Activation of AT₂ Receptors by Endogenous Angiotensin II Is Involved in Flow-Induced Dilation in Rat Resistance Arteries

Khalid Matrougui, Laurent Loufrani, Christophe Heymes, Bernard I. Lévy, Daniel Henrion

Abstract—Pressure-induced tone (myogenic, MT) and flow (shear stress)–induced dilation (FD) are potent modulators of resistance artery tone. We tested the hypothesis that locally produced angiotensin II interacts with MT and FD. Rat mesenteric resistance arteries were perfused in situ. Arterial diameter was measured by intravital microscopy after a bifurcation on 2 distal arterial branches equivalent in size (150 μm, n=7 per group). One was ligated distally and thus submitted to pressure only (MT, no FD). The second branch was submitted to flow and pressure (MT and FD). The difference in diameter between the 2 vessels was considered to be FD. Flow-diameter-pressure relationship was established in the absence and then in the presence of 1 of the following agents. In the nonligated segment (MT + FD), angiotensin II type 1 (AT₁) receptor blockade (losartan) had no significant effect, whereas angiotensin II type 2 (AT₂) receptor blockade (PD123319) or saralasin (AT₁ + AT₂ blocker) decreased the diameter significantly, by 9±1 and 10±0.8 μm, respectively. Angiotensin II in the presence of losartan increased the diameter by 18±6 μm (inhibited by PD123319). PD123319 or saralasin had no effect after NO synthesis blockade or after endothelial disruption. In the arterial segment ligated distally (MT only), AT₁ or AT₂ receptor blockade had no significant effect. AT₂-dependent dilation represented 20% to 39% of FD (shear stress from 22 to 37 dyn/cm²), and AT₂-receptor mRNA was found in mesenteric resistance arteries. Thus, resistance arterial tone was modulated in situ by locally produced angiotensin II, which might participate in flow-induced dilation through endothelial AT₂ receptor activation of NO release. (Hypertension. 1999;34:659-665.)

Key Words: blood vessels ■ myogenic ■ arteries ■ stress, mechanical ■ angiotensin II ■ bradykinin

Flow (shear stress)–induced vasodilation and pressure (tensile stress)–induced tone (myogenic tone) play a key role in the control of vascular tone.1–6 In resistance arteries, pressure or stretch induces myogenic tone,6–8 which is opposed by flow-induced dilation, in vitro as well as in vivo.3,5,6,8,9 Whereas myogenic tone is mainly independent of endothelial factors,6,8 shear stress has been widely shown to induce the release of endothelium-derived vasoactive agents.1,4,5,10,11 The local tissue renin-angiotensin system 12–14 is another potent regulator of vascular tone. At physiological concentrations, angiotensin II amplifies agonist-induced contractions.13–15 Angiotensin II also activates nitric oxide (NO) production by vascular endothelial cells through angiotensin II type 1 receptor (AT₁) activation16 or through angiotensin II type 2 (AT₂) receptor activation.17,18 Although no relation between flow-induced dilation and angiotensin II is known as yet, angiotensin II production may be activated by stretch in cardiac myocytes.19,20 Moreover, in the aorta, stretch and angiotensin II synergistically activate DNA and protein synthesis.21 Nevertheless, the role of angiotensin II in the functional response of resistance arteries to pressure and to flow is not yet known. We hypothesized that locally produced angiotensin II might play a role in the acute response (changes in diameter) of resistance arteries to pressure (myogenic tone) and/or to flow (flow-induced dilation).

In rat mesenteric resistance arteries perfused in situ, arterial diameter was measured after a bifurcation on 2 distal branches equivalent in size. One branch was perfused, and the other was ligated distally, so that it was submitted to an identical pressure but not to flow. Thus, in 1 branch (ligated), myogenic tone developed only on pressure stimulation, and in the other branch (left open), myogenic tone was opposed by flow-induced dilation. The difference in diameter between the 2 branches can be considered to be due to flow-induced dilation.

Methods

Mesenteric Artery In Situ

Twelve-week-old normotensive rats were anesthetized (sodium pentobarbital, 50 mg/kg IP), a medial laparotomy was performed, and
where (10 m branch was perfused (154 were dissected free of fat and connective tissues (Figure 1). One Two segments of a mesenteric resistance artery, equivalent in size, 

\[ \text{Flow (F) to the third-order mesenteric artery (3)} \]

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the last loop of the small intestine was exposed and placed into a container allowing the superfusion of the preparation. The following physiological salt solution (PSS) was used (mmol/L): NaCl 130, NaHCO3 14.9, KCl 3.7, CaCl 2 1.2, MgSO4 1.2, glucose 11, and HEPES 10, and albumin 4%. Temperature was 37°C to 38°C, pH 7.4, Po2 160 mm Hg, and PCO2 37 mm Hg. Two segments of a mesenteric resistance artery, equivalent in size, were dissected free of fat and connective tissues (Figure 1). One branch was perfused (154:6.4 µm OD, n=28), and the other was ligated distally, so that it was submitted to pressure but not to flow (158:6.8 µm OD before ligation, n=28). A video camera (Pulnix, CCIR) mounted on a binocular lens allowed recording and analysis of the image of the isolated arterial segments. A polyethylene catheter (OD 0.6 mm, ID 0.28 mm) was placed in the first-generation branch of the mesenteric artery and connected to a syringe infusion pump (Harvard Apparatus) driving a 10-mL syringe (Becton Dickinson). One second-order branch was left open, and a 10-cm-long catheter, connected to a pressure transducer (Gould P10EZ), was inserted into an arterial branch located upstream of the observed segment of artery to record the mesenteric arterial pressure. Flow was measured in the perfused arterial segment with a Transonic T106 small-animal blood flowmeter (Transonic systems Inc). Step increases in flow from 75 to 298 mL/min 11,14,16 (n=7) or captopril (10 µmol/L, n=7) as a nonspecific AT1 receptor blocker, and HOE 140 (0.1 µmol/L) as a specific bradykinin B2 receptor blocker. Only 1 drug was used per experiment. Drugs were added to the superfuse so that both the ligated and the nonligated vessels were submitted to the same concentration of drug. Drugs added extraluminally to the bath of isolated vessels diffuse rapidly through the vascular wall.13

The effect of PD 123319 (1 µmol/L, 30 minutes) on the arterial diameter was also investigated in vessels in which the endothelium had been removed by a perfusion of 1 mL of CO2 at a flow rate of 150 μL/min 11,14,16 (n=7) or in vessels pretreated with the NO synthesis blocker N0-nitro L-arginine methyl ester (L-NAME, 10 µmol/L, 30 minutes, n=7).

In a separate series of experiments (n=7), angiotensin II (0.1 nmol/L) was perfused in mesenteric arteries pretreated with candesartan cilexetil (10 nmol/L).

At the end of each experiment, flow steps and diameter measurements were conducted in the presence of sodium nitroprusside (1 mmol/L) or EGTA (2 mmol/L) and in the absence of extracellular Ca2+ to determine artery passive diameter.10,11,13

Mesenteric Resistance Artery: In Vitro Preparation 

Mesenteric artery segments ~100 µm in ID were isolated and cannulated at both ends and mounted in a video-monitored perfusion system as previously described. 11,12,22,23 Briefly, the artery segment was bathed in a 5-mL organ bath containing PSS (changed at a rate of 4 mL/min). Intraluminal pressure (100 mm Hg) was controlled by a servo perfusion system, and the arterial diameter was measured and recorded continuously with a video monitoring system (Living System Instrumentation Inc). Arteries were submitted to flow (0 to 150 µL/min), and this was subsequently repeated after addition of PD 123319 (1 µmol/L, n=7). Passive diameter was then determined. Results are given in micrometers for artery diameters and flow-induced relaxation.22–24 In another series of experiments (n=6), angiotensin II (0.1 nmol/L) was perfused in mesenteric arteries after pretreatment of the vessels with the AT1 receptor blocker candesartan cilexetil (10 nmol/L).

Reverse Transcription–Polymerase Chain Reaction Analysis of AT2 Receptors 

AT2 receptor mRNA expression was measured by reverse transcription–polymerase chain reaction (RT-PCR). Total RNA was extracted from mesenteric arteries (n=6) with Trizol (Gibco Life Technologies). RT was performed in a reaction volume of 20 µL containing 100 ng RNA, 0.4 µmol/L reverse primer, 2.5 µmol/L dNTP, 6 µL of BRL 5× buffer, 0.6 µL oligo(dT) 12 to 18 primer (0.5 µg/µL), 200 U Moloney murine leukemia virus reverse transcriptase, RNase inhibitor 50 U, and dithiothreitol 10 mmol/L at 39°C for 90 minutes. The reaction was inactivated at 70°C for 10 minutes. The resulting cDNA was amplified with specific primers. For amplification of AT2, receptor cDNA, the sense primer was 5’TGAGTCCGCAATTAACTGCGT-3′ (extension from base 86 to 105), and the antisense primer was 5’ACCACTGAGCATATTCTGCAGG-3′ (extension from base 600 to 622). For GAPDH, the sense primer was 5’TGAAGGTCGTCTTCACTGCGT-3′, and the antisense primer was 5’CATGAGGACATATTCTGCAGG-3′. The amplification profile involved denaturation at 94°C, annealing at 53°C, and extension at 72°C for 30 cycles. The PCR products were separated on a 5% polyacrylamide gel, and radioactivity was measured.

Western Blot Analysis of AT2 Receptors

Mesenteric arteries (n=4) were homogenized with Vibra-Cell (Bioblock Scientific). The homogenate was incubated on ice for 30 minutes in ice-cold lysis buffer B (20 mmol/L Tris-HCl [pH 7.5], 5 mmol/L EGTA, 150 mmol/L NaCl, 20 mmol/L glyc erophosphate, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1 µmol/L Triton X-100, 0.1% Tween-20, 1 µg/mL aprotinin, 1 mmol/L PMSF, 0.5 mmol/L TPCK, and 0.5 mmol/L TLCK) at a ratio of 0.3 mL/10 mg wet wt. Extracts were incubated on ice for 15 minutes and then centrifuged (15 000g, 15 minutes, 4°C). The protein concentration was determined with Micro BCA Protein Assay Kit (Pierce). After denaturation at 100°C for 5 minutes, equal amounts of proteins (20 µg) were loaded on a 9% polyacrylamide gel and transferred to nitrocellulose.
membranes for 12 hours at 65 V at 4°C. Membranes were blocked with 5% nonfat dry milk in TBST (20 mM Tris [pH 8.0], 150 mM NaCl, and 0.1% Tween-20) for 1 hour and were then incubated with rabbit polyclonal antibody against AT2 receptors (50 kDa) (Eurogentec) diluted 1:100 or 1:200 in washing solution at room temperature for 1 hour. The membranes were then washed, incubated with anti-rabbit horseradish peroxidase–conjugated second antibody 1:2000 for 1 hour at room temperature, and washed extensively. An enhanced chemiluminescence system was used as the detection method. Blots were washed and subjected to autoradiography. Molecular weights of proteins were estimated by use of prestained markers (Bio-Rad, 161-0324). Each lane presented in a single panel of the gel picture was from the same gel and the same exposure of the autoradiogram.

Immunofluorescence Analysis of AT2 Receptors

Segments of a mesenteric resistance artery were mounted in embedding medium (Miles, Inc.), frozen in isopentane previously cooled in liquid nitrogen, and stored at −80°C. Immunostaining analysis was performed as previously described23 on transverse cross sections 5 μm thick incubated overnight at 4°C with anti-AT2 antibodies (Eurogentec) at a dilution of 1:200 and then incubated for 30 minutes at 37°C with anti-rabbit antibodies conjugated to FITC (Amersham). Fluorescence staining was visualized with an epifluorescence microscope (Leica). Control experiments were performed to verify the specificity of the antibodies in which the second antibody only was present. In these series of controls, no autofluorescence was observed.

Drugs

Losartan was provided by Dupont-Merck Pharmaceutical, PD 123319 by Parke-Davis, perindopril by Servier, HOE 140 by Hoechst AG, and candesartan cilexetil by Astra Hässle. Other reagents were purchased from Sigma Chemical Co.

Statistical Analysis

Results are expressed as mean±SEM. Significance of the differences between groups was determined by 2-way ANOVA for consecutive measurements (flow rates) or by 1-way ANOVA followed by a Dunnett’s test when appropriate. Values of P<0.05 were considered to be significant.

Results

Phenylephrine-induced tone and acetylcholine-induced dilation were performed before the experimental protocol to test smooth muscle contractility and the presence of the endothelium. Phenylephrine (1 μmol/L) induced a significant decrease in arterial diameter from 158±5 to 86±3 μm (n=35). After this preconstriction with phenylephrine, acetylcholine (10 μmol/L) induced a significant increase in arterial diameter, from 86±3 to 155±8 μm (n=35).

Flow-diameter and pressure-diameter relationships determined in mesenteric resistance arteries under control conditions, in situ, are shown in Figure 2. OD and intraluminal pressure increased when flow was raised by steps. Diameter values were significantly lower in the distally ligated arterial segments (submitted to pressure but not to flow) than in the segments left open and submitted to both pressure and flow. Flow-induced dilation could be estimated as the difference between the diameter in the vessel submitted to both pressure and flow and the diameter of the vessel submitted only to pressure (Figure 2, bottom). Flow rates measured in the arterial segment left open (with flow) ranged from 75±4.5 to 298±4.6 μL/min (n=35). These flow rates corresponded to mesenteric arterial pressures ranging from 48±4 to 71±4 mm Hg. Passive diameter (maximal dilation), determined for each flow step (as given above) in the presence of EGTA (2 mmol/L) or sodium nitroprusside (0.1 mmol/L) and in the absence of extracellular calcium, ranged from 172±3 to 197±4 μm.

The ACEI perindopril (10 μmol/L, n=7) induced a significant increase in diameter in the mesenteric arterial branch submitted to pressure without flow and in the branch submitted to flow and pressure (Figure 3, top). Captopril (10 μmol/L, n=3) had an effect similar to that of perindopril (Figure 3, middle).

Bradykinin B2 receptor blockade (HOE 140, 0.1 μmol/L, n=7), after pretreatment with the ACEI perindopril (10 μmol/L), induced a significant decrease in diameter in the mesenteric arterial branches submitted to flow and pressure and in the mesenteric arterial branches submitted to pressure only (Figure 3, bottom). When bradykinin B2 receptor blockade (HOE 140, 0.1 μmol/L) was used without pretreatment with perindopril, no significant change in diameter occurred in the mesenteric artery branches submitted to flow and/or pressure (0.5±1.0- to 1.8±1.6-μm change in diameter, vessels with flow, n=5, P=NS and 1.2±0.9- to 2.2±1.8-μm change in diameter, vessels with flow, n=5, P=NS).

AT1 receptor antagonism with losartan (10 μmol/L) or with candesartan cilexetil (10 nmol/L) induced no significant change
in diameter in the mesenteric arterial branches submitted either to flow and pressure or to pressure only (Figure 4).

AT\textsubscript{2} receptor antagonism with PD 123329 (0.1, 1, or 10 \(\mu\)mol/L) induced a concentration-dependent decrease in diameter in mesenteric arterial branches submitted to flow and pressure (Figure 5). The effect of PD 123319 (10 \(\mu\)mol/L) was not affected by a pretreatment of the vessels with losartan (10 \(\mu\)mol/L) (1 ± 1.1- to 0.5 ± 1.2-\(\mu\)m change in diameter, \(n=5\)). In the mesenteric arterial branches submitted to pressure only, PD 123319 (1 or 10 \(\mu\)mol/L) induced no significant changes in diameter (Figure 5). Nonselective AT\textsubscript{1} and AT\textsubscript{2} receptor blockade with saralasin (10 \(\mu\)mol/L) significantly decreased the arterial diameter in the mesenteric branches submitted to flow and pressure, not in the arterial branches submitted to pressure only (Figure 5). Similarly, saralasin (10 \(\mu\)mol/L) significantly decreased the arterial diameter (9.2 ± 1.6- to 7.5 ± 1.4-\(\mu\)m decrease in diameter for flow rates ranging from 75 to 298 \(\mu\)L/min) in the mesenteric branches submitted to flow and pressure in presence of losartan (10 \(\mu\)mol/L).

The AT\textsubscript{2} receptor antagonist PD 123329 (10 \(\mu\)mol/L) had no significant effect when used in a vessel pretreated with L-NAME (Figure 6, top) or after endothelium removal (Figure 6, bottom). Inhibition of NO synthesis (L-NAME, 10 \(\mu\)mol/L) induced a decrease in diameter in arteries submitted to pressure and flow (Figure 6) and did not affect

**Figure 3.** Effect of ACE inhibition with perindopril (10 \(\mu\)mol/L, top, \(n=7\)) or captopril (10 \(\mu\)mol/L, middle, \(n=3\)) and effect of bradykinin \(B_2\) receptor blockade (HOE, 0.1 \(\mu\)mol/L, bottom, \(n=7\)) on the diameter of rat resistance mesenteric arteries with or without flow. Results are expressed as change in diameter induced by perindopril, captopril, or HOE 140 (mean ± SEM). Two-way ANOVA: no significant difference (with flow vs without flow).

**Figure 4.** Effect of the AT\textsubscript{1} receptor blocker losartan (10 \(\mu\)mol/L) or candesartan cilexetil (10 \(\mu\)mol/L) on the diameter of mesenteric arteries with or without flow (\(n=7\) per group). Values are mean ± SEM. Two-way ANOVA: no significant difference (with flow vs without flow).

**Figure 5.** Effects of AT\textsubscript{2} receptor blockade (PD 123319, 0.1, 1, or 10 \(\mu\)mol/L) and of the nonselective angiotensin II receptor blocker saralasin (10 \(\mu\)mol/L) on mesenteric artery diameter, with flow or without flow (\(n=7\) per group). Results are expressed as change in diameter induced by PD 123319 or saralasin (mean ± SEM). *\(P<0.001\), 2-way ANOVA for repeated measures, with flow vs without flow.

**Figure 6.** Effect of AT\textsubscript{2} receptor blockade (PD 123319, 1 \(\mu\)mol/L) on mesenteric artery diameter (only arteries submitted to flow are shown, \(n=7\) per group). Arteries were pretreated with the NO synthesis blocker L-NAME (10 \(\mu\)mol/L, top), or the endothelium was removed (bottom). Inhibition of NO synthesis (L-NAME, 10 \(\mu\)mol/L) induced a decrease in diameter in arteries submitted to pressure and flow (Figure 6) and did not affect.
arteries submitted to pressure only (1.5±2.0- to 2.1±2.2-μm change in diameter, n=7).

Perfusion of angiotensin II (0.1 μmol/L) in mesenteric arteries after pretreatment of the vessel with losartan (10 μmol/L) induced a significant dilation (diameter from 90±3 to 108±4 μm, n=7, P<0.001). Angiotensin II–induced dilation was absent when the vessels were pretreated with losartan plus PD 123329 (10 μmol/L) (diameter from 104±6 to 102±7 μm, n=7, P=NS) or when the endothelium was disrupted in the presence of losartan (diameter from 84±9 to 88±11 μm, n=57, P=NS).

In mesenteric arteries isolated in an arteriograph, in vitro, flow induced a dilation (Figure 7) that was significantly less pronounced in vessels pretreated for 30 minutes with the AT1 receptor antagonist PD 123329 (1 μmol/L, Figure 7). In isolated mesenteric arteries pretreated with candesartan (0.1 μmol/L), the perfusion of angiotensin II (0.1 nmol/L) induced a significant dilation (Figure 8).

RT–PCR of AT2 receptors revealed the presence of AT2 receptor mRNA (Figure 9), whereas Western blot analysis showed the presence of the AT2 receptors in the wall of mesenteric resistance arteries (Figure 10). Immunofluorescence analysis indicated that AT2 receptors are present in the endothelium of mesenteric resistance arteries (Figure 11).

Discussion
The major new finding of the present study is that endogenous angiotensin II, through the activation of endothelial AT2 receptors, participated in flow-induced dilation in situ.

In resistance arteries, myogenic origin6–8 is counteracted by flow-induced dilation.3,5,6–7,9 The design of the experiments performed in the present study allowed us to measure a basal tone in the presence or in the absence of flow in 2 daughter arterial branches similar in size. In the branch distally ligated, only pressure was acting on the vessel wall, so that myogenic tone developed without opposition by flow-induced dilation. In the arterial branch submitted to both flow and pressure, myogenic tone was opposed by flow-induced dilation, so the resulting diameter was higher.

Blockade of AT1 receptors (PD 123319) decreased the diameter of the arteries submitted to pressure and flow but did not affect the diameter of the vessels ligated distally. Blockade of AT2 receptor blockers was concentration-dependent. In addition, because AT1 blockade had no effect on the diameter, we used saralasin (AT1 and AT2 blocker) to confirm the effect of PD 123319. Moreover, the dilation depending on the AT2 receptor stimulation by flow could be increased by the infusion of exogenous angiotensin II (in the presence of an AT1 receptor blocker) and could be reproduced in vitro. These observations suggest that locally produced angiotensin II participates in flow-induced dilation through AT2 receptor activation.

Previous studies support our observation that AT2 receptor activation might be involved in flow-induced dilation. Acti-
vation of AT2 receptors induces a dilation in cerebral arteries and in renal afferent arterioles. The AT2 receptors mediate cGMP production and NO synthesis. Also in support of our observation, it has been shown that AT2 receptor gene disruption increases blood pressure. Our results allow at least 2 possibilities to link shear stress and AT2-induced dilation: (1) flow might induce the local release of angiotensin II, which would activate AT2 receptors. This supposes that angiotensin II locally produced acts on endothelial cells only; or (2) flow might also allow a better diffusion of endogenous angiotensin II to its receptors. In agreement with the first possibility, a recent work has shown that short-term increases in shear stress enhance ACE activity. But in this case, angiotensin II produced should activate both AT1 and AT2 receptors, and AT2 receptor blockade had no effect in the present study. In support of the second possibility, it has been shown that flow increases the availability of agonists by influencing their mass transport. Nevertheless, the precise mechanism of AT2-dependent flow dilation remains to be elucidated. In the present study, AT2-dependent vasorelaxation represented a 10-μm increase in diameter. Such a change in a vascular tree might represent a considerable decrease in vascular resistance. In addition, AT2-dependent dilation represented 20% to 39% of the total flow-induced dilation (for shear stress values ranging from 22 to 37 dyn/cm²), obtained in vessels with a spontaneous tone mainly of myogenic origin. No exogenous drug was added. The presence of AT1 receptor and of its mRNA in the wall of mesenteric resistance arteries, evidenced by Western blot analysis, histoimmunofluorescence, and RT-PCR, is in agreement with a previous study performed in the same vessels. In addition, we provided evidence by immunofluorescence that AT2 receptors were present in the endothelium, which is in agreement with the pharmacological study described above.

Dilation induced by ACEIs in both branches (with or without flow) indicates that endogenous angiotensin II is released by the wall of small arteries in the presence or absence of flow. This result extends our previous studies, extending the vascular synthesis of angiotensin II participating in the local control of smooth muscle tone. ACE inhibition induced similar relaxation in the vessel submitted to flow and in the vessel not exposed to flow. This dilatation was in part reversed by bradykinin B2 receptor blockade. HOE 140 alone had no significant effect on the arterial diameter. These results are in agreement with previous studies showing that ACE inhibition induces a relaxation in part mediated by bradykinin, although in some vascular territories, it might not play an important role in basal tone.

Thus, the present work shows that in rat resistance mesenteric arteries in situ, flow-induced dilation involves locally produced angiotensin II via endothelial AT2 receptor activation. Flow-induced AT2-dependent activation resulted in NO synthesis by the endothelium.

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