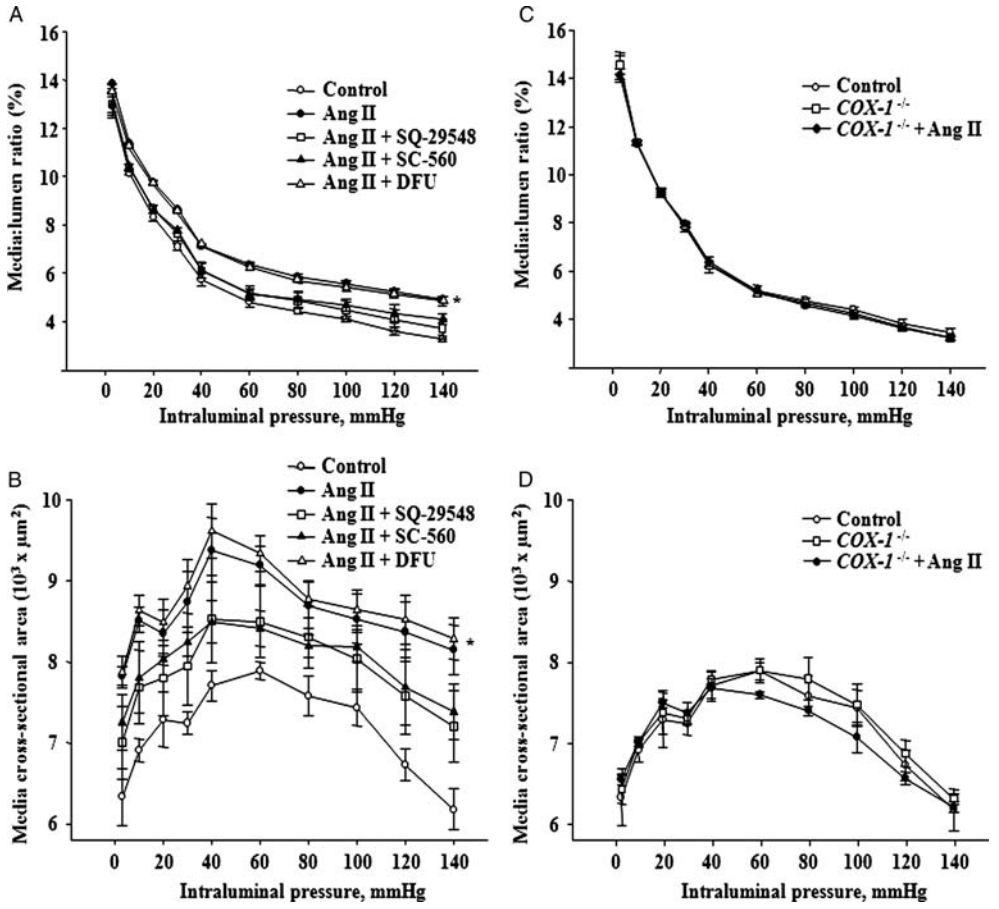


Figure 1 Mechanical properties of mesenteric resistance arteries. The media:lumen ratio and media CSA at different intraluminal pressures in WT (Control, *A*–*B*) and *COX-1*$^{-/-}$ mice (*C*–*D*) under different treatments. Data are presented as means ± SEM ($n = 8$ per group). *$P < 0.001$ vs. other groups (whole curve).

a similar extent by SC-560 and SQ-29548, but not statistically modified by DFU (*Figure 4C* and *D*; *Figure 5C* and *D*; *Table 2*). Likewise, fibronectin vascular deposition was markedly increased by Ang II (*Figure 4F* and *G*; *Table 2*), and this effect was prevented by SC-560 or SQ-29548 to a similar extent, but not significantly modified by DFU (*Figure 4H* and *I*; *Table 2*). The elastin-rich basal wavy pattern of the internal and external elastic membranes in control arteries was significantly reduced and deranged in Ang II-infused WT mice (*Figure 5A* and *B*; *Table 2*) and fully prevented by SC-560 and SQ-29548 treatments, but not significantly modified by DFU (*Figure 5C* and *D*; *Table 2*). As a consequence, the collagen-to-elastin ratio, which was significantly increased by Ang II, returned to normal values after SC-560 and SQ-29548 administrations, and was not ameliorated by DFU (*Table 2*).

In vessels from *COX-1*$^{-/-}$ mice, Ang II did not affect type I collagen and fibronectin depositions (*Figure 6*; *Table 2*). Similarly, vascular elastin content was not modified by Ang II infusion (*Figure 5E* and *F*; *Table 2*). As a consequence, the collagen-to-elastin ratio secondary to Ang II was similar to controls (*Table 2*).

Real-time polymerase chain reaction analysis of COX-1 and COX-2 expression

A basal expression of mRNA encoding COX-1 and COX-2 was detected in mesenteric vessels from WT mice. Angiotensin II infusion significantly induced COX-1 expression, which was not affected by treatment with SC-560, DFU, or SQ-29548 (*Figure 7A*, upper panel). In contrast, the expression of COX-2 mRNA was down-regulated in vessels from Ang II-infused animals, and not significantly modified by any concomitant treatment (*Figure 7A*, middle panel). In *COX-1*$^{-/-}$ mice, the expression of mRNA encoding COX-1 was not detected, and not significantly induced by Ang II. Expression of COX-2 mRNA was basally detected, and down-regulated by Ang II (*Figure 7A*, lower panel).

Western blot analysis of COX-1 and COX-2

A basal expression of COX-1 was detected in vessels from WT mice. Angiotensin II significantly induced COX-1 expression, which was not significantly affected by SC-560, DFU, or SQ-29548 treatments (*Figure 7B*). The expression of COX-2 was

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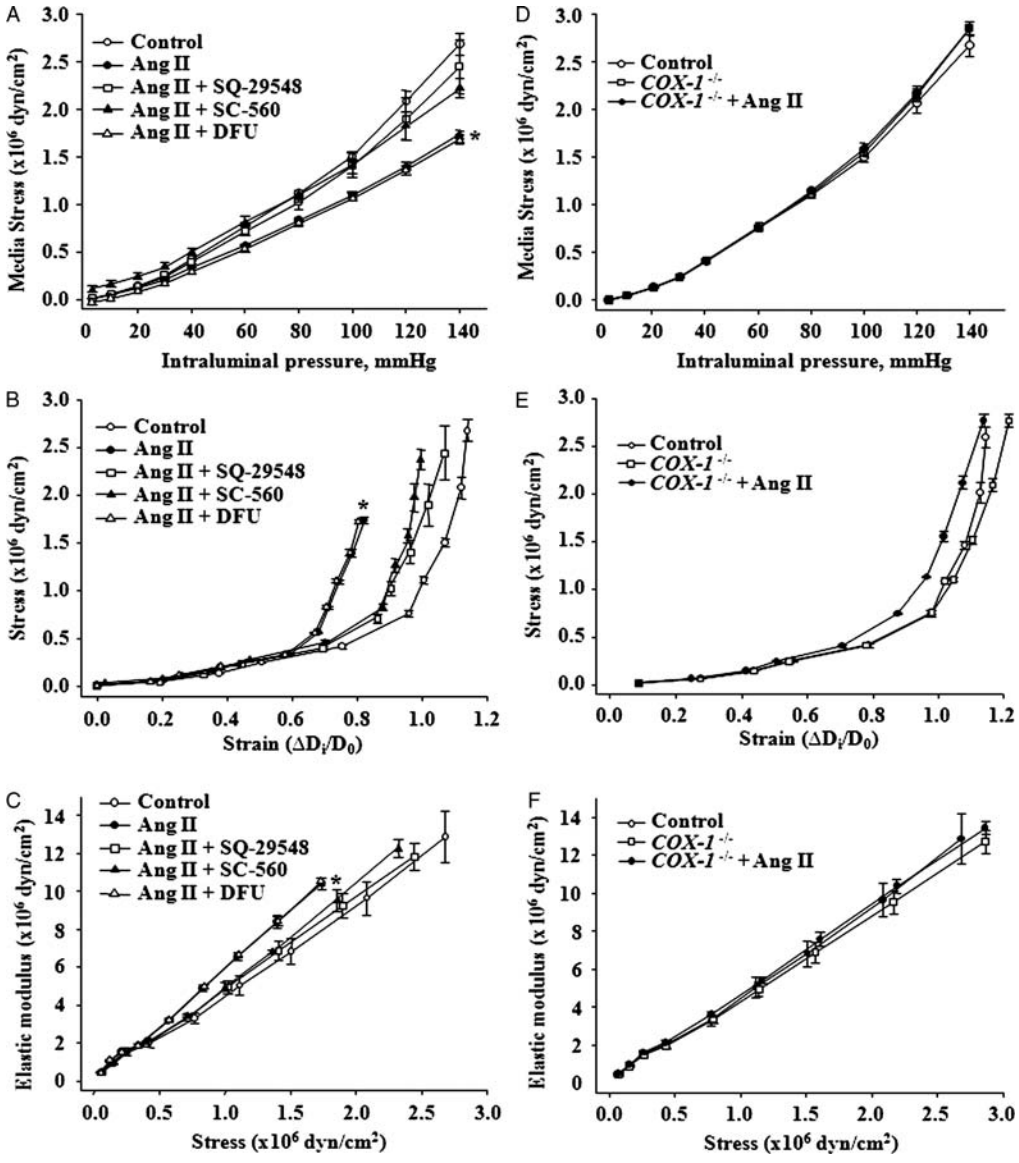


Figure 2 Mechanical properties of mesenteric resistance arteries. Media stress at different intraluminal pressures, media stress–strain relationship, and incremental elastic modulus plotted against media stress in WT (Control, *A*–*C*) and *COX-1*$^{-/-}$ mice (*D*–*F*) under different treatments. Data are presented as means ± SEM ($n = 8$ per group). *$P < 0.001$ vs. other groups (whole curve).

down-regulated in vessels from Ang II-infused animals, and not significantly modified by any concomitant drug treatment (*Figure 7B*).

6-Keto-prostaglandin $F_{1\alpha}$ and 8-isoprostane production

At baseline, the release of 6-keto-$PGF_{1\alpha}$ was higher in the incubation medium of mesenteric vessels from Ang II-treated WT mice when compared with controls (*Table 2*). This increased production was not statistically different in the DFU or SQ-29548 groups, but it was significantly prevented by SC-560. In *COX-1*$^{-/-}$ mice, 6-keto-$PGF_{1\alpha}$ production was not significantly induced by Ang II (*Table 2*). At baseline, 8-isoprostane production was detected in WT. This production was slightly reduced by Ang II, not affected by SC-560 or SQ-29548, and significantly reduced by DFU. In *COX-1*$^{-/-}$ mice, basal 8-isoprostane production was not significantly affected by Ang II (*Table 2*).

Angiotensin II-induced COX-1 expression and collagen synthesis in HCASMCs: effect of SC-560 and SQ-29548

Incubation of HCASMCs with Ang II for 24 h resulted in a significantly increased protein expression of COX-1 (*Figure 8A*). TP receptor protein expression, which was present under basal conditions, was not significantly modified by Ang II (*Figure 8A*).

Procollagen I gene expression, detected at baseline, was markedly enhanced by Ang II. This response was prevented by

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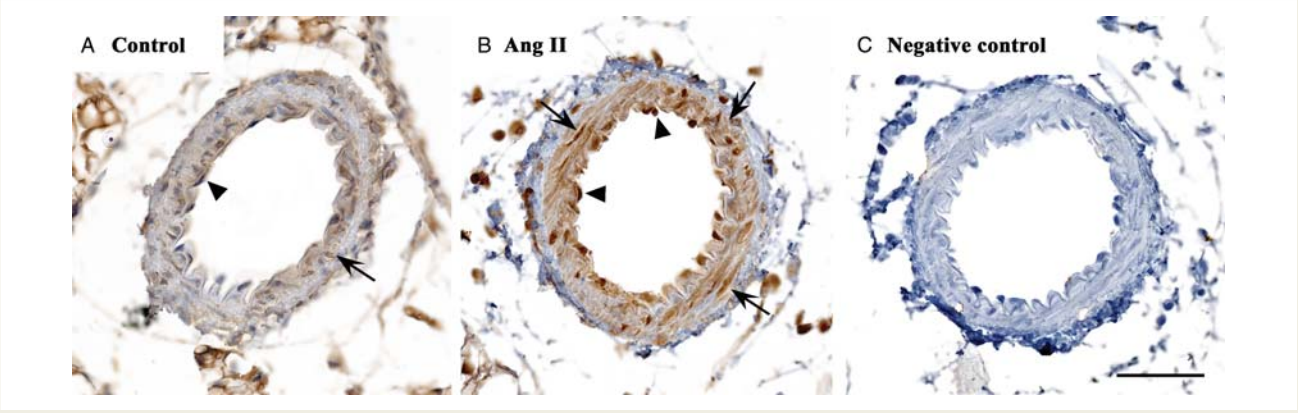


Figure 3 Immunostaining of COX-1 in small mesenteric arteries from control (*A*) and angiotensin II-infused (*B*) WT mice. The basal COX-1 expression detected in endothelium and muscle cells is enhanced by angiotensin II infusion. Negative control for COX-1 (*C*). Scale bar: 50 μm.

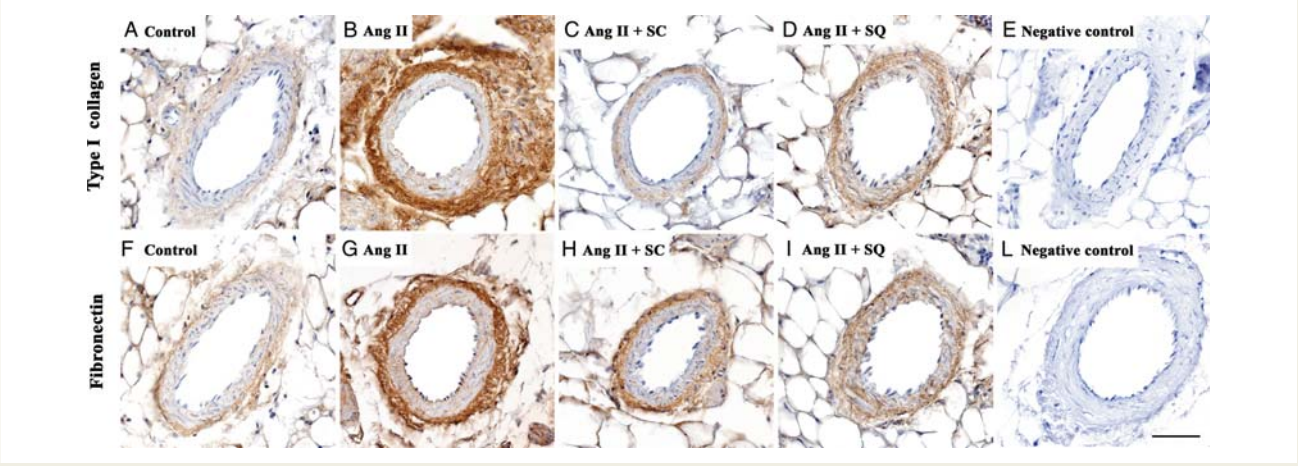


Figure 4 Immunostaining of type I collagen (*A–D*, brown staining) and fibronectin (*F–I*, brown staining) in small mesenteric arteries from controls, angiotensin II-infused, angiotensin II + SC-560 (SC)-treated and angiotensin II + SQ-29548 (SQ)-treated wild-type mice. Type I collagen (A) and fibronectin (F) in control walls were enhanced by angiotensin II (B, G, respectively), and prevented by SC (C, H, respectively) or SQ (D, I, respectively). Negative controls for type I collagen (E) and fibronectin (*L*). Scale bar: 50 μm.

SC-560 or SQ-29548 to a similar extent (*Figure 8B*). Moreover, procollagen I expression was dramatically increased by the TP receptor agonist U46619, and this effect was abrogated by SQ-29548 (*Figure 8B*).

Discussion

The first major novel finding of our study consists of the demonstration that Ang II-induced changes in structure and mechanical properties of small resistance arteries depend on the activation of COX-1 pathway. Angiotensin II-induced hypertrophic remodelling was partly reduced by administration of SC-560, but not DFU, thus indicating that COX-1, but not COX-2, contributes to the pathogenesis of vascular structural changes elicited by Ang II. Of note, SC-560 and DFU were employed at a fixed standard dose known to ensure a selective blockade of COX-1 and COX-2, respectively,[10,11] and dose–response curves were not constructed for either drug, since a selectivity loss was expected to occur when testing these drugs at increasing doses. Nevertheless, the present findings, obtained by a pharmacological approach, were strongly substantiated by our results from COX-1-KO mice, in which Ang II failed to elicit vascular remodelling.

Our data extend to the vascular structure our previous observations, indicating a significant involvement of COX-1 in the development of endothelial dysfunction induced by Ang II in resistance arteries.[9] In the present study, we demonstrate that smooth muscle cells within the media layer are an important target of several Ang II-induced vascular deleterious effects, and that these cells represent a major source of COX-1, as documented by immunostaining results.

With regard for the COX-1-derived product implicated in the vascular alterations elicited by Ang II, we observed that the

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Table 2 Percent relative amounts of media ECM components, 6-keto-$PGF_{1\alpha}$ and 8-isoprostane levels

Mesenteric arteries	Wild-type mice					*COX-1*$^{-/-}$ mice	
	Ctrl ($n = 8$)	Ang II ($n = 8$)	Ang II + SC-560 ($n = 8$)	Ang II + DFU ($n = 8$)	Ang II + SQ ($n = 8$)	Ctrl ($n = 8$)	Ang II ($n = 8$)
Type I collagen ($\times 10^{-7}$)	21.6 ± 0.8	34.9 ± 1.6*	22.8 ± 1.6	30.2 ± 1.8*	21.0 ± 1.1	20.9 ± 0.9	21.2 ± 1.0
Fibronectin ($\times 10^{-7}$)	21.3 ± 1.2	34.6 ± 2.0*	21.5 ± 1.2	29.8 ± 1.6*	21.8 ± 1.4	18.6 ± 2.1	19.2 ± 1.3
Elastin ($\times 10^{-7}$)	34.1 ± 2.1	17.9 ± 1.4*	27.8 ± 1.3	18.6 ± 0.4*	30.2 ± 1.5	31.8 ± 1.9	31.3 ± 1.4
Collagen/Elastin	0.63	1.94*	0.82	1.62*	0.69	0.65	0.67
6-Keto-$PGF_{1\alpha}$ (μg/g/mL)	19.6 ± 0.4	40.2 ± 0.7*	10.3 ± 0.4**	38.2 ± 0.4*	37.3 ± 1.6*	8.2 ± 1.2	8.5 ± 0.3
8-Isoprostane (pg/g/mL)	10.3 ± 0.1	8.6 ± 1.0	8.3 ± 0.8	5.4 ± 0.6*	8.2 ± 0.7	10.0 ± 0.1	10.1 ± 0.9

Values are expressed as relative intensity/area (pixels). Ctrl, controls; SQ, SQ-29548.
*$P < 0.05$ vs. other groups.
**$P < 0.05$ vs. Ang II.

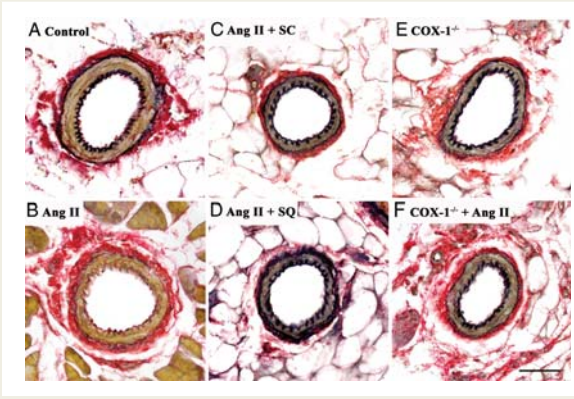


Figure 5 Verhoeff-van Giesson histochemistry for elastic (black) and collagen (red) fibres in small mesenteric arteries from WT and *COX-1*$^{-/-}$ mice. In wild-type mice, the elastic fibres of internal and external elastic membranes in control arteries (*A*) were reduced by angiotensin II (*B*) and maintained by SC-560 (*C*) and SQ 29548 (*D*). In contrast, collagen fibres (A) were increased by angiotensin II (B) and normalized by SC-560 (C) or SQ-29548 (D). In *COX-1*$^{-/-}$ mice the basal expression of elastin and collagen fibres (*E*) was not affected by angiotensin II (*F*). Scale bar: 50 μm.

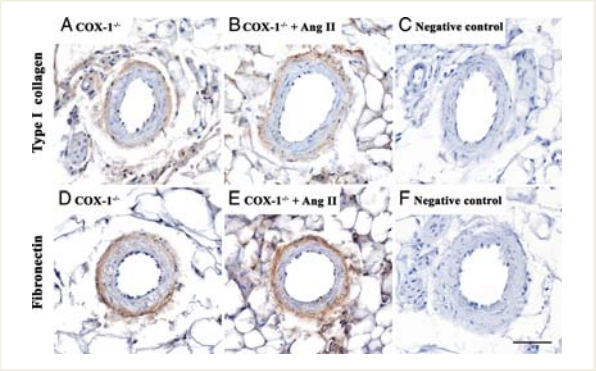


Figure 6 Immunostaining of type I collagen (*A*, *B*) and fibronectin (*D*, *E*), in small mesenteric arteries from control and angiotensin II-infused *COX-1*$^{-/-}$ mice. Basal expression of these arterial components was not affected by the angiotensin II infusion. Negative controls for type I collagen (C) and fibronectin (*F*). Scale bar: 50 μm.

selective TP receptor antagonist SQ-29548 prevented the Ang II-induced structural alterations to a similar extent as that obtained with COX-1 blockade. Moreover, we chose to evaluate 6-keto-$PGF_{1\alpha}$ as a stable metabolite of prostacyclin, which is involved in the control of vascular tone and is mainly produced by COX-1.[13,16] 8-Isoprostane was assayed as a main product of arachidonic acid, which has been detected in vascular tissues from hypertensive animals, and for its ability to activate contracting TP receptors.[17,18] 6-keto-$PGF_{1\alpha}$ levels were found to be higher in the Ang II-treated group and they were not affected by DFU, but being abrogated by SC-560. In *COX-1*$^{-/-}$ mice, 6-keto-$PGF_{1\alpha}$ production was almost absent, and not stimulated by Ang II. In contrast, 8-isoprostane was not stimulated by Ang II, and it was reduced by DFU. Taken together, these data allow us to propose the novel concept that a COX-1-derived prostanoid acting on TP receptors, likely prostacyclin, plays a major role in the pathogenesis of Ang II-induced vascular remodelling. This proposal is in line with previous convincing demonstrations that prostacyclin is a kind of 'good guy' which, under certain circumstances, turns 'bad' causing vascular contraction via TP receptor activation.[19]

In concomitance with structural changes, Ang II evoked alterations in resistance artery wall mechanics. The vessel walls of Ang II-infused mice were significantly stiffer, a condition which was ameliorated by administration of SC-560 or SQ-29548, but not DFU. Angiotensin II failed also to increase vascular stiffness in *COX-1*$^{-/-}$ mice. Taken together, these findings strengthen the concept of a predominant role played by the COX-1 pathway in Ang II-induced alterations of vascular mechanics. Of note, the prevention of vascular mechanical alterations by the COX-1 inhibitor resulted in a modest decrease in SBP, thus confirming the

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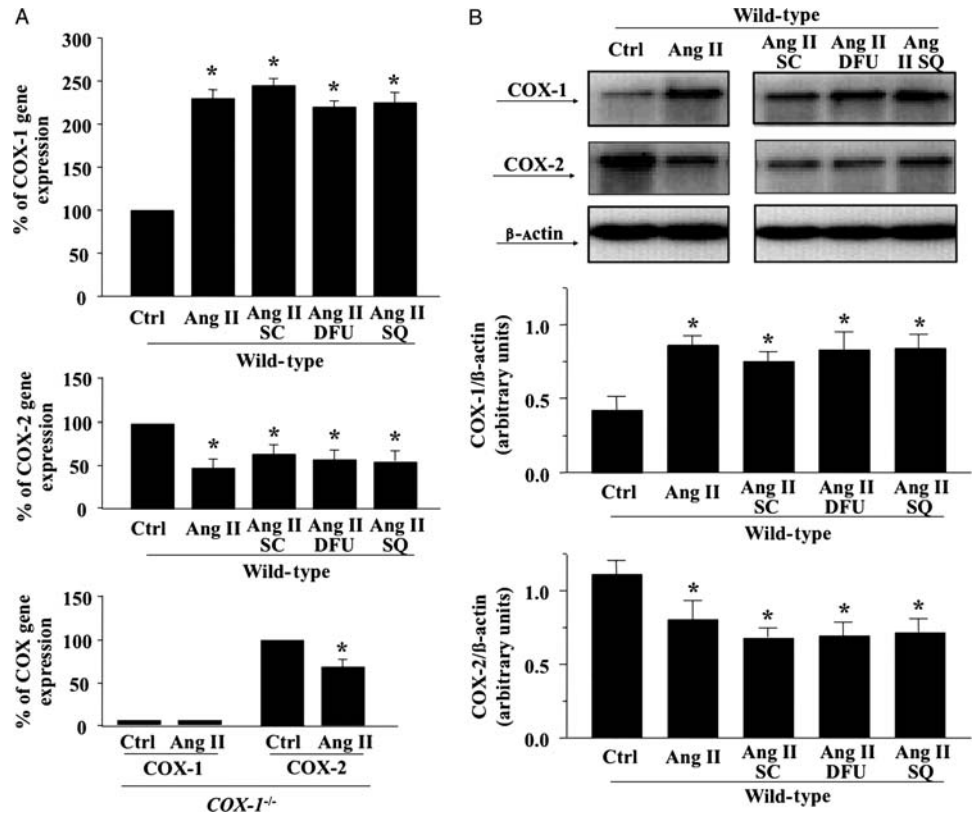


Figure 7 (*A*) Real-time polymerase chain reaction analysis of COX-1 (upper panel) and COX-2 (medium panel) mRNA expression in mesenteric vessels from wild-type mice under control conditions (Ctrl) or treatment with angiotensin II ± SC-560 (SC), DFU or SQ-29548 (SQ). Lower panel shows real-time polymerase chain reaction analysis of COX-1 and COX-2 in *COX-1*$^{-/-}$ mice ± angiotensin II. Each column represents the mean of 3–5 experiments ± SEM. *$P < 0.05$ vs. Ctrl. (*B*) Western blot analysis of protein products for COX-1, COX-2, and β-actin in mesenteric vessels from wild-type mice under control conditions (Ctrl) or treatment with angiotensin II ± SC, DFU, or SQ. Panels display a representative blot and column graphs referring to the densitometric analysis of immunoreactive bands normalized to the expression of β-actin. Each column represents the mean of five experiments ± SEM. *$P < 0.05$ vs. Ctrl.

contention that the resistance vascular bed is a major determinant of diastolic blood pressure, while contributing only in part to the systolic component.

The stiffness of arterial wall depends on balance between more distensible components, such as elastin, and less distensible elements, such as collagen and fibronectin.[3,20] Our results from immunohistochemical and histochemical assays confirm the ability of Ang II to increase collagen type I and fibronectin depositions and decrease elastic fibres in small resistance arteries, as previously demonstrated.[4,6,21] In this regard, the second novel finding of the present study deals with a significant role of the COX-1 pathway in Ang II-induced vascular ECM changes. Indeed, the enhanced deposition of collagen and fibronectin was reduced by SC-560 and, to a similar extent, SQ-29548, but not affected by DFU. In parallel, the vascular content of elastin was completely restored by COX-1 and TP receptor blockade, while being not ameliorated by COX-2 inhibition. In vessels from *COX-1*$^{-/-}$ mice, collagen, fibronectin, and elastin contents did not differ from WT, and they were not modified by Ang II. Overall, these findings support a relevant role of COX-1-dependent TP receptor activation in the development of Ang II-induced changes in ECM components, leading to vascular fibrosis.

Our proposed causal relationship between COX-1, TP receptor activation and altered ECM composition is strongly supported by the present findings in cultured HCASMCs. Such cells were chosen because coronary arteries represent a critical target of atherosclerotic disease. They display also a wall composition and structure not substantially different from mouse mesenteric arteries, and express TP receptors. These experiments showed that Ang II elicited a marked stimulation of COX-1 and procollagen I expression, and that the collagen synthesis was prevented by COX-1 or TP receptor blockade. In addition, a marked collagen induction, similar to that evoked by Ang II, was observed also when TP receptors were activated by a selective agonist. The latter finding underlines in a conclusive manner the pivotal role of TP receptor activation in promoting collagen synthesis at level of smooth muscle cells in resistance arteries. However, it must be noted also that, based on our immunohistochemical and histochemical results, Ang II infusion to WT mice promoted a marked accumulation of type I collagen in the adventitial layer of arterial

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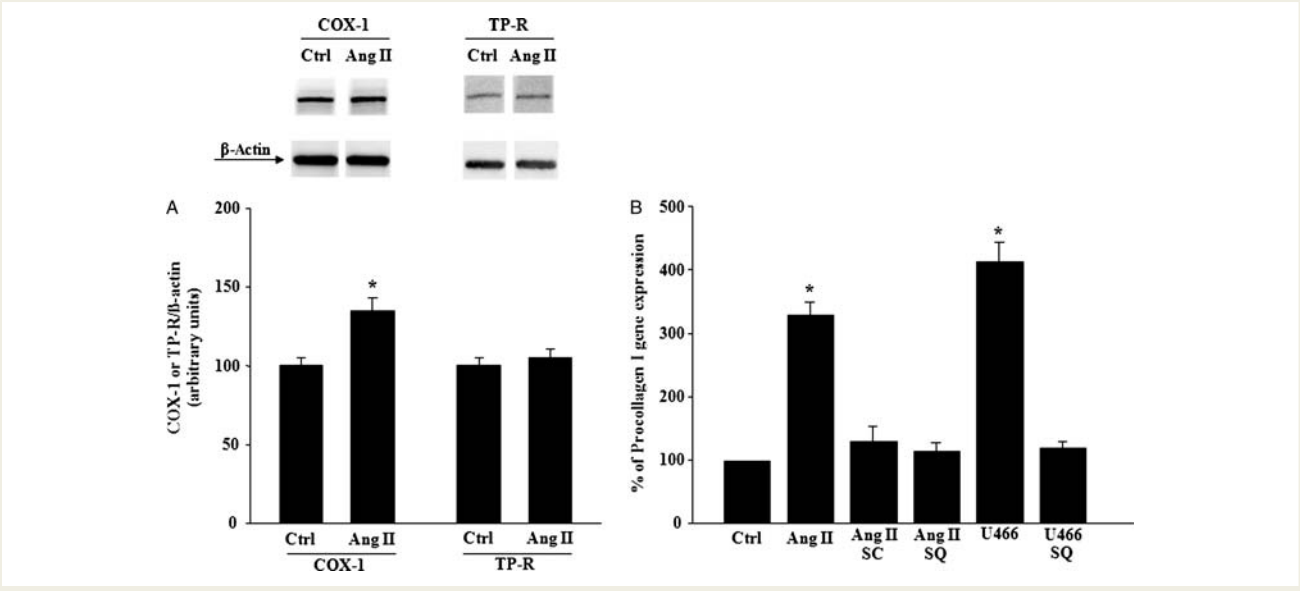


Figure 8 (*A*) Western blot analysis of COX-1 and TP receptor (TP-R) protein expression in human coronary artery smooth muscle cells at baseline (Ctrl) and after treatment with angiotensin II. Panels display a representative blot of three repeated experiments. Housekeeping β-actin expression is also reported. Column graphs refer to the densitometric analysis of three experiments ± SEM. *$P < 0.05$ vs. Ctrl. (*B*) Real-time polymerase chain reaction analysis of procollagen I mRNA expression in human coronary artery smooth muscle cells at baseline (Ctrl) and after incubation with angiotensin II ± SC-560 (SC) or SQ-29548 (SQ), or after incubation with U46619 ± SQ. Each column represents the mean of three to five experiments ± SEM. *$P < 0.05$ vs. Ctrl.

vessels. This observation suggests that, beside the smooth muscle cells, other cell types, such as fibroblasts and/or myofibroblasts, are called into play by Ang II as determinants of vascular remodelling. The specific mechanisms whereby the adventitial remodelling contributes to Ang II-induced arterial fibrosis remain to be established and deserve future investigations.

Our findings, showing that COX-1 contributes to Ang II-induced vascular alterations in structure, mechanics and composition agree with previous evidence, obtained in other animal models of atherosclerosis, where the pharmacological blockade of COX-1, or the TP receptor antagonist S18886 was able to inhibit aortic atherosclerotic injury.[7,8] Taken together, these observations strongly suggest a predominant role of COX-1-derived activators of TP receptors in the development/progression of vascular atherosclerosis. In line with this concept, the results of the present study provide the first demonstration that, at the level of murine small resistance arteries, intravascular COX-1 overexpression, and its derived prostanoids acting on TP receptors represent a major mechanism whereby Ang II induces vascular remodelling and promotes fibrosis. However, the pharmacological blockade of COX-1 was able to reverse only in part vascular remodelling, thus suggesting that other COX-1-independent mechanisms may account for Ang II-induced vascular changes, such as vascular inflammation and aldosterone activity, as previously documented.[22] In this respect, the lack of arterial remodelling in Ang II-treated *COX-1*$^{-/-}$ mice might depend on the fact that, in the absence of COX-1 mediated effects, the COX-1 independent actions require a more prolonged exposure to Ang II to become evident.

Previous papers have demonstrated that some effects of Ang II on vascular wall are mediated by reactive oxygen species (ROS) generation, via vascular NAD(P)H oxidase activation.[22,23] Moreover, a cross-talk between NADPH oxidase and COX-1 was previously documented.[9] Although the molecular mechanisms whereby Ang II modulates the expression/activity of COX isoforms were not specifically investigated in the present study, in preliminary experiments we have observed that chronic administration of the NAD(P)H oxidase inhibitor apocynin prevented Ang II-induced mechanical changes (Virdis *et al.*, unpublished data). These findings, suggesting the possibility that Ang II-mediated vascular remodelling is triggered by NAD(P)H-induced ROS generation, deserve more extensive investigations.

In conclusion, the present study indicates that, in murine mesenteric small arteries, hypertrophic vascular remodelling, changes in mechanics and composition secondary to Ang II infusion are partly dependent on the activation of COX-1 pathway. It is proposed that Ang II is associated with COX-1 overexpression at the level of smooth muscle cells to stimulate the production of prostanoid(s), including prostacyclin, acting as agonist on TP receptors. Such mechanisms are very likely to contribute to the atherosclerotic damage elicited by Ang II.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Conflict of interest: none declared.

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