Angiotensin II Type 2 Receptor–Mediated Vasodilation in Human Coronary Microarteries

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Background—Angiotensin (Ang) II type 2 (AT2) receptors are believed to mediate vasodilation, although data to support this concept in humans are not available. Neither in vitro studies investigating Ang II–induced vasoconstriction in isolated human coronary arteries1 and saphenous veins2 nor in vivo studies investigating Ang II–induced vasoconstriction in the forearm vascular bed of healthy volunteers3,4 provided evidence for AT2 receptor–mediated vasodilation. In contrast, both in vitro and in vivo studies in rats and mice support this notion.5–10 One explanation for the discrepancy between the lack of AT2 receptor–mediated vasodilation in human coronary arteries1 and the occurrence of such dilation in the rat coronary vascular bed8 is that AT2 receptors are located in coronary microarteries only. In the present study, we therefore investigated AT2 receptor–induced vasodilation in human coronary microarteries (HCMAs) mounted in Mulvany myographs. We also investigated whether endothelial NO and/or bradykinin type 2 (B2) receptors mediate such vasodilation in HCMAs, because studies in animals support this possibility.10–13 Finally, we verified, both through radioligand binding studies and reverse transcription–polymerase chain reaction (RT-PCR), whether HCMAs express AT2 receptors.

Methods

HCMAs (diameter, 160 to 500 μm) were obtained from 49 heart valve donors (age, 3 to 65 years). Ang II constricted HCMAs, mounted in Mulvany myographs, in a concentration-dependent manner (pEC50, 8.6±0.2; maximal effect [Emax], 79±13% of the contraction to 100 mmol/L K+). The Ang II type 1 receptor antagonist irbesartan prevented this vasoconstriction, whereas the AT2 receptor antagonist PD123319 increased Emax to 97±14% (P<0.05). The increase in Emax was larger in older donors (correlation ΔEmax versus age, r=0.47, P<0.05). The PD123319-induced potentiation was not observed in the presence of the NO synthase inhibitor L-NAME, the bradykinin type 2 (B2) receptor antagonist Hoe140, or after removal of the endothelium. Ang II relaxed U46619-preconstricted HCMAs in the presence of irbesartan by maximally 49±16%, and PD123319 prevented this relaxation. Finally, radioligand binding studies and reverse transcription–polymerase chain reaction confirmed the expression of AT2 receptors in HCMAs.

Conclusions—AT2 receptor–mediated vasodilation in the human heart appears to be limited to coronary microarteries and is mediated by B2 receptors and NO. Most likely, AT2 receptors are located on endothelial cells, and their contribution increases with age. (Circulation. 2004;109:2296-2301.)

Key Words: angiotensin ■ bradykinin ■ microcirculation ■ nitric oxide ■ vasodilation

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separated 6-mL organ baths containing oxygenated Krebs at 37°C. The Krebs was continuously aerated with 95% O2 and 5% CO2, and tissue responses were measured in changes in isometric force, with the use of a Harvard isometric transducer. After a 30-minute stabilization period, the optimal internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 100 mm Hg effective transmural pressure, as described by Mulvany and Halpern.14 In some vessels, the endothelium was removed by gently rubbing a hair through the lumen of the mounted artery. Endothelial integrity or removal was verified by observing relaxation (or lack thereof) to 10 mmol/L substance P after preconstriction with 10 mmol/L of the thromboxane A2 (TxA2) analogue U46619 (Sigma). Subsequently, to determine the maximum contractile response, the tissue was exposed to 100 mmol/L KCl. The segments were then allowed to equilibrate in fresh organ bath fluid for 30 minutes. Next, segments were preincubated for 30 minutes with the Ang II type 1 (AT1) receptor antagonist irbesartan (1 μmol/L, a gift of Bristol-Myers Squibb),28 the AT2 receptor antagonist PD123319 (1 μmol/L, a gift of Parke-Davis),19 the B2 receptor antagonist Hoe140 (1 μmol/L, a gift of Hoechst)10 and/or L-NAME (100 μmol/L, Sigma). Thereafter, concentration-response curves (CRCs) were constructed to Ang II, either directly or after preconstriction with 10 mmol/L U46619 to 60% of the maximum contractile response. A higher concentration of U46619 (30 mmol/L) was required in segments that had been preincubated with irbesartan because irbesartan antagonizes TxA2 receptors.17 The cyclo-oxygenase inhibitor indomethacin (5 μmol/L) was present during the entire experiment to suppress spontaneously occurring contractions and relaxations.

Cyclic GMP Measurement

To study Ang II–induced cGMP production, vessel segments (5 to 10 mg) were exposed to 1 μmol/L Ang II in 10 mL oxygogenated Krebs bicarbonate solution for 1 minute at 37°C in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (100 μmol/L), after a 30-minute preincubulation without (control) or with 1 μmol/L PD123319 or irbesartan. Tissues were then frozen in liquid nitrogen and stored at −80°C. To determine cGMP, frozen tissues were homogenized in 0.5 mL 0.1 mol/L HCl with the use of a stainless steel ultraturrax (Polytron). Homogenates were centrifuged at 3300g, and cGMP was measured in 300 μL supernatant by ELISA (R&D Systems). Results are expressed as picomoles per milligram of protein. The lower limit of detection was 0.1 pmol/mg protein.

Radioligand Binding Studies

Sarcolemmal membrane fractions were prepared from HCMAs and porcine adrenal glands as described before.18 The adrenergals were obtained from three 2- to 3-month-old pigs that had been used in in vivo experiments investigating the effects of calcitonin gene–related peptide receptor (ant)agonists.19 125I–Ang II, prepared with the chloramine T-method (specific activity, 2200 Ci/mmol),20 was used as the radioligand. Assays were run for 60 minutes at 18°C in 30 μL Tris buffer (50 mmol/L), 40 μL membrane fraction (containing 100 μg protein, determined by the Bradford assay as described before18), and 30 μL radioligand (final volume, 100 μL). Nonspecific binding, AT1 receptor–specific binding, and AT2 receptor–specific binding were determined by repeating the experiment in the presence of Ang II (at a concentration 100 times the concentration of 125I–Ang II), irbesartan (0.3 μmol/L to 0.3 μmol/L), and PD123319 (0.3 μmol/L to 0.3 μmol/L), respectively. Incubation was stopped by adding 4 mL ice-cold PBS (pH 7.4). Samples were then filtered through a Whatman GF/B filter. Filters were washed twice with 4 mL ice-cold PBS, and filter-bound radioactivity was measured in a gamma-counter.

AT1 and AT2 Receptor mRNA

Total RNA was isolated from HCMAs, right epicardial coronary arteries, and left ventricular tissue through the use of the Trizol reagent (Gibco-BRL). RT-PCR was performed according to standard procedures and 35 cycles of amplification, using primer sequences as

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Contractions of HCMAs to Ang II in the absence (control, circles) or presence of irbesartan (triangles) or PD123319 (squares). Contractions (mean±SEM, n=5 to 22) are expressed as a percentage of the response to 100 mmol/L K+.* P<0.05 vs control.

follows: AT1 receptor sense 5’-CTT TTC CTG GAT TCC CCA C-3’, and antisense 5’-CTT CTG GGT GGA TGA GCT TAC-3’. AT2 receptor sense 5’-GTG ACC AAG TCA TGA AGA TG-3’ and antisense 5’-CAC AAA GGT CTC CAT TTC TC-3’, resulting in amplification products of 304 and 335 bp, respectively. Positive and negative controls were mRNAs extracted from human liver, a human breast carcinoma cell line (MCF7), and a human colon carcinoma cell line (SW480).21 The absence of nonspecific amplification was verified by running RT-PCR and PCR amplifications without adding tissue extracts. As controls for RNA quality, amplification reactions were performed by using pairs of primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).22 Amplified transcripts were analyzed on 2% agarose gels.

Data Analysis

Data are given as mean±SEM. Contractile or relaxant responses are expressed as a percentage of the contraction to 100 mmol/L K+ or U46619. CRCs were analyzed as described to obtain pEC50 (−logEC50) values.1 Statistical analysis was made by paired t test, once 1-way ANOVA, followed by Dunnett’s post hoc evaluation, had revealed that differences existed between groups. A value of P<0.05 was considered significant.

Results

**Myography Studies**

Ang II constricted HCMAs in a concentration-dependent manner (pEC50=8.6±0.2, n=22), with a maximal response (Emax) of 79±13% (Figure 1). Irbesartan nearly completely blocked the Ang II–mediated constriction. PD123319 increased Emax to 97±14% (P<0.05). PD123319 did not affect the potency of Ang II (pEC50=8.7±0.2, n=22), although in 11 experiments a leftward shift of the Ang II CRC (ie, an increase in the pEC50 value of ≥0.2) was observed in the presence of the AT1 receptor antagonist. The PD123319-dependent increase in Emax was larger in older donors (r=0.47, P<0.05; Figure 2). The increase in Emax was largest in the 11 experiments in which PD123319 induced a leftward shift of the Ang II CRC: +34±10% versus +2.2±8.4% in the experiments in which PD123319 induced either no (ie,
The total amount of protein in the HCMA sarcolemmal membrane fraction (∼500 µg), prepared from vessel segments of 19 subjects, was too small to study a wide range of conditions. We therefore used sarcolemmal membrane fractions prepared from 6 porcine adrenal glands to obtain the most optimal conditions to demonstrate the presence of AT2 receptors in HCMAs. After a 1-hour incubation with 125I-Ang II (final concentration in the incubation mixture, 0.5 nmol/L), total and nonspecific 125I-Ang II binding to porcine adrenal membranes amounted to 4660±150 and 2100±80 cpm/100 µg protein (n=8), respectively. PD123319 and irbesartan abolished specific binding in a concentration-dependent manner (Figure 6A). The inhibitor concentration required to reduce specific binding by 50% (IC50) was 50±1 nmol/L for PD123319. This value mimics the IC50 of PD123319 obtained in previous experiments with cells expressing AT2 receptors only.23 In contrast, the IC50 of irbesartan in the present study (20±1 µmol/L) exceeded its IC50 in cells exclusively expressing AT1 receptors by 3 orders of magnitude.24 Taken together, these data suggest that our porcine adrenal membrane fraction contained predominantly AT2 receptors. A PD123319 concentration of 10 µmol/L is required to fully block 125I-Ang II binding to these receptors.

On the basis of these findings, as well as on previous studies investigating irbesartan concentrations that selectively block AT1 receptors,24,25 we incubated HCMA membranes with 0.5 nmol/L 125I-Ang II in the absence or presence of 50 nmol/L Ang II, 10 µmol/L PD123319, or 1 µmol/L irbesartan. Ang II reduced 125I-Ang II binding from 1813 to 1175 cpm/100 µg protein. PD123319 and irbesartan both reduced specific binding by ∼50%, thereby indicating that HCMAs contain AT1 as well as AT2 receptors (Figure 6B).

AT1 and AT2 Receptor mRNA
RT-PCR revealed expression of AT1 and AT2 receptors in HCMAs, large epicardial coronary arteries, and/or left ventricular tissue from 5 hearts (Figure 6C). Similar data were obtained in additional HCMAs from 7 hearts (data not shown).

Discussion
This study is the first to show AT2 receptor–mediated vasodilation in human blood vessels. Evidence for this effect...
was obtained in two ways. First, the AT₂ receptor antagonist PD123319, at a concentration that has been reported to result in complete blockade of AT₂ receptor–mediated effects, increased the maximal contractile response to Ang II, thereby indirectly demonstrating that AT₂ receptor stimulation counteracts AT₁ receptor–mediated vasoconstriction. Second, during AT₁ receptor blockade with irbesartan (allowing selective AT₂ receptor stimulation), Ang II relaxed preconstricted HCMAs, and this was prevented by PD123319. Such vasodilation was not observed in quiescent HCMAs in the presence of irbesartan, probably because vasodilator responses are more difficult to detect without preconstriction. On the basis of these data, it is clear that at least in HCMAs, the net contractile effect of Ang II is determined by the magnitude of the response mediated through AT₁ (contraction) and AT₂ (relaxation) receptors.

In addition to its effect on Eₘ₉, PD123319 caused a leftward shift of the Ang II CRC in ~50% of the experiments. Such increased potency of Ang II in the presence of PD123319 is not due to an effect of the AT₂ receptor antagonist on Ang II metabolism. It could point to more efficient AT₁ receptor signal transduction during AT₂ receptor blockade. Furthermore, a recent study has suggested that AT₁ and AT₂ receptors form heterodimers. An alternative explanation for the increased potency might therefore be that in some donors AT₁ receptor–AT₂ receptor heterodimers exist that bind Ang II with higher affinity during AT₂ receptor blockade. The underlying assumption for this explanation is, however, that AT₁ and AT₂ receptors in these donors are located on the same cell.

The increase in Eₘ₉ was larger in older donors, suggesting that the contribution of AT₂ receptors increases with age. Although AT₂ receptor density increases under pathological conditions, the donors in the present study died of noncardiac causes and did not use cardiovascular medication. Thus, it is unlikely that the increased Eₘ₉ during AT₂ receptor blockade in older donors simply reflects the occurrence of cardiovascular disorders in these subjects. It might reflect a general decrease of vascular function with age.

In an earlier study in large epicardial human coronary arteries, we were unable to detect AT₂ receptor–mediated vasodilation, whereas vasodilation did occur in the rat coronary vascular bed. The present study solves this discrepancy by demonstrating that AT₂ receptor–mediated vasodilation is limited to coronary microarteries. It is notable that AT₂ receptor expression in HCMAs could be demonstrated by both RT-PCR and radioligand binding experiments. Unexpectedly, AT₂ receptor mRNA was also detected by RT-PCR in large coronary arteries. This would imply that either the AT₂ receptor density in large coronary arteries is too low to allow detection of vasodilation in the organ bath setup or that AT₂ receptors in these arteries mediate other (nondilatory) effects, for example, effects on vascular growth and remodeling. AT₂ receptor expression has been demonstrated before in the human myocardium, including the coronary vascular bed.

The mechanism underlying AT₂ receptor–mediated vasodilation in HCMAs is currently unknown. AT₂ receptors themselves may act as AT₁ receptor antagonists independent
of Ang II.\textsuperscript{27} This would require their occurrence on the same cell, as discussed above. Furthermore, B₂ receptors, NO, cGMP, Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels, and/or phosphatases have been implicated in AT\textsubscript{2} receptor–induced effects.\textsuperscript{6,7,10–13,32,33} Our data with L-NAME and Hoe140 in HCMAs support a role for B₂ receptors and NO. Because the vasodilator effects in HCMAs were observed in the presence of indomethacin, prostaglandins do not appear to be involved. The lack of effect of PD123319 in deendothelialized segments confirms the contribution of endothelial B₂ receptor–induced NO release and simultaneously suggests that AT₂ receptors in HCMAs are located on endothelial cells. In agreement with this concept, cultured human coronary artery endothelial cells do express AT\textsubscript{2} receptors.\textsuperscript{34}

Taken together, the most likely scenario to explain our results is that Ang II stimulates endothelial AT\textsubscript{2} receptors in HCMAs. This results in endothelial B₂ receptor activation and NO release. NO subsequently activates guanylyl cyclase in vascular smooth muscle cells, thereby counteracting the contractile responses mediated by the AT\textsubscript{1} receptors on these cells. Guanylyl cyclase generates cGMP, and although the Ang II–induced (AT\textsubscript{2} receptor–mediated) increase in the microvascular cGMP content in the present study was not significant, the tendency of PD123319 (but not irbesartan) to block this increase mimics similar observations in rat aorta and rat uterine arteries.\textsuperscript{10,33} The lack of significance in the present experiments probably relates to the modest (\textlesssim30\%) increase in cGMP content induced by Ang II as compared with other agonists. For instance, in our experimental setup, 1 \textmu mol/L bradykinin increased microvascular cGMP 7\textsuperscript{2}–fold (\textit{n} = 4, data not shown).

In conclusion, AT\textsubscript{2} receptor–mediated vasodilation occurs in the coronary microcirculation of nondiseased human hearts in an endothelium-dependent manner and is mediated by B₂ receptors and NO. This finding could be of clinical relevance, not only because cardiac AT\textsubscript{2} receptors are upregulated under pathological conditions,\textsuperscript{30} but also because animal studies have shown that the beneficial effects of AT\textsubscript{1} receptor antagonists, in contrast to those of ACE inhibitors, depend on AT\textsubscript{2} receptor stimulation.\textsuperscript{35,36}

**References**


![Image](http://circ.ahajournals.org/)

Figure 6. A and B, Displacement of specifically bound [125I]Ang II by irbesartan or PD123319 in sarcosomal membrane fractions prepared from 6 porcine adrenal glands (A) and 19 HCMAs (B). C, Results from RT-PCR amplification of AT\textsubscript{1} receptor mRNA (304 bp), AT\textsubscript{2} receptor mRNA (335 bp), and GAPDH mRNA in HCMAs (lanes 1 to 3), large epicardial human coronary arteries (lanes 4 to 6) and human left ventricular tissue (lanes 7 to 9) obtained from 5 hearts. Positive controls (T+) for AT\textsubscript{1} and AT\textsubscript{2} receptor mRNA are extracts of human liver and human breast carcinoma cells (MCF7), respectively. Negative controls (T-) for AT\textsubscript{1} and AT\textsubscript{2} receptor mRNA are extracts of human breast carcinoma cells (MCF7) and colon carcinoma cells (SW480), respectively. Bl RT-PCR and Bl PCR represent the results of RT-PCR or PCR amplifications performed in the absence of added tissue extracts (to exclude contamination).


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