

**AT2 Receptor-Mediated Relaxation Is Preserved After Long-Term AT1 Receptor Blockade**

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**Abstract**—Angiotensin II type 2 receptor (AT2R) stimulation may cause vasodilation per se and may contribute to the antihypertensive effect produced by Angiotensin II type 1 receptor (AT1R) antagonists, given that AT1R blockade increases endogenous levels of Ang II, suggesting a physiological role for the unblocked AT1R. Thus, we first directly assessed whether or not there is desensitization to AT2R-mediated vasorelaxation because this is an important consideration, given the raised Ang II levels and the marked desensitization that is known to occur after AT1R stimulation. Second, we examined if AT2R-mediated vasorelaxation is preserved after long-term treatment with the AT1R antagonist candesartan cilexetil. Consecutive concentration-response curves to AT2R stimulation, with either Ang II (with AT1R blockade) or the selective agonist CGP42112, were studied in rat isolated mesenteric resistance arteries mounted in an arteriograph. AT1R stimulation with Ang II induced a concentration-dependent relaxation without desensitization. Similarly, CGP42112 evoked highly reproducible relaxation, which, like Ang II, was abolished by the AT2R antagonist PD123319. By contrast, AT1R-mediated contraction exhibited marked desensitization. In rats treated with candesartan cilexetil (2 mg/kg per day for 2 weeks), AT1R-mediated contraction was abolished, whereas AT2R-mediated relaxation evoked by either Ang II or CGP42112 was highly reproducible, PD123319-sensitive, and of a magnitude similar to that observed in naïve animals. Therefore, this study has provided unequivocal evidence for the reproducible nature of AT2R-mediated vasorelaxation during short-term and long-term AT1R blockade. Such preservation of AT1R function is a prerequisite for the consideration of physiological role(s) of AT2R during AT1R blockade. *(Hypertension. 2002;40:516-520.)*

**Key Words:** receptors, angiotensin • muscle, smooth, vascular • vasodilation

Angiotensin II Type 1 receptors (AT1R) mediate well-established physiological effects of angiotensin II (Ang II), including vasoconstriction, aldosterone release, and trophic effects. The physiological role of the AT2 receptor (AT2R), on the other hand, is more controversial. Increasingly, evidence suggests that AT2R stimulation opposes the actions of AT1R stimulation.1 However, recent data with AT1R knockout mice have provided conflicting data regarding the trophic effects of AT1R, in that AT1R in fact mediated Ang II–evoked cardiovascular growth,2,3 as opposed to conventional dogma of a growth-inhibitory effect of AT1R.4 Interestingly, the former studies are consistent with earlier findings in rats with pharmacological AT1R blockade.5 Although the trophic effects of AT1R are open to debate, the potential vasodilator signaling pathways of AT1R stimulation are much more accepted. AT1R are thought to signal through cGMP/nitric oxide and/or bradykinin, at least in the vasculature and kidney.6–9 Indeed, it has recently been shown that short-term in vivo AT1R stimulation in spontaneously hypertensive rats will lower blood pressure.10 In addition, AT1R-mediated relaxation of isolated mesenteric resistance arteries has been reported,11–13 as well as AT2 receptor mRNA and protein expression in this tissue.11,13–15

In vivo, AT1R antagonists are associated with a rise in plasma Ang II concentration as the result of inhibition of the AT1R-mediated negative feedback on renin release. Therefore, it has been suggested that at therapeutic doses of AT1R antagonists, endogenous Ang II may stimulate unopposed AT2R and so contribute to the decrease in blood pressure.9,16 Initial evidence for this hypothesis was mainly indirect, based on enhanced Ang II–mediated vasoconstriction in the presence of AT2R blockade or AT1R knockouts.17–19 Other in vivo evidence for an inhibitory role of the AT2R was provided in chronic heart failure by demonstrating that long-term treatment with the AT1R antagonist PD123319 reversed the beneficial cardiac effects of losartan treatment.20 More recently, it has been reported that short-term administration of PD123319 reversed both the short-term antihypertensive effect and elevated levels of cGMP, NO, and bradykinin in renal interstitial fluid caused by AT1R blockade in renal wrap and salt-restricted rats.21,22 Although these studies are persuasive, they have not directly assessed whether or not Ang II is...
capable of causing AT₂R-mediated vasodilation per se in a reproducible manner.

Ang II–evoked contractions, mediated through AT₁R, in isolated vasculature are notorious for undergoing desensitization after repeat exposure.²³ However, there are no data on the reproducibility of vasorelaxation effects of AT₁R stimulation. If any credence is to be given to the hypothesis that AT₁R-mediated vasodilation contributes to the antihypertensive effects of AT₁R blockade, one has to demonstrate, first, that AT₂R-evoked vasorelaxation does not desensitize, and second, that this mechanism is preserved during AT₁R blockade.

Therefore, the aims of this study were 2-fold: (1) to determine whether or not concentration-response curves (CRC) to AT₂R stimulation in vitro were reproducible, and (2) to determine if AT₂R vasorelaxation did in fact occur After long-term treatment with the AT₁R antagonist candesartan cilexetil.²⁴²⁵

Methods

Rat Mesenteric Artery

Male Wistar-Kyoto rats (Ifla Credo), 8 to 10 weeks old, were anesthetized (sodium pentobarbital, 50 mg/kg), and the gut was excised. The protocol used was in accordance with the European Community standards on the care and use of laboratory animals (authorization No. 00577). A segment of mesenteric artery (389±21 μm, internal diameter, 3 to 5 mm in length) was dissected, cannulated at both ends, and mounted in a video-monitored perfusion system,²⁶ as we have previously described.¹¹²⁷ Briefly, arteries were bathed in a physiological salt solution maintained at 37°C, pH 7.4. The pO₂ was 160 mm Hg and the pCO₂ 37 mm Hg.¹¹²⁷ The artery was superfused (4 mL/min) and perfused (100 mL/min). The intraluminal pressure was set at 75 mm Hg. Arterial diameter was measured (Living Systems Instrumentations) and recorded (Biopac) continuously. Vessels were allowed to stabilize for at least 30 minutes. The integrity of the endothelium was assessed by testing the relaxant effect of acetylcholine (1 μmol/L) after a precontraction with phenylephrine (1 to 3 μmol/L). Thereafter, the tissues were reexposed to phenylephrine (1 to 3 μmol/L) to cause ~20% to 30% reduction in resting diameter, and when this response had reached a plateau, the protocols listed below were performed. Ang II, CGP42112, and PD123319 were purchased from Sigma. Candesartan and candesartan cilexetil were generously provided by Dr Peter Morsing (AstraZeneca).

AT₂R-Mediated Relaxation

Candesartan (100 nmol/L) was incubated for 30 minutes before the construction of two CRCs to Ang II superfusion. Analogous experiments were also performed in which the AT₂R antagonist PD123319 (100 nmol/L) was also added 30 minutes before Ang II CRCs. In another series of experiments, the ability of selective AT₂R stimulation to cause concentration-dependent relaxation was examined. To this end, two CRCs to CGP42112 (in the presence of candesartan) were constructed, and the effect of PD123319 was also tested.

AT₁R-Mediated Contraction

In these vessels, there was no precontraction to phenylephrine, and a CRC to Ang II was initially performed (n=8). In a subgroup of these tissues (n=3), a second CRC to Ang II was repeated. In the remaining tissues (n=5), candesartan was again added (30 minutes' equilibration), and the tissue was precontracted to phenylephrine to perform a relaxation CRC to Ang II.

Figure 1. Typical recordings obtained in mesenteric arteries isolated from rats and perfused under pressure of 75 mm Hg, flow of 100 μL/min, and in the presence of candesartan (100 nmol/L). After precontraction with phenylephrine (PE), cumulative concentrations of Ang II were added to the bath containing the artery. Finally, acetylcholine (ACh, 1 μmol/L) was added to fully relax the artery (top). After washout, the same protocol was repeated in the same artery (middle). In a different artery, a time-control experiment was performed, in which the PE contraction was not followed by the addition of Ang II (bottom).

AT₂R-Mediated Relaxation After Long-Term AT₁R Blockade

Wistar-Kyoto rats were given candesartan cilexetil (2 mg/kg per day in drinking water) for 2 weeks, which is a dose we have used previously to block AT₁ receptor–mediated constriction.²³ Blood pressure in anesthetized animals was measured before removal of mesenteric arteries, as previously described.²⁸

Statistical Analysis

Results are expressed as mean±SEM. The significance of the different treatments was determined by ANOVA or 2-tailed Student paired t test. Probability values <0.05 were considered significant.

Results

In precontracted mesenteric arteries (23±5% decrease in resting diameter), Ang II evoked concentration-dependent relaxation in the presence of AT₁R blockade (Figure 1). This effect of Ang II, significant from 0.1 nmol/L (P=0.04) and maximum at 100 nmol/L (maximum change=23±3 μm or 24% of acetylcholine dilation, n=10, Figure 2), was attributed to AT₁R stimulation since it was abolished by the AT₂R antagonist PD123319 (n=4, Figure 2). Time control experiments, in which tissues were precontracted but not exposed to
Ang II, exhibited negligible change in vascular tone over the time taken to perform a CRC (Figure 1). This AT1R-mediated vasorelaxation was highly reproducible (Figure 1), evidenced by a virtually identical second CRC to Ang II (Figure 2). In addition, the AT1R agonist CGP42112 also caused highly reproducible relaxation of mesenteric arteries (n=6), from a threshold response at 0.1 nmol/L (P=0.02), which was also PD123319-sensitive (n=4) (Figure 2). By contrast, the AT2R-mediated contraction evoked by Ang II (n=8) underwent marked desensitization, since a second CRC to Ang II did not cause contraction (n=3, Figure 2). By contrast, a subset of these tissues (n=5) did subsequently exhibit AT2R-mediated vasorelaxation, which was identical to that observed previously in tissues without any prior exposure to Ang II (Figure 2).

Rats were also treated with the AT2R antagonist candesartan cilexetil (2 mg/kg per day) for 2 weeks. Before treatment,

systolic blood pressure was 115±4 mm Hg (n=5), whereas after treatment, systolic blood pressure was 105±6 mm Hg (n=4). As expected, in mesenteric arteries (resting diameter=369±25 µm) obtained from these animals, AT1R-mediated contraction was abolished (n=4). Strikingly, AT2R-mediated relaxation evoked by Ang II was fully preserved (n=6), with similar changes to those obtained in tissues from naïve animals. Moreover, the AT2R CRC to Ang II was again highly reproducible (n=6) and was blocked by PD123319 (n=4) (Figure 3). Similarly, CGP42112 evoked vasorelaxation (n=6), as seen in tissues taken from naïve rats, which was abolished by PD123319 (n=4) (Figure 3).

Discussion
The novel finding from the current study was that AT2R-mediated vasorelaxation is a highly reproducible phenomenon, as directly assessed by constructing two CRCs to Ang II and measuring vessel diameter. Moreover, this effect was preserved during long-term AT1R blockade. Indeed, this is the first study that has demonstrated that AT2R-mediated vasorelaxation can occur during long-term AT1R blockade with candesartan cilexetil.
Much has previously been inferred from blood pressure changes that do not directly assess vascular relaxation.6–10 These current data confirm previous in vitro studies that have demonstrated AT2R-mediated vasorelaxation with Ang II in mesenteric arteries,11–13 and we have now also shown that CGP42112 behaves as a full AT2R agonist, as has previously been shown in cell culture.29 Consistent with this finding, short-term AT2R stimulation in vivo with CGP42112, in the presence of candesartan, lowered blood pressure in spontaneously hypertensive rats.10 More recently, we have also observed widespread regional vasodilator effects of CGP42112 by using identical protocols in a conscious rats instrumented with Doppler flow probes.30

There is, however, very little known about the pharmacodynamics of AT1R stimulation. Although functional effects of AT1R stimulation in vitro are well known to desensitize in response to prolonged and/or repeated stimulation,25 no information is available concerning AT2R. As expected, AT1R-evoked contraction underwent marked desensitization after a single CRC to Ang II, presumably because of receptor internalization.31 By contrast, for the first time, we have shown that AT2R-mediated vasorelaxation is highly reproducible and occurs at low concentrations of either Ang II or CGP42112, irrespective of whether the tissue was first exposed to either relaxant (AT1R) or contractile (AT1R) activity. Moreover, AT2R-mediated relaxation evoked by either Ang II or CGP42112 was similar in rat vessels taken from naïve animals or animals with long-term AT1R blockade. The reproducible nature of AT2R function is consistent with previous studies that have reported a lack of AT2R internalization52,33 and hence lack of desensitization. In these studies, cellular trafficking of both AT1 and AT2 receptors expressed in human embryonic kidney 293 cells was examined. AT2 receptor cell-surface binding was not altered after prolonged exposure to Ang II,32 and fluorescently labeled AT2 receptors were also not internalized after agonist exposure.33 By contrast, AT1 receptors were rapidly internalized,31,33 which is consistent with the functional data in the present study.

A number of experimental studies have reported full or partial reversal of AT1R antagonist-mediated depressor activity by PD123319,20–22 which indirectly suggests a depressor role for AT1R. The postulated mechanism involves the action of endogenously raised Ang II levels, after AT1R blockade, at the unopposed AT2.9,16 However, implicit in this hypothesis is the assumption that AT1 receptor stimulation is still functionally operative despite chronically elevated plasma Ang II levels.

In the current study, we have shown that this was the case. Although we did not measure Ang II levels in the rats treated with candesartan cilexetil, it would be expected that this treatment would have elevated Ang II levels at least 5-fold.34 As expected, candesartan cilexetil virtually abolished Ang II–evoked contraction in mesenteric arteries, which is consistent with the prolonged duration of action of this compound, both in vivo25 and ex vivo.24 Although it was not expected that AT1R blockade would directly affect AT2R-mediated responses (because different receptor subtypes), it was not known if increased circulating levels of Ang II could desensitize the action of Ang II at AT1Rs, as occurred at AT2Rs. Importantly, at this time, relaxation of mesenteric arteries in response to Ang II or CGP42112 was fully preserved to the extent that these changes were of a similar magnitude and occurred over the same concentration range as observed in tissues taken from untreated rats. The fact that desensitization did not occur under conditions of long-term AT1 receptor blockade, where there are raised Ang II levels, underpins a potential physiological role for AT2Rs. As demonstrated for the first time, we believe this represents “proof of principle” for the concept that AT2R can be activated to cause vasodilation without desensitization, during short-term or long-term AT1 receptor blockade.

Thus, it is likely that AT2R-mediated vasodilation is an important modulatory mechanism during AT1R blockade. Indeed, AT1R blockade alone elevates Ang II and raises cGMP/NO in vasculature and renal interstitial fluid, which can be blocked by PD123319.6–8 Furthermore, it is now also appreciated that AT2R are located in vasculature such as mesenteric artery,11,13–15 as in the current study. Moreover, recent evidence suggests that AT2R density is actually increased with elevated Ang II levels,15 which emphasizes the potentially important physiological role of AT2R.

Collectively, the results from the current study provide unequivocal evidence for the ability of vascular AT2R to directly modulate vascular tone during short-term and long-term AT1R blockade. Given the highly reproducible vasodilation mediated by the AT1R at low concentrations, the AT2R should be considered as a novel target for the treatment of cardiovascular disease such as hypertension.10

**Perspectives**

AT2 receptor stimulation evoked concentration-dependent vasorelaxation without desensitization, which contrasted with AT1 receptor-mediated contraction. Moreover, this AT2R-mediated vasodilator effect was preserved during long-term AT1 receptor blockade. These data provide “proof of principle” for the concept that AT2R can be activated to cause vasodilation without desensitization, which is consistent with the hypothesis that long-term AT1R stimulation by Ang II may contribute to the antihypertensive effects of AT2R antagonists. Clearly, analogous experiments are required with human isolated blood vessels to determine if these results can be extrapolated to humans. Moreover, these findings also raise the question of whether or not other angiotensin peptide fragments, which are reported to cause vasodilation, also exhibit a lack of desensitization.

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