Angiotensin II Type 2 Receptors Stimulate Collagen Synthesis in Cultured Vascular Smooth Muscle Cells

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Abstract—Previously, we and others have shown that angiotensin II enhances vascular smooth muscle cell extracellular matrix synthesis via stimulation of the angiotensin II type 1 (AT1) receptor. Recently, expression of the type 2 (AT2) receptor has been confirmed in the adult vasculature, but its role has not yet been fully defined. The aim of the present study was to examine the effects of stimulation of AT2 receptors on collagen synthesis in vascular smooth muscle cells. Retroviral gene transfer was used to supplement adult vascular smooth muscle cells with AT2 receptors to mimic the vasculature in vivo. The treatment of these cells with the AT2 receptor agonist CGP42212A (10^-7 mol/L) alone did not cause a significant change in p42/p44 MAP kinase activity but caused a modest (30% to 50%) decrease in protein tyrosine phosphatase activity. Treatment with CGP42112A also caused a dose- and time-dependent increase in both cell-associated and secretory collagen synthesis (148±17% of control at 48 hours, P<0.05), which was completely inhibited by the AT2 receptor antagonist PD123319, unaffected by the AT1 receptor antagonist losartan, and attenuated by treatment with pertussis toxin or G-alpha antisense oligonucleotides. Interestingly, studies in other cell lines demonstrated that CGP42112A caused similar results in transfected mesangial cells but had essentially opposite effects in fibroblasts (NIH-3T3-AT2). These results suggest that AT2 receptor stimulation can increase collagen synthesis in vascular smooth muscle cells via a G-alpha-mediated mechanism and provide evidence for heterogeneity in the effects of AT2 receptor stimulation in different tissues. (Hypertension. 2000;36:845-850.)

Key Words: angiotensin II receptor, angiotensin II collagen muscle, smooth, vascular fibroblasts

Angiotensin II (Ang II) plays a central role in the regulation of systemic blood pressure and fluid homeostasis through its multiple effects on the vasculature, adrenals, kidneys, and brain. These pleiotropic actions of Ang II are mediated by specific receptors located on target tissues. The presence of 2 subtypes of angiotensin receptors (AT1 and AT2) has been demonstrated through pharmacological and molecular biology studies. Most of the “classic” hypertensive actions of the Ang II, namely vasoconstriction, stimulation of aldosterone secretion, and increased renal tubular sodium reabsorption, have been shown to be mediated by the AT1 receptor. On the other hand, several lines of evidence have suggested that the AT2 receptor has predominantly antagonistic functions to the AT1 receptor.

Although the AT1 receptor is well known to be widely expressed in fetal tissues, histological confirmation of the expression of these receptors in the vasculature of normal human adults as well as rats has been provided by several groups with immunohistochemistry, in situ hybridization, and receptor binding studies. A recent study with adult hypertensive rats demonstrated that the stimulation of these receptors can increase the hypertensive actions of AT1 receptor antagonists, providing evidence for a role for these receptors in blood pressure control in adults.

In addition to being a vasoconstrictor and pressor hormone, Ang II is thought to play a direct role in the development of vascular hypertrophy and fibrosis and, consequently, of the vascular thickening that is a hallmark of hypertensive and atherosclerotic vascular disease. In regard to this relationship between Ang II and vascular remodeling, previous studies from our and other laboratories have shown that the stimulation of AT1 receptors causes hypertrophy and increased extracellular matrix synthesis in vascular smooth muscle cells (VSMCs). In contrast, the effects of AT2 receptor stimulation on extracellular matrix synthesis in the vasculature have not been well defined. In vivo studies have produced only discrepant results, with some groups reporting that AT2 receptor blockade increases vascular hypertrophy, whereas other groups have reported that AT2 receptor blockade inhibits vascular hypertrophy and fibrosis in vivo. It is particularly important to characterize the effects of prolonged AT2 receptor stimulation on the vasculature at this time in view of the recent increase in the use of AT1 receptor antagonists for the treatment of hypertension. The use of these agents is associated with a feedback activation of the renin-angiotensin system, which results in increased stimulation of the “unprotected” vascular AT2 receptors. Thus, an understanding of the
effects of AT₂ receptor stimulation on different tissues is increasing in clinical relevance.

Because in vivo experiments have produced confusing results regarding the role of AT₂ receptors in vascular extracellular matrix synthesis, our aim in the present study was to use a direct approach to examine the effects of AT₂ receptor stimulation on collagen synthesis by VSMCs in vitro. We also examined the signal transduction mechanisms involved in the AT₂ receptor-mediated control of collagen synthesis and then compared the effects of AT₂ receptor stimulation on collagen synthesis in 4 different cell types.

Methods

VSMC Culture and Transfection of AT₂ Receptor

Rat VSMCs were prepared from the thoracic aortas of 6-week-old male Wistar rats and cultured in DMEM supplemented with 10% FCS. For transfection of the AT₂ receptor, a retrovirus construct (pLXSN-AT₂) was constructed through ligation of the rat AT₂ receptor cDNA into the EcoRI-XhoI site of the retroviral vector plXSN (Stratagene). plXSN-AT₂ was stably transfected into the packaging cell line GP+E-86 (kindly provided by Dr Arthur Bank, Columbia University, New York, NY) through calcium phosphate transfection followed by G418 selection to produce the AT₂ receptor retrovirus-producing cell line GP+E-86-AT₂. Supernatant from these cells was used to transfect the VSMCs in the presence of Polybrene (4 μg/mL); then, the transfectants were isolated through selection with G418 (400 μg/mL).

Analysis of AT₁ and AT₂ Receptor mRNA and Binding

Expressions of AT₁ and AT₂ receptor mRNA and binding in VSMC-AT₂ cells were assessed with RT-PCR and whole cell receptor binding studies as described in detail previously.11,13

p42/p44 Mitogen-Activated Protein Kinase and Protein Tyrosine Phosphatase Assays

Mitogen-activated protein kinase (MAPK) activity in CGP42112A- or Ang II–treated cells was assessed with the p42/p44 MAPK enzyme assay system (Amersham) as described previously.14 Protein tyrosine phosphatase (PTP) activity was assessed through dephosphorylation of a PTP-specific synthetic phosphotyrosyl peptide with a commercially available kit (Takara).

Determination of Cell Proliferation

VSMCs on 24-well plates were made quiescent through placement on collagenase-sensitive proline incorporation.15 In brief, quiescent VSMCs on 24-well plates were stimulated with CGP42112A (10⁻⁷ mol/L) for 48 hours, and cell counts were determined with a cytometer. In parallel experiments, cells were treated in a similar manner, and then thymidine incorporation was determined with a cytometer. In parallel experiments, cells were treated in a similar manner, and then thymidine incorporation was determined with a cytometer.

Determination of Collagen Synthesis

Synthesis of cell-associated and secreted collagen was determined as described for VSMCs.11,13 Protein tyrosine phosphatase (PTP) activity was assessed through dephosphorylation of a PTP-specific synthetic phosphotyrosyl peptide with a commercially available kit (Takara).

Antisense Oligonucleotide Experiments

Gₐₐ and Gₐᵢ antisense experiments were performed with the primers and protocols described by Wang et al.5 The sequences of the oligonucleotides were Gₐᵢ sense, 5'-GGCGACGGCTCGCCACCATG-3'; Gₐᵢ antisense, 5'-CATGGTGGCCGAGGCTGC-3'; Gₐₑ sense, 5'-CCTCTCGGCGCGCATG-3'; and Gₐₑ anti-

Results

Establishment of AT₂ Receptor–Expressing VSMCs

As shown in Figure 1, neither AT₂ receptor mRNA nor binding was detectable in wild-type adult VSMCs. However, in VSMC-AT₂ cells that had been transfected with AT₂ receptor retrovirus, both AT₂ receptor mRNA and binding were clearly detectable. Relative levels of AT₁ and AT₂ receptor expression in these cells were ≈3:1 on binding assay (AT₁ 664 ± 64, AT₂ 280 ± 48 fmol/mg protein).

Effects of CGP42112A on MAPK and PTP Activity in VSMC-AT₂

The treatment of VSMC-AT₂ with CGP42112A (10⁻⁷ mol/L) did not cause significant changes in MAPK activity (MAPK

Statistical Analysis

Results are expressed as the mean±SEM. Statistical comparisons were made by ANOVA followed by Fisher’s PLSD test for comparison between groups. Values of P<0.05 were considered statistically significant.

Studies on Mesangial, NIH-3T3, and NRK49F Cells

Mesangial cells from Sprague-Dawley rats were obtained through enzymatic digestion14 and cultured in RPMI 1640 supplemented with 10% FCS. The kidney interstitial fibroblast cell line NRK-49F was obtained from American Type Culture Collection and cultured in DMEM plus 10% FCS. NIH-3T3 cells stably transfected with the AT₂ receptor6 were cultured in DMEM plus 10% FCS. The transfection of mesangial and NRK-49F cells with the AT₂ receptor was performed as described for VSMCs.

Materials

CGP42112A was obtained from Research Biochemicals International. Cell culture reagents were obtained from Gibco BRL. RT-PCR reagents were from Perkin–Elmer Cetus. Radiochemicals were from Amersham. All other chemicals were from Sigma Chemical Co, unless otherwise stated.

Figure 1. AT₁ and AT₂ receptor expression in wild-type and AT₂ receptor–transfected VSMC. a, Analysis of AT₁ and AT₂ receptor mRNA with RT-PCR. b, Analysis of AT₁ and AT₂ receptor binding with receptor binding assay. Data represent mean±SEM (n=4). ND indicates no significant difference from nontarget binding.
activity at 0 minutes 456±442, 5 minutes 477±276, 10 minutes 485±106 cpm/μg protein; n=4). On the other hand, the treatment of VSMC-AT2 with the AT2 receptor antagonist PD123319 did cause a significant increase in Ang II–stimulated MAPK activity, as has been reported by other groups17 (Figure 2a). A small (30% to 50%), dose-dependent decrease in PTP activity was also detected after CGP42112A stimulation in these cells, which was inhibited by pretreatment with the AT2 receptor antagonist PD123319 but not the AT1 antagonist losartan (Figures 2b and 2c). Further studies showed that the CGP42112A-induced decrease in PTP activity was not seen when the cells were pretreated with pertussis toxin (PTX) (without PTX: control 42.9±2.1, CGP42112A treated 29.9±4.0×105 U/μg; with PTX: control 37.4±4.4, CGP42112A treated 38.2±4.3×105 U/μg; n=4; *P<0.05 versus control).

Effects of CGP42112A on Cell Proliferation in VSMC-AT2
Treatment of VSMC-AT2 with CGP42112A caused only small, statistically insignificant changes in cell numbers (from 22 900±900 to 21 600±1400 cells per well, n=4) and thymidine incorporation (from 3294±210 to 3568±106 cpm/well, n=4), suggesting that CGP42112A did not have a major effect on proliferation in these cells.

Effects of CGP42112A on Collagen Synthesis in VSMC-AT2
In control VSMCs transfected with the vector alone, CGP42112A treatment did not cause any change in collagen synthesis, as shown in Figure 3a. On the other hand, the stimulation of VSMC-AT2 with CGP42112A caused a significant increase in collagen synthesis that was completely inhibited by the AT2 receptor antagonist PD123319 and unaffected by the AT1 receptor antagonist losartan, as shown in Figure 3b, confirming AT2 receptor–mediated enhancement of collagen synthesis. For comparison, experiments were also performed with the agonist Ang II in the presence of AT1 and AT2 receptor antagonists (Figure 3c). These experiments confirmed that an increase in collagen synthesis (~1.4-fold) was seen in cells treated with an alternate method of the AT2 receptor stimulation, namely a combination of Ang II and losartan.
II and losartan. Stimulation of the AT1 receptors alone, with a combination of Ang II and PD123319, caused a 1.7-fold increase in collagen synthesis. Interestingly, the stimulation of both receptors with Ang II in the absence of antagonists caused a 2.6-fold increase in collagen synthesis, suggesting a synergistic effect of the 2 receptor subtypes on collagen synthesis in these cells.

Studies on the time course and dose dependency of the effect of CGP42112A on collagen synthesis showed a dose- and time-dependent effect on both cell-associated and secreted collagen synthesis (Figure 4). The AT1 receptor–mediated effect on collagen synthesis was unaffected by pretreatment of cells with the tyrosine phosphatase inhibitor okadaic acid or the serine/threonine phosphatase inhibitor sodium orthovanadate. On the other hand, the CGP42112A-mediated enhancement of collagen synthesis was attenuated in cells pretreated with PTX (Figure 4c). To further clarify the role of G\(\alpha_i\) in the AT1-mediated effect, antisense experiments were performed with the method of Wang et al. As shown in the Table, the CGP42112A-induced enhancement of collagen synthesis was seen in cells pretreated with G\(\alpha_i\) or G\(\alpha_\text{at}\) sense oligonucleotides but not in cells pretreated with the corresponding antisense oligonucleotides.

**Discussion**

The vasoactive peptide Ang II is known to act on 2 types of receptor: AT1 and AT2. The AT1 receptor is widely expressed in tissues that are known physiological targets for Ang II.
action, such as the heart, kidneys, adrenal, and brain, as well as the vasculature.

The AT$_2$ receptor has also been shown to be expressed in adult blood vessels, both in the rat and in humans. In particular, expression of the AT$_2$ receptor in the small to medium-sized arteries of the kidney and mesenteric vasculature was confirmed by several groups through the use of in situ hybridization, immunohistochemistry, and binding assays. Physiological studies have suggested that these vascular AT$_2$ receptors may be involved in vasodilation and control of regional hemodynamics, as well as blood pressure control.

Although the AT$_2$ receptor is also a member of the superfamily of G protein–coupled heptahelical receptors, its signal transduction appears to be quite different from that of other receptors of this family. Interestingly, several studies have shown that the actions of the AT$_2$ receptor can be abolished with PTX pretreatment, suggesting that the receptor is coupled to G$_i$.$^1$ Further support for AT$_2$ receptor–G$_i$ coupling is provided in studies that show the intracellular application of anti-G$_i$ antibodies can attenuate AT$_2$ receptor–induced activation of potassium$^{19}$ as well as T-type calcium currents.$^{20}$ Further downstream, AT$_2$ receptor stimulation has been shown to modulate PTP activity (for a review, see Inagami$^1$).

In terms of the effects of Ang II on extracellular matrix, previous studies from our and other laboratories have shown that Ang II causes hypertrophy as well as increased collagen synthesis in VSMC via an AT$_1$ receptor–mediated mechanism.$^{7,8}$ It is puzzling that the AT$_2$ receptor has been suggested to inhibit vascular hypertrophy in vivo by some investigators$^9$ and to enhance vascular hypertrophy and collagen accumulation by others.$^{4,10}$ These findings were based on experiments conducted with the AT$_2$ receptor antagonist PD123319 in vivo.

In the present study, we designed an in vitro strategy to examine the direct effects of AT$_2$ stimulation on collagen synthesis in VSMCs. To circumvent problems caused by potential incomplete inhibition of receptors at low concentrations of losartan and PD123319 on the one hand and by potential cross-inhibition of AT receptor subtypes at high concentrations on the other, we stimulated our cells with AT$_2$ receptorspecific ligand CGP42112A. The fact that simultaneous stimulation with Ang II and losartan produced the same effect as CGP42112A suggested that the latter acted as an agonist, as reported previously.$^{16}$ This was underscored in the present study by the fact that the AT$_2$ receptor antagonist PD123319 inhibited the CGP42112A-induced effects in our cells.

Although AT$_2$ receptors have been reported to be expressed in the vasculature of adult rats,$^5$ their expression was undetectable with RT-PCR in cultured VSMCs, presumably because AT$_2$ receptors are easily lost after subculturing.$^{21}$ We therefore supplemented the VSMCs with AT$_2$ receptors through gene transfer to mimic the situation of the vasculature in vivo. Previous studies have shown that pretreatment with AT$_2$ receptor antagonist PD123319 can enhance AT$_1$ receptor–stimulated MAPK activation,$^{17}$ and we noted the same phenomenon in the present study with these cells. AT$_2$ receptor stimulation has been reported to be able to cause both an increase and a decrease in PTP activity$^1$; in the present study, we detected a small decrease in PTP activity after CGP42112A stimulation.

In the VSMC–AT$_2$ cells, stimulation of the AT$_2$ receptor caused a dose- and time-dependent increase in collagen synthesis. The mechanism was different from AT$_1$ receptor–induced enhancement of collagen synthesis, which has been shown to involve p42/p44 MAPK, because no significant change in p42/p44 MAPK activity was seen after CGP42112A stimulation. We found that the AT$_2$ receptor–mediated enhancement of collagen synthesis was attenuated by PTX treatment, suggesting the involvement of G$_i$ in the AT$_2$-induced effects. Moreover, pretreatment of cells with anti-G$_i$ antisense oligonucleotides provided similar results in response to PTX pretreatment, further supporting a role for G$_i$ in the AT$_2$ receptor–mediated effect on collagen synthesis. PTX treatment was also effective in attenuating the CGP42112A-induced decrease in PTP activity that we noted in these cells. Interestingly, the experiments that compared the effects of AT$_1$ and AT$_2$ receptor stimulation suggested a synergistic effect of the 2 receptor subtypes on collagen synthesis in these cells. These data point to the need for further studies to clarify the intracellular signaling events downstream of G$_i$, which may be involved in the observed effects of the AT$_2$ receptor on collagen synthesis.

Recently, the effect of the AT$_2$ receptor on extracellular matrix synthesis in a nonvascular tissue was studied by another group. Ohkubo et al$^{22}$ examined cardiac fibrosis in myopathic hamsters and found clear evidence that AT$_2$ receptor stimulation is associated with decreased collagen synthesis in the cardiac fibroblasts of these rats. To reconcile the conflicting reports concerning the effects of AT$_2$ receptor stimulation on extracellular matrix synthesis in vivo, we went on to examine the effects of AT$_2$ receptor stimulation in different cell types. In the case of mesangial cells, we found similar results to those with VSMCs. However, in fibroblasts, our results were markedly different from the results in VSMCs and mesangial cells and revealed tissue specificity in the actions of the AT$_2$ receptor on extracellular matrix synthesis. One caveat in the interpretation of these results is that the data reflect the situation in vitro with an "artificial" overexpression system. However, the fact that our in vitro results are in good agreement with in vivo reports that PD123319 decreased fibrosis in the rat vasculature and increased fibrosis in the hearts of cardiomyopathic hamsters$^{4,10,22}$ suggests that our in vitro results are relevant to the in vivo situation.

What are the implications of the findings of the present study? First, as noted in the introduction, an increasing number of patients are starting on AT$_1$ receptor antagonists for the treatment of hypertension. The feedback activation of the renin-angiotensin system results in enhanced AT$_2$ receptor stimulation. The results of the present study suggest that this could have multiple effects on remodeling in different tissues.

Second, the results of the study have suggested the existence of an AT$_2$ receptor–induced and G$_i$–mediated pathway for the control of vascular collagen synthesis. Increased extracellular matrix synthesis plays an important role in the progression not only of hypertensive and atherosclerotic vascu-
lar disease but also of heart disease. Specific examples include myocardial scarring after myocardial infarction, cardiomyopathy associated with connective tissue diseases such as systemic sclerosis, and other causes of restrictive cardiomyopathy. Therefore, further study of the mechanisms involved may help to design new strategies to control the pathological accumulation of extracellular matrix in disease states.

Third, AT₂ receptor stimulation was found to cause apparently opposite effects on collagen synthesis in different tissues. To our knowledge, this is the first description of bidirectional control of extracellular matrix synthesis by a G protein–coupled receptor. At the present, we do not know the reason for the striking tissue variability in the effect of the AT₂ receptor, but we speculate that AT₂ receptor–mediated signal transduction may be strongly modulated by interaction with other tissue-specific factors. In conclusion, our results provide strong evidence that further study of the AT₂ receptor will provide us with a unique opportunity to understand previously unrecognized facets of the control of collagen synthesis in different tissues.

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