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

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***Background***—Angiotensin (Ang) II type 2 ($AT_2$) receptor stimulation results in coronary vasodilation in the rat heart. In contrast, $AT_2$ receptor–mediated vasodilation could not be observed in large human coronary arteries. We studied Ang II–induced vasodilation of human coronary microarteries (HCMAs).

***Methods and Results***—HCMAs (diameter, 160 to 500 $\mu$m) 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 ($pEC_{50}$, 8.6±0.2; maximal effect [$E_{max}$], 79±13% of the contraction to 100 mmol/L $K^+$). The Ang II type 1 receptor antagonist irbesartan prevented this vasoconstriction, whereas the $AT_2$ receptor antagonist PD123319 increased $E_{max}$ to 97±14% ($P<0.05$). The increase in $E_{max}$ was larger in older donors (correlation $\Delta E_{max}$ 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 ($B_2$) 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 $AT_2$ receptors in HCMAs.

***Conclusions***—$AT_2$ receptor–mediated vasodilation in the human heart appears to be limited to coronary microarteries and is mediated by $B_2$ receptors and NO. Most likely, $AT_2$ 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



Angiotensin (Ang) II type 2 ($AT_2$) 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 arteries[1] and saphenous veins[2] nor in vivo studies investigating Ang II–induced vasoconstriction in the forearm vascular bed of healthy volunteers[3,4] provided evidence for $AT_2$ 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 $AT_2$ receptor–mediated vasodilation in human coronary arteries[1] and the occurrence of such dilation in the rat coronary vascular bed[8] is that $AT_2$ receptors are located in coronary microarteries only. In the present study, we therefore investigated $AT_2$ receptor–induced vasodilation in human coronary microarteries (HCMAs) mounted in Mulvany myographs. We also investigated whether endothelial NO and/or bradykinin type 2 ($B_2$) 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 $AT_2$ receptors.

## Methods

### Human Tissue Collection

HCMAs were obtained from 49 heart-beating organ donors (22 men, 27 women; age, 3 to 65 years; mean, 45 years) who died of noncardiac causes (3 cerebrovascular accident, 9 head trauma, 21 subarachnoid bleeding, 4 post–anoxic encephalopathy, 12 intracranial bleeding) <24 hours before the heart was taken to the laboratory. Hearts were provided by the Rotterdam Heart Valve Bank after removal of the heart valves for transplantation purposes. The Ethics Committee of the Erasmus MC approved the study. The hearts were stored in an ice-cold sterile organ-protecting solution after circulatory arrest. After arrival at the laboratory, a tertiary branch of the left anterior descending coronary artery (diameter, 160 to 500 $\mu$m; mean, 360 $\mu$m) was removed and stored overnight in a cold (4°C), oxygenated Krebs bicarbonate solution of the following composition (mmol/L): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25, and glucose 8.3; pH 7.4. In addition, HCMAs, right epicardial coronary arteries, and/or pieces of left ventricular tissue from 29 hearts were frozen in liquid nitrogen for mRNA determinations or radioligand binding studies.

### Myograph Studies

After overnight storage, HCMAs were cut into segments of ≈2 mm length and mounted in a Mulvany myograph (J.P. Trading) with

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separated 6-mL organ baths containing oxygenated Krebs at 37°C. The Krebs was continuously aerated with 95% $O_2$ and 5% $CO_2$, and tissue responses were measured as 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 nmol/L substance P after preconstriction with 10 nmol/L of the thromboxane $A_2$ ($TxA_2$) 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 ($AT_1$) receptor antagonist irbesartan (1 $\mu$mol/L, a gift of Bristol-Myers Squibb),[1] the $AT_2$ antagonist PD123319 (1 $\mu$mol/L, a gift of Parke-Davis),[15] the $B_2$ receptor antagonist Hoe140 (1 $\mu$mol/L, a gift of Hoechst)[16] and/or L-NAME (100 $\mu$mol/L, Sigma). Thereafter, concentration-response curves (CRCs) were constructed to Ang II, either directly or after preconstriction with 10 nmol/L U46619 to 60% of the maximum contractile response. A higher concentration of U46619 (30 nmol/L) was required in segments that had been preincubated with irbesartan because irbesartan antagonizes $TxA_2$ receptors.[17] The cyclo-oxygenase inhibitor indomethacin (5 $\mu$mol/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 $\mu$mol/L Ang II in 10 mL oxygenated Krebs bicarbonate solution for 1 minute at 37°C in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (100 $\mu$mol/L), after a 30-minute preincubation without (control) or with 1 $\mu$mol/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 3300*g*, and cGMP was measured in 300 $\mu$L supernatant by ELISA after acetylation (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 adrenals 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] $^{125}$I-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 $\mu$L Tris buffer (50 mmol/L), 40 $\mu$L membrane fraction (containing 100 $\mu$g protein, determined by the Bradford assay as described before[15]), and 30 $\mu$L radioligand (final volume, 100 $\mu$L). Nonspecific binding, $AT_1$ receptor–specific binding, and $AT_2$ receptor–specific binding were determined by repeating the experiment in the presence of Ang II (at a concentration 100 times the concentration of $^{125}$I-Ang II), irbesartan (0.3 pmol/L to 0.3 mmol/L), and PD123319 (0.3 pmol/L to 0.3 mmol/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.

## $AT_1$ and $AT_2$ 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 follows: $AT_1$ receptor sense 5′-CTT TTC CTG GAT TCC CCA C-3′, and antisense 5′-CTT CTT GGT GGA TGA GCT TAC-3′, $AT_2$ receptor sense 5′-GTG ACC AAG TCC 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.

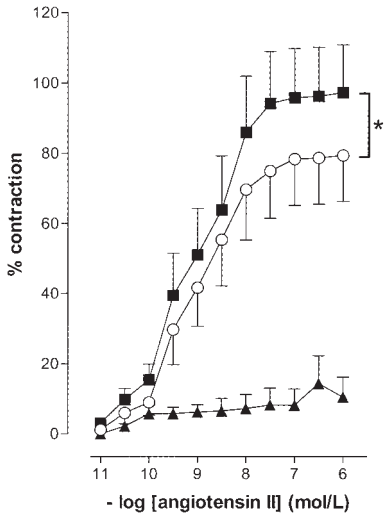


**Figure 1.** Contractions of HCMAs to Ang II in the absence (control, circles) or presence of irbesartan (triangles) or PD123319 (squares). Contractions (mean±SEM, n=5 to 22) are expressed as a percentage of the response to 100 mmol/L $K^+$. *$P<0.05$ vs control.

## 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 $pEC_{50}$ ($-^{10}\log EC_{50}$) 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

## Myograph Studies

Ang II constricted HCMAs in a concentration-dependent manner ($pEC_{50}=8.6\pm0.2$, n=22), with a maximal response ($E_{max}$) of 79±13% (Figure 1). Irbesartan nearly completely blocked the Ang II–mediated constriction. PD123319 increased $E_{max}$ to 97±14% ($P<0.05$). PD123319 did not affect the potency of Ang II ($pEC_{50}=8.7\pm0.2$, n=22), although in 11 experiments a leftward shift of the Ang II CRC (ie, an increase in the $pEC_{50}$ value of ≥0.2) was observed in the presence of the $AT_2$ receptor antagonist. The PD123319-dependent increase in $E_{max}$ was larger in older donors ($r=0.47$, $P<0.05$; Figure 2). The increase in $E_{max}$ 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,

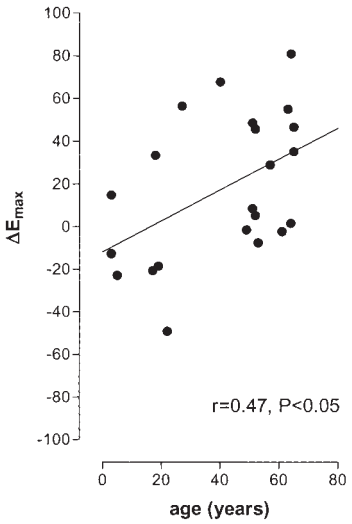


**Figure 2.** Correlation between donor age and the change in $E_{max}$ ($\Delta E_{max}$) of the Ang II CRC observed after addition of PD123319 to the organ bath (n=22).

$\Delta pEC_{50}$ <0.2; n=7) or a rightward (ie, $pEC_{50}$ decreased by ≥0.2; n=4) shift of the Ang II CRC.

L-NAME increased baseline contraction to 20% to 30% of the maximum response to 100 mmol/L $K^+$ and prevented the PD123319-induced potentiation of Ang II (Figure 3). Potentiation was also not observed after removal of the endothelium and in the presence of Hoe140 (Figure 3).

After preconstriction with U46619 (to ≈60% of the maximum response to 100 mmol/L $K^+$), Ang II caused a marginal further increase ($P$=NS) in contraction (Figure 4). This response was unaltered by PD123319 and reversed into a relaxation (by maximally 49±16%) in the presence of irbesartan. PD123319 fully prevented the latter relaxation. Without Ang II, U46619-induced preconstrictions in the presence of irbesartan remained stable for at least 60 minutes (data not shown). Thus, the Ang II–induced relaxations under these conditions cannot be attributed to $TxA_2$ receptor antagonism by irbesartan.[17]

## Cyclic GMP Measurement

Ang II did not significantly increase microvascular cGMP (Figure 5; n=8, $P$=0.11, versus control) either alone or in the presence of PD123319 or irbesartan.

## Radioligand Binding Studies

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 $AT_2$ receptors in HCMAs. After a 1-hour incubation with $^{125}$I-Ang II (final concentration in the incubation mixture, 0.5 nmol/L), total and nonspecific $^{125}$I-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% ($IC_{50}$) was 50±1 nmol/L for PD123319. This value mimics the $IC_{50}$ of PD123319 obtained in previous experiments with cells expressing $AT_2$ receptors only.[23] In contrast, the $IC_{50}$ of irbesartan in the present study (20±1 μmol/L) exceeded its $IC_{50}$ in cells exclusively expressing $AT_1$ receptors by 3 orders of magnitude.[24] Taken together, these data suggest that our porcine adrenal membrane fraction contained predominantly $AT_2$ receptors. A PD123319 concentration of 10 μmol/L is required to fully block $^{125}$I-Ang II binding to these receptors.

On the basis of these findings, as well as on previous studies investigating irbesartan concentrations that selectively block $AT_1$ receptors,[24,25] we incubated HCMA membranes with 0.5 nmol/L $^{125}$I-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 $^{125}$I-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 $AT_1$ as well as $AT_2$ receptors (Figure 6B).

## $AT_1$ and $AT_2$ Receptor mRNA

RT-PCR revealed expression of $AT_1$ and $AT_2$ 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 $AT_2$ receptor–mediated vasodilation in human blood vessels. Evidence for this effect

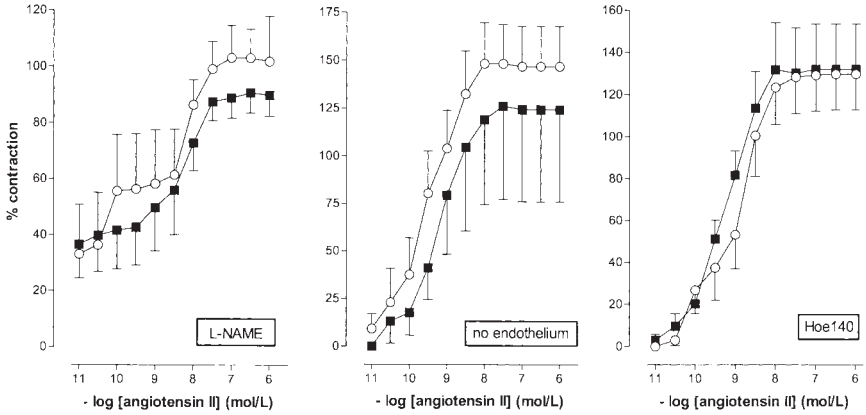


**Figure 3.** Contractions of HCMAs to Ang II in the absence (circles) or presence of PD123319 (squares) after pretreatment with L-NAME, endothelium removal, or pretreatment with Hoe140. Contractions (mean±SEM, n=3 to 7) are expressed as a percentage of the response to 100 mmol/L $K^+$.

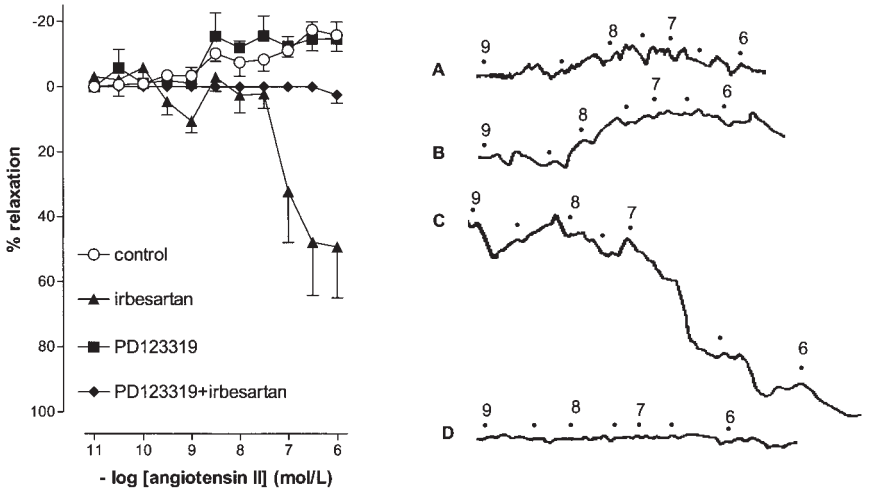


**Figure 4.** Left, Response of U46619-preconstricted HCMAs to Ang II in the absence or presence of irbesartan, PD123319, or irbesartan+PD123319. Data (mean±SEM, n=2 to 5) are expressed as a percentage of the response to U46619. Right, Original tracing of an experiment in which a U46619-preconstricted HCMA was exposed to Ang II under control conditions (A) or after preincubation with PD123319 (B), irbesartan (C), or irbesartan+PD123319 (D). Ang II concentrations were increased with half log steps, starting at 1 nmol/L (9) and ending at 1 μmol/L (6).

was obtained in two ways. First, the AT$_2$ receptor antagonist PD123319, at a concentration that has been reported to result in complete blockade of AT$_2$ receptor–mediated effects,[15] increased the maximal contractile response to Ang II, thereby indirectly demonstrating that AT$_2$ receptor stimulation counteracts AT$_1$ receptor–mediated vasoconstriction. Second, during AT$_1$ receptor blockade with irbesartan (allowing selective AT$_2$ 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 AT$_1$ (contraction) and AT$_2$ (relaxation) receptors.

In addition to its effect on E$_{max}$, PD123319 caused a leftward shift of the Ang II CRC in ≈50% of the experiments.

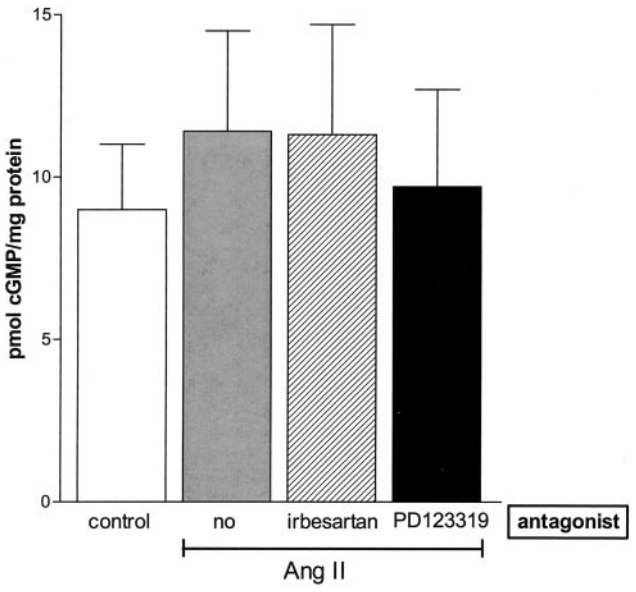


**Figure 5.** Cyclic GMP levels (mean±SEM, n=8) in HCMAs at baseline and after 1-minute exposure to Ang II under control conditions (no antagonist) and in the presence of irbesartan or PD123319.

Such increased potency of Ang II in the presence of PD123319 is not due to an effect of the AT$_2$ receptor antagonist on Ang II metabolism.[25,26] It could point to more efficient AT$_1$ receptor signal transduction during AT$_2$ receptor blockade. Furthermore, a recent study has suggested that AT$_1$ and AT$_2$ receptors form heterodimers.[27] An alternative explanation for the increased potency might therefore be that in some donors AT$_1$ receptor–AT$_2$ receptor heterodimers exist that bind Ang II with higher affinity during AT$_2$ receptor blockade. The underlying assumption for this explanation is, however, that AT$_1$ and AT$_2$ receptors in these donors are located on the same cell.

The increase in E$_{max}$ was larger in older donors, suggesting that the contribution of AT$_2$ receptors increases with age. Although AT$_2$ receptor density increases under pathological conditions,[11] the donors in the present study died of noncardiac causes and did not use cardiovascular medication. Thus, it is unlikely that the increased E$_{max}$ during AT$_2$ 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 AT$_2$ receptor–mediated vasodilation,[1] whereas vasodilation did occur in the rat coronary vascular bed.[8] The present study solves this discrepancy by demonstrating that AT$_2$ receptor–mediated vasodilation is limited to coronary microarteries. It is notable that AT$_2$ receptor expression in HCMAs could be demonstrated by both RT-PCR and radioligand binding experiments. Unexpectedly, AT$_2$ receptor mRNA was also detected by RT-PCR in large coronary arteries. This would imply that either the AT$_2$ receptor density in large coronary arteries is too low to allow detection of vasodilation in the organ bath setup or that AT$_2$ receptors in these arteries mediate other (nondilatory) effects, for example, effects on vascular growth and remodeling.[28,29] AT$_2$ receptor expression has been demonstrated before in the human myocardium, including the coronary vascular bed.[30,31]

The mechanism underlying AT$_2$ receptor–mediated vasodilation in HCMAs is currently unknown. AT$_2$ receptors themselves may act as AT$_1$ receptor antagonists independent

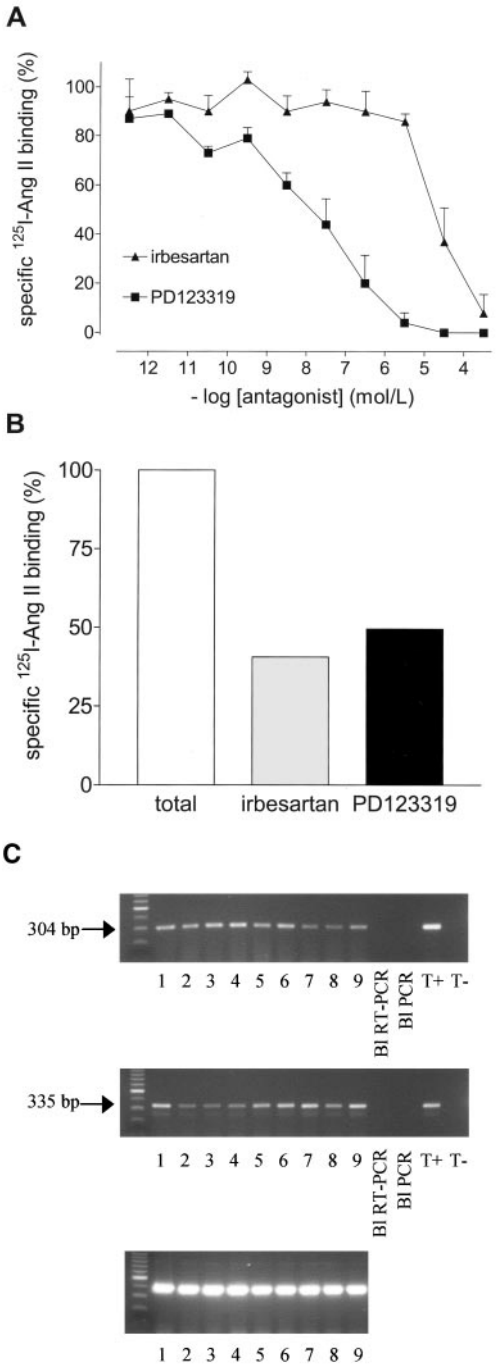


**Figure 6.** A and B, Displacement of specifically bound $^{125}$I-Ang II by irbesartan or PD123319 in sarcolemmal membrane fractions prepared from 6 porcine adrenal glands (A) and 19 HCMAs (B). C, Results from RT-PCR amplification of $AT_1$ receptor mRNA (304 bp), $AT_2$ receptor mRNA (335 bp), and 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 $AT_1$ and $AT_2$ receptor mRNA are extracts of human liver and human breast carcinoma cells (MCF7), respectively. Negative controls (T-) for $AT_1$ and $AT_2$ 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).

of Ang II.[27] This would require their occurrence on the same cell, as discussed above. Furthermore, $B_2$ receptors, NO, cGMP, $Ca^{2+}$-activated $K^+$ channels, and/or phosphatases have been implicated in $AT_2$ receptor–induced effects.[6,7,10–13,32,33] Our data with L-NAME and Hoe140 in HCMAs support a role for $B_2$ 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 $B_2$ receptor–induced NO release and simultaneously suggests that $AT_2$ receptors in HCMAs are located on endothelial cells. In agreement with this concept, cultured human coronary artery endothelial cells do express $AT_2$ receptors.[34]

Taken together, the most likely scenario to explain our results is that Ang II stimulates endothelial $AT_2$ receptors in HCMAs. This results in endothelial $B_2$ receptor activation and NO release. NO subsequently activates guanylyl cyclase in vascular smooth muscle cells, thereby counteracting the contractile responses mediated by the $AT_1$ receptors on these cells. Guanylyl cyclase generates cGMP, and although the Ang II–induced ($AT_2$ 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.[10,33] The lack of significance in the present experiments probably relates to the modest (≈30%) increase in cGMP content induced by Ang II as compared with other agonists. For instance, in our experimental setup, 1 μmol/L bradykinin increased microvascular cGMP 7±2-fold (n=4, data not shown).

In conclusion, $AT_2$ receptor–mediated vasodilation occurs in the coronary microcirculation of nondiseased human hearts in an endothelium-dependent manner and is mediated by $B_2$ receptors and NO. This finding could be of clinical relevance, not only because cardiac $AT_2$ receptors are upregulated under pathological conditions,[30] but also because animal studies have shown that the beneficial effects of $AT_1$ receptor antagonists, in contrast to those of ACE inhibitors, depend on $AT_2$ receptor stimulation.[35,36]

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