Evidence for a Functional Role of Angiotensin II Type 2 Receptor in the Cardiac Hypertrophic Process In Vivo in the Rat Heart

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Background—The precise function of angiotensin II type 2 receptor (AT₂-R) in the mammalian heart in vivo is unknown. Here, we investigated the role of AT₂-R in cardiac pressure overload.

Methods and Results—Rats were infused with vehicle, angiotensin II (Ang II), PD123319 (an AT₂-R antagonist), or the combination of Ang II and PD123319 via subcutaneously implanted osmotic minipumps for 12 or 72 hours. Ang II–induced increases in mean arterial pressure, left ventricular weight/body weight ratio, and elevation of skeletal α-actin and β-mysin heavy chain mRNA levels were not altered by PD123319. In contrast, AT₂-R blockade resulted in a marked increase in the gene expression of c-fos, endothelin-1, and insulin-like growth factor-1 in Ang II–induced hypertension. In parallel, Ang II–stimulated mRNA and protein expression of atrial natriuretic peptide were significantly augmented by AT₂-R blockade. Moreover, PD123319 markedly increased the synthesis of B-type natriuretic peptide. Furthermore, the expression of vascular endothelial growth factor and fibroblast growth factor-1 was downregulated by Ang II only in the presence of AT₂-R blockade.

Conclusions—Our results provide evidence that AT₂-R plays a functional role in the cardiac hypertrophic process in vivo by selectively regulating the expression of growth-promoting and growth-inhibiting factors. (Circulation. 2003;108: 2414-2422.)

Key Words: angiotensin receptors | hypertrophy | growth substances | natriuretic peptides
Methods

Experimental Design
Male Sprague-Dawley rats (n = 132; body weight, 313 ± 3 g) from the Center for Experimental Animals at the University of Oulu were used. Vehicle, Ang II (33 μg · kg⁻¹ · h⁻¹, Sigma), PD123319 (1.25 mg · kg⁻¹ · h⁻¹)¹²⁻¹⁴ (a gift from Dr Joan A. Keiser, Parke-Davies Co), losartan (400 μg · kg⁻¹ · h⁻¹)¹²⁻¹⁴ (a gift from Dr Ronald D. Smith, DuPont Merck Pharmaceutical Co), or Ang II + PD123319 or Ang II + losartan was administered via subcutaneously implanted osmotic minipumps (Alzet). PD123319 has high affinity (Kᵦ > 12 nmol/L) for the AT₁-R but a low affinity (Kᵦ > 100 μmol/L) for the AT₂-R. Therefore, administration of PD123319 at a dose of 1.25 mg · kg⁻¹ · h⁻¹, yielding a plasma concentration of ~500 nmol/L, would result in an effective AT₂-R blockade without affecting the AT₁-R. After 12 or 72 hours of treatment, LVs were weighed, frozen in liquid nitrogen, and stored at −80°C, as described previously.¹⁶

Monitoring of Blood Pressure and Heart Rate
Telemetric monitoring of mean arterial pressure (MAP) and heart rate was performed as described previously.¹⁶

Echocardiography
LV function and chamber dimensions were assessed by transthoracic echocardiography using the Acuson Ultrasound System (Sequoia 512) and a 15-MHz linear transducer (15L8) as described previously.¹⁷

Isolation of Cytoplasmic RNA and Northern Blot Analysis
Total RNA was isolated from LV tissue by the guanidine thiocyanate CsCl method, and 20-μg samples of RNA were transferred to nylon membranes (Osmonics) for Northern blot analysis as described previously.¹⁶ Full-length rat atrial natriuretic peptide (ANP) cDNA probe (a gift from Dr Peter Davies, Queen’s University, Kingston, Canada), a 390-bp rat B-type natriuretic peptide (BNP) cDNA probe, and an 18S cDNA probe were prepared as previously reported.¹⁶ cDNA probes for rat α-actin and myosin isoforms were made by reverse transcription–polymerase chain reaction (RT-PCR) and ligated to dT-tailed pCR2.1 vector by use of a TA Cloning Kit (Invitrogen). Sequencing with ABI PRISM 310 Genetic Analyzer (Applied Biosystems) confirmed that the probes correspond to bases 2950 to 3184 of rat skeletal α-actin (GenBank Accession Number v01218), 289 to 447 of rat cardiac α-actin (x00306), 5794 to 5923 of rat β-myosin heavy chain (β-MHC) (x15939), and 5830 to 5921 of rat α-myosin heavy chain (α-MHC) (x15938). cDNA probes were labeled, and the membranes were hybridized, washed, and quantified as previously described.¹⁶

Real-Time Quantitative RT-PCR Analysis
The cDNA was synthesized from 0.5 μg total RNA derived from LV tissue (First-Strand cDNA Synthesis Kit, Amersham). Rat c-fos, endothelin-1 (ET-1), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), fibroblast growth factor-1 and -2 (FGF-1 and -2), transforming growth factor-β₁ (TGF-β₁), and 18S RNA levels were measured by real-time quantitative RT-PCR analysis using TaqMan chemistry on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) as described previously.¹⁶ The sequences of the forward and reverse primers and probes for RNA detection are presented in the Table.

Radioimmunoassay for ANP and BNP
Immunoreactive ANP (ir-ANP) and ir-BNP levels were measured by radioimmunoassays from extracted LV tissue samples as described previously.¹⁶

Statistical Analysis
Results are expressed as mean ± SEM. The data were analyzed with 1-way ANOVA followed by Bonferroni post hoc test. The hemodynamic variables were analyzed with repeated-measures ANOVA. Differences at the level of P<0.05 were considered statistically significant.

Results

Hemodynamic Parameters
MAP, measured by telemetry, was similar in all groups before the beginning of the infusions (Figure 1A). Ang II infusion produced a rapid increase in MAP, reaching maximum values at 14 hours, after which it remained elevated until the end of the experiment. In agreement with previous studies,¹²⁻¹⁵ PD123319 infusion alone had no effect on MAP; moreover, it did not modify Ang II–induced increase in blood pressure (Figure 1A), showing that AT₂-R is not involved in the regulation of blood pressure. As described previously, the increase in MAP by Ang II was associated with a significant decrease in heart rate,¹⁶ remaining unaffected by PD123319 (Figure 1B). Echocardiographic measurements showed that LV ejection fraction (Figure 1C) and LV fractional shortening (Figure 1D) were similar in all groups, suggesting that LV function was preserved in Ang II–induced hypertension.

LVH and Contractile Protein Gene Expression
LV weight to body weight (LVW/BW) ratio increased by 9% at 12 hours (P<0.01) and 16% at 72 hours (P<0.001; Figure 1E) in Ang II–infused rats compared with controls. Administration of AT₂-R antagonist alone had no effect on LVW/BW ratio and did not significantly change the Ang II–induced increase in LVW/BW ratio (Figure 1E). Echocardiographic measurements revealed similar changes in LV mean wall thickness (Figure 1F). Furthermore, upregulation of skeletal α-actin and β-MHC mRNA levels in the LV was similar in the Ang II and Ang II + PD123319-infused groups at 72 hours (Figure 2, C and D). Moreover, α-MHC mRNA levels decreased similarly in Ang II–infused rats with or without AT₂-R blockade (Figure 2B). PD123319 treatment alone did not influence the gene expression of α-actin and MHC isoforms (Figure 2, A and B).

LV c-fos Gene Expression
Upregulation of c-fos gene is a hallmark of cardiac hypertrophy induced by mechanical stretch or growth-promoting factors.¹⁹ Ang II treatment caused a transient increase (5.1-fold; P<0.01 versus sham) in c-fos mRNA at 12 hours (Figure 3A). In the presence of AT₂-R antagonist, Ang II induced a more marked and sustained increase in c-fos mRNA levels, the increase being 10-fold at 12 hours (P<0.01 versus sham; P<0.05 versus Ang II alone; Figure 3A) and 6-fold at 72 hours (P<0.01 versus sham; P<0.05 versus Ang II alone; Figure 3B). PD123319 treatment alone had no effect on LV c-fos gene expression.

LV Gene Expression of ET-1 and Peptide Growth Factors
The increase in c-fos expression in the presence of AT₂-R blocker could be mediated by increased expression of growth-promoting factors such as ET-1 and peptide growth factors.¹⁹ Ang II alone had no effect on ET-1 mRNA levels; however, it upregulated ET-1 gene expression in the presence
of PD123319 by 2.2-fold \((P<0.05)\) and 3-fold \((P<0.001)\) at 12 and 72 hours, respectively (Figure 4, A and B). IGF-1 mRNA levels were elevated 1.5-fold \((P<0.05)\) at 72 hours only in the Ang II + PD123319 group (Figure 4C). Moreover, Ang II reduced VEGF and FGF-1 mRNA levels by 49% and 46% \((P<0.01)\), respectively, only in the presence of AT2-R blockade. In contrast to these changes, FGF-2 mRNA levels increased similarly during Ang II infusion alone and in combination with AT2-R antagonist (Figure 4F), whereas TGF-\(\beta\) gene expression was not modified by the treatments (data not shown). PD123319 infusion alone had no effect on the LV gene expression of ET-1 or peptide growth factors.

### LV Expression of ANP and BNP

Because AT2-R blockade resulted in an augmented expression of growth-promoting factors in response to Ang II without an increase in LV mass, we measured expression of natriuretic peptides, which exert antihypertrophic effect in vivo.\(^{20,21}\) Ang II infusion resulted in 2.9-fold \((P<0.001)\) and 10.5-fold \((P<0.001)\) increases in LV ANP mRNA levels at 12 and 72 hours, respectively (Figure 5, A and B). Interestingly, PD123319 significantly augmented the Ang II–induced increase in ANP mRNA levels at 12 hours \((P<0.01)\) (Figure 5A). In addition, LV ir-ANP levels increased 2.6-fold in Ang II–infused rats compared with those in control animals at 72 hours \((P<0.01)\), and combined administration of Ang II and PD123319 further increased ir-ANP levels \((P<0.05)\) versus Ang II (Figure 5D). Moreover, PD123319 increased ir-BNP levels in Ang II–infused animals by 23% \((P<0.05)\) and 36% \((P<0.05)\) at 12 and 72 hours (Figure 6, C and D), respectively; meanwhile, the transient Ang II–induced increase in BNP mRNA levels was not modified during AT2-R blockade (Figure 6, A and B). Administration of PD123319 alone had no effect on ANP and BNP mRNA or ir-ANP and ir-BNP levels (Figures 5 and 6).

### Blockade of Ang II–Induced Hypertrophic Effects by Losartan

AT1-R blockade by losartan completely abolished Ang II–induced changes in the cardiac gene expression at both time points (Figure 2, A and B, and Figure 7). When given alone, losartan did not modify mRNA levels. Losartan also abolished the Ang II–induced increase in LVW/BW ratio \((2.47\pm0.09\) versus \(2.12\pm0.03, P<0.001)\) and hemodynamics (data not shown).

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### Primer and Probe Sequences Used for Real-Time Quantitative RT-PCR Analysis

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Figure 1. Effect of Ang II with or without AT$_2$-R blockade on MAP (A), heart rate (B), LV ejection fraction (C), LV fractional shortening (D), LVW/BW ratio (E), and LV mean wall thickness (F). Rats were infused with vehicle, PD123319, Ang II, or Ang II+PD123319 via subcutaneously implanted osmotic minipumps for 72 hours. Results are expressed as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 vs control.
The precise role of AT₂-R in the development of LVH is controversial.²²,²³ Previously, in vitro studies have shown that AT₂-R stimulation inhibits the growth of various cell types, including cardiomyocytes,⁹ by counteracting AT₁-R signaling. However, in 2 different lines of AT₂-R knockout mice, either pressure overload failed to generate LVH¹¹,²⁴ or the increases in ventricular mass and myocyte cross-sectional area were comparable to those in nontransgenic littermates.²⁵,²⁶ Our results show that in vivo Ang II–induced hypertrophic process is regulated by AT₂-R, because PD123319, a selective AT₂-R antagonist,¹² markedly upregulated the gene expression of various hypertrophy-inducing (eg, ET-1, IGF-1) and growth-inhibiting (eg, ANP) factors in the rat heart.

**Figure 2.** Northern blot analysis showing effect of administration of vehicle, Ang II, PD123319, Ang II+PD123319, losartan, or Ang II+losartan for 12 (A) and 72 (B) hours on LV skeletal (sk) and cardiac (ca) α-actin, α- and β-MHC, ANP, and BNP mRNA levels. 18S ribosomal RNA signals were similar in different groups. Effect of AT₂-R blockade on Ang II–induced increase in LV skeletal α-actin mRNA levels at 12 hours (C) and β-MHC mRNA levels at 72 hours (D). Results are expressed as ratio of skeletal α-actin or β-MHC mRNA to 18S RNA determined by Northern blot analysis. Data are mean±SEM. *P<0.05, ***P<0.001 vs control.
AT$_2$-R is expressed at very high levels in the heart of the developing fetus, and it may play a significant role during embryogenesis. In the present experiments, to avoid the possible embryogenic effects of the lack of AT$_2$-R, we inhibited AT$_2$-R in adult hearts by means of a pharmacological approach. Telemetry and echocardiographic measurements documented that AT$_2$-R blockade with PD123319 had no effect on Ang II–induced pressor response or LV function, respectively. Moreover, Ang II–induced increase in LVW/BW ratio, an index of LVH, and elevation of skeletal $\alpha$-actin and $\beta$-MHC mRNA levels were not altered by PD123319. These observations suggest that AT$_2$-R blockade has no impact on vascular tone or LV function and it does not trigger additional increase in cardiac mass.

Although LVH was not affected during AT$_2$-R blockade in Ang II–induced hypertension, the LV gene expression of...
c-fos, a hallmark of ventricular hypertrophy, was markedly upregulated. Moreover, Ang II was able to induce a prominent increase in ET-1 mRNA levels only in the presence of AT$_2$-R blockade. ET-1 induces cardiac hypertrophy, myocardial cellular injury, and ventricular arrhythmias, and increased ET-1 synthesis contributes to the progression of chronic heart failure. Long-term ET$_A$ receptor blockade significantly ameliorates LV dysfunction, prevents histological and electrical ventricular remodeling, suppresses ventricular arrhythmias, and therefore greatly improves survival in
various models of experimental heart failure. In addition to ET-1, combined administration of Ang II and PD123319 significantly elevated the mRNA level of IGF-1, a peptide growth factor involved in cardiomyocyte proliferation and differentiation. It was demonstrated previously that Ang II-induced arteriolar growth was blunted by PD123319 in the hypertrophied heart. The marked downregulation of the pivotal angiogenic factors, VEGF and FGF-1, by Ang II/PD123319 treatment may offer an explanation for the impairment of AT2-R-regulated angiogenesis. In contrast to the above-mentioned changes, FGF-2 mRNA levels increased similarly, whereas TGF-β1 gene expression was not changed during Ang II infusion alone or in combination with PD123319. These data demonstrate that although the degree of Ang II-induced pressure overload was comparable in the presence and absence of AT2-R blockade, the gene expression of peptide growth factors was differentially regulated in the heart, suggesting that AT2-R has a specific role in the cardiac hypertrophic process in vivo.

Notably, there was an apparent discrepancy between the marked upregulation of growth-promoting factors and the lack of effect on ventricular mass during AT2-R blockade in Ang II-induced hypertension. Such dissociation could be explained by the counterbalancing effect of increased synthesis of substances with antihypertrophic properties. Indeed, levels of ANP mRNA, ir-ANP, and ir-BNP were significantly higher in Ang II/PD123319-treated animals, suggesting that a new equilibrium has been established between the production of growth-promoting and growth-inhibiting factors. Importantly, plasma ET-1 and BNP levels have been demonstrated to be powerful predictors of mortality in patients with heart failure characterized by mechanical dysfunction or arrhythmic instability. Thus, increased production of these factors in the presence of AT2-R blockade may be associated with a poor prognosis in the long term.

The present observations may have important clinical implications for the treatment of human heart failure. The expression level of AT2-R is much higher in human hearts...
than in rodent hearts; moreover, the ratio of AT₂-R to AT₁-R increases in failing human hearts.⁴–⁶ Thus, AT₂-R–related beneficial effects could be further activated under these pathophysiological conditions by drugs with simultaneous AT₁-R antagonist and AT₂-R agonist properties, and therefore, they may be more favorable than AT₂-R blockers alone. Of note, losartan, a selective AT₁-R antagonist, was not superior to captopril, an ACE inhibitor, in improving survival in elderly heart-failure patients.⁶⁶

In summary, the present study provides evidence that AT₂-R plays a functional role in the cardiac hypertrophic process in vivo by counterbalancing AT₁-R–mediated growth effects. Our results show that AT₂-R blockade selectively regulates the gene expression of c-fos, ET-1, IGF-1, FGF-1, VEGF, and ANP in the LV during the development of myocardial hypertrophy. Taken together with previous findings, the changes in gene transcription observed during AT₂-R blockade might aggravate LVH and lead to cardiac dysfunction and arrhythmic instability in the long run. These data support the hypothesis that stimulation of the AT₂-R has a beneficial effect during pressure overload.

Acknowledgments
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References
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