Induction of Connective Tissue Growth Factor by Angiotensin II
Integration of Signaling Pathways

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Objective—Angiotensin II is recognized as one of the major mediators of cardiovascular pathology. Because connective tissue growth factor (CTGF) is involved in the pathophysiologic processes underlying fibrotic diseases, its regulation by angiotensin II was investigated.

Methods and Results—In the 2-kidney, 1-clip model of renovascular hypertension, increased expression of CTGF was detectable in the hypertrophic left ventricle. By activation of angiotensin II type 1 receptors, angiotensin II caused rapid expression of CTGF mRNA and protein in a human fibroblast cell line. Activation of the p42/44 mitogen-activated protein (MAP) kinase signaling pathway proved to be essential for angiotensin II–stimulated CTGF expression. Inhibition of MAP kinase activation by forskolin prevented CTGF induction. Inhibition of the isoprenylation of small GTPases by simvastatin or pretreatment of the cells with toxin B reduced basal CTGF expression below detection limits and prevented induction by angiotensin II. Specific interference with RhoA signaling by Y27632 primarily reduced basal CTGF expression. There was no significant reduction of expression of angiotensin II type 1 receptors by simvastatin. These data indicate cooperation between the Rho signaling and the angiotensin II–activated MAP kinase pathways.

Conclusions—Direct induction of CTGF by angiotensin II is indicative of a role for CTGF in angiotensin II–mediated fibrosis and might be a target of antifibrotic interventions. (Arterioscler Thromb Vasc Biol. 2003;23:1782-1787.)

Key Words: connective tissue growth factor ■ angiotensin II ■ Rho proteins ■ mitogen-activated protein kinase ■ statins

Angiotensin II is the primary effector molecule of the renin-angiotensin system, and as such, it plays a central role in the regulation of arterial blood pressure and the etiology of hypertension. Apart from its pressure effects, angiotensin II exerts a variety of nonhemodynamic effects that are linked to cardiovascular and renal pathology.1 By binding to and activation of angiotensin II type 1 (AT1) receptors, angiotensin II mediates renal or cardiac fibrosis.2 The fibrogenic effects of angiotensin II are often related to the cytokine transforming growth factor-β (TGF-β). In vivo and in vitro data show the high capacity of TGF-β to induce the synthesis of extracellular matrix proteins and to prevent their degradation, thus leading to excess deposition of extracellular matrix and fibrosis (summarized in Eddy3). It was shown in various cellular systems and animal studies that interference with TGF-β signaling reduced angiotensin II–mediated synthesis of matrix molecules (reviewed in Kim and Iwao1 and Williams4).

Connective tissue growth factor (CTGF) is a member of the CYR61, CTGF, and NOV protein family, structurally characterized by their cysteine-rich sequence.5 Functionally, CTGF was characterized as a downstream mediator of TGF-β, mediating many but not all of the profibrotic actions of this cytokine.6 TGF-β was shown to be the strongest inducer of CTGF in most cells, but other factors such as bioactive lipids have also been implicated in CTGF induction.7,8 In injury-induced animal models of cardiac hypertrophy, increased levels of CTGF were observed.9,10 In cyclosporin A–induced myocardial lesions, CTGF was reduced when the renin–angiotensin II system was blocked.11 CTGF was similarly reduced in diabetic nephropathy when the animals were treated with an angiotensin II receptor blocker,12 suggesting a link between angiotensin II and CTGF expression. These animal models, however, did not allow elucidation of the molecular mechanisms of this relation.

Angiotensin II exerts its diverse biologic effects by binding to 2 types of receptors, AT1 and AT2, both of which belong to the group of heptahelical transmembrane receptors. Depending on the cell type and stimulus, coupling to various different intracellular pathways mediates the physiologic and pathophysiologic actions of angiotensin II (summarized in Touyz and Schiffrin13). Given the profibrotic effects of angiotensin II, we hypothesized that
angiotensin II, by activation of AT receptors, might be a direct inducer of CTGF expression.

Methods
Methods are presented online at http://atvb.ahajournals.org.

Results
Upregulation of CTGF in an Angiotensin II–Dependent In Vivo Model
The 2-kidney, 1-clip (2K,1C) model of renovascular hypertension is one of the classic models for investigating angiotensin II–mediated effects in vivo. Mice were treated as described in the Methods section. Cardiac hypertrophy was assessed by determination of the wet weight of the heart relative to whole body weight (cardiac weight index). After 8 weeks, the relative heart weight was significantly increased, from 3.30 ± 0.09 to 4.14 ± 0.20 mg/g (sham-operated n = 9; 2K,1C, n = 7; P < 0.005, unpaired t test). Expression of CTGF mRNA was determined by real-time reverse transcription–polymerase chain reaction (RT-PCR). As shown in Figure 1, CTGF was significantly upregulated in left ventricular tissue. To discriminate between direct and indirect effects of angiotensin II, regulation of CTGF was further investigated in vitro in a human fibroblast cell line.

Induction of CTGF by Angiotensin II
The fibroblast cell line TK173 was incubated with angiotensin II (10⁻⁷ mol/L) for up to 4 hours, and CTGF mRNA expression was determined by Northern blot analysis. CTGF levels were transiently increased, with maximal expression after ~1 hour (Figure 2A and B), returning to background levels after 4 hours. For comparison, incubation of the cells with TGF-β led to a more long-lasting expression of CTGF. Induction of CTGF mRNA by angiotensin II was detectable at 10⁻⁸ mol/L angiotensin II and was further increased at higher concentrations (analyzed up to 10⁻⁶ mol/L; data not shown). All experiments presented were performed with 1 or 2 × 10⁻⁷ mol/L angiotensin II, which did not differ significantly. Incubation of the cells with cycloheximide, an inhibitor of protein synthesis, increased the basal levels of CTGF, indicative of ongoing mRNA synthesis (Figure 2C). Concomitant incubation with angiotensin II further enhanced CTGF mRNA levels, showing that angiotensin II–mediated induction of CTGF expression was independent of protein synthesis. Transient expression of CTGF protein was detectable in the cellular homogenates (Figure 2D) and followed kinetics similar to that of mRNA.

The extent of angiotensin II–mediated CTGF mRNA induction varied with the basal levels of CTGF observed in the cells cultured under standard conditions. Stimulation rates were higher with low background levels (mean ± SD, 4.86 ± 2.12-fold increase; n = 7 independent experiments, P < 0.01, Student’s t test).
Role of RhoA Signaling in CTGF Induction

Statins interfere with the isoprenylation of Rho and Ras proteins and thereby might affect their activity. Preincubation of the cells with various concentrations of simvastatin for 18 hours caused a concentration-dependent decrease in basal and angiotensin II–induced expression of CTGF (Figure 3A). At 3 µmol/L simvastatin, basal levels were still detectable and were stimulated by angiotensin II to degree similar to that in the absence of simvastatin (inset; data of n=3 experiments). Complete inhibition of the basal expression of CTGF prevented induction by angiotensin II. Mevalonate, the precursor of the isoprenoid phosphates, prevented the inhibition of CTGF by simvastatin, indicating that the effect of simvastatin had to be attributed to inhibition of the hydroxymethylglutaryl coenzyme A reductase (Figure 3B). To further characterize the signaling proteins affected by simvastatin, the cells were incubated with geranylgeranylpyrophosphate (GGPP, 5 µmol/L) or farnesylpyrophosphate (FPP, 5 µmol/L). FPP only marginally affected CTGF expression (blot and graph, Figure 3B). GGPP, in contrast, increased basal CTGF levels, irrespective of coinubcation with simvastatin. Angiotensin II–mediated induction of CTGF was not further increased by GGPP, but the reduction by simvastatin was prevented. These data indicated a role for geranylgeranylated proteins, most likely of the Rho family.

Inhibition of the small GTPases RhoA, Rac, and Cdc42 by toxin B (1 ng/mL, 90 minutes) completely abrogated basal and induced expression of CTGF, as determined after a total incubation time of 150 minutes (Figure 4A). Even 0.1 ng/mL caused a strong decrease in basal CTGF expression but still allowed stimulation by angiotensin II. To differentiate between the different proteins of the Rho family, a downstream RhoA–associated kinase was inhibited by the compound Y27632 (Fig 4A–C). A concentration-dependent inhibition of CTGF expression was observed, indicating a role for RhoA in the regulation of CTGF expression. As in the case of low concentrations of toxin B, angiotensin II stimulated CTGF expression in the presence as well as the absence of Y27632 (Figure 4C).

Induction of CTGF via AT1 Receptors

To characterize the endogenous receptor involved in angiotensin II signaling, the cells were preincubated with Exp3174, an active metabolite of isosartan and a specific inhibitor of the AT1 receptor, or PD123319, a specific inhibitor of the AT2 receptor. Preincubation with Exp3174 prevented the induction of CTGF by angiotensin II, whereas PD123319 did not interfere with CTGF induction (Figure 1A available online at http://atvb.ahajournals.org). These data indicate that interaction of angiotensin II with AT1 receptors was responsible for the angiotensin II–mediated increase in CTGF expression.

In vascular smooth muscle cells, statins interfere with the expression of AT1 receptors.14,15 Therefore, the fibroblast cell line was preincubated with simvastatin, and the expression of AT1 receptors was analyzed by semiquantitative RT-PCR and Western blot analysis. At the mRNA level, partial reduction was observed at extremely high concentrations of simvastatin (30 µmol/L, Figure 1B). AT1 receptor protein was detected in a membrane fraction containing plasma membranes and microsomal membranes (Figure 1C). There was some variability in the AT1 protein detected in the presence or absence of simvastatin but no significant downregulation.

Inhibition of CTGF Expression by Interference With p42/44 MAP Kinase Signaling

Depending on the cell type, AT1 receptors couple to different types of trimeric G proteins and might activate multiple signaling pathways. As a possible intracellular mediator, activation of the p42/44 mitogen-activated protein (MAP) kinase (ie, extracellular signal–regulated kinase) was investigated. Treat-
ment of the cells with angiotensin II activated p42/44 MAP kinase, as detected by Western blot analysis of the phosphorylated and thus, activated form of the kinase (Figure 5A).

Interference with p42/44 MAP kinase activation by the MAP kinase kinase inhibitor by PD98059 partially reduced basal expression and almost prevented angiotensin II–stimulated CTGF induction (Figure 5B; PD98059-treated vs untreated cells, \( P < 0.05 \)). The stimulation rate in the absence of PD98059 (1.5 \( \pm \) 0.2-fold) was reduced to 1.1 \( \pm \) 0.1-fold (\( P < 0.01 \)) in the presence of PD98059, indicating a substantial contribution of MAP kinase signaling to the induction of CTGF.

To assess cross-talk between the MAP kinase and the Rho protein signaling pathways, the effect of simvastatin on p42/44 MAP kinase activation was determined. Even very high concentrations of simvastatin (up to 30 \( \mu \)mol/L) did not significantly reduce p42/44 MAP kinase activation in these cells (data not shown), indicating cross-talk between MAP kinase signaling and Rho signaling below the level of p42/44 MAP kinase. Similarly,

Figure 4. Regulation of basal CTGF expression by RhoA signaling. A, Fibroblasts were preincubated with toxin B (ToxB, 1 and 0.1 ng/mL) for 90 minutes or Y27632 (Y27, 10 and 1 \( \mu \)mol/L) for 30 minutes and then stimulated with angiotensin II for 60 minutes. mRNA expression was detected by Northern blot expression. B, Fibroblasts were preincubated with Y27632 (Y27, 10 \( \mu \)mol/L) for 30 minutes and then stimulated with angiotensin II (2 \( \times \) 10\(^{-7}\) mol/L) for 90 minutes. Whole-cell lysates were analyzed by Western blotting with an antibody from Santa Cruz. To control for equal loading, the blot was also analyzed for tubulin. C, Fibroblasts were preincubated with Y27632 (Y27, 1 and 10 \( \mu \)mol/L) for 30 minutes and then stimulated with angiotensin II for 60 minutes. To compare different experiments, stimulation with angiotensin II was set to 100%, corresponding to 1.9-fold stimulation. Data are mean \( \pm \) SD of 4 independent experiments. \( * P < 0.05 \), Mann-Whitney U test.

Figure 5. Regulation of angiotensin II–mediated CTGF expression by the 42/44 MAP kinase pathway; interference by cAMP. A, Fibroblasts were stimulated with angiotensin II (10\(^{-7}\) mol/L) for the times indicated. The phosphorylated form of p42/44 MAP kinase was detected in cellular homogenates by Western blot analysis. The blot is representative of 2 experiments with comparable results. B, Fibroblasts were preincubated with PD 98059 (PD, 10 \( \mu \)mol/L) for 30 minutes and then stimulated with angiotensin II (10\(^{-7}\) mol/L) for 60 minutes. Data are mean \( \pm \) SD of 4 independent experiments. Stimulation with angiotensin II was set to 100%, corresponding to 1.6-fold stimulation. NS indicates not significant. C, After preincubation with toxin B (ToxB, 1 and 5 ng/mL), cells were stimulated with angiotensin II (2 \( \times \) 10\(^{-7}\) mol/L) for 5 minutes. MAP kinase (MAPK) and the phosphorylated form of MAPK (P-MAPK) were detected on the same blot. The blot is representative of 2 independent experiments. D, Fibroblasts were preincubated with forskolin (FK, 10 \( \mu \)mol/L) for 45 minutes and then stimulated with angiotensin II (2 \( \times \) 10\(^{-7}\) mol/L) for 60 minutes. Data are mean \( \pm \) half range of 2 experiments; stimulation of CTGF by angiotensin II was 2.5-fold. To detect the phosphorylation of MAP kinase, cells were stimulated with angiotensin II (2 \( \times \) 10\(^{-7}\) mol/L) for 5 minutes. Whole-cell lysates were analyzed by Western blotting with an antibody directed against the phosphorylated form of p42/44 MAP kinase. To control for equal loading, the blot was also analyzed for anti-tubulin. Parallel blot was analyzed for p42/44 MAP kinase. E, Fibroblasts were preincubated with the cAMP analogue cBIMPs (10 \( \mu \)mol/L) for 90 minutes and then stimulated with angiotensin II (2 \( \times \) 10\(^{-7}\) mol/L) for 60 minutes to detect CTGF mRNA and for 5 minutes to detect phosphorylation of MAP kinase (P-MAPK) by Western blot analysis. The blot is representative of 3 independent experiments.
MAP kinase activation was reduced by high concentrations of toxin B (5 ng/mL) but not by 1 ng/mL, which was sufficient to prevent CTGF induction (Figure 5C).

**Interference of cAMP With the MAP Kinase–Mediated Induction of CTGF**

CTGF expression has been shown to be negatively regulated by elevated levels of cAMP. When the levels of cAMP were increased by stimulation of adenylate cyclase by forskolin, angiotensin II–mediated induction of CTGF expression was prevented (Figure 5D, left). Forskolin strongly interfered with the activation of p42/44 MAP kinase (Figure 5D, right), defining the molecular target for the interference of cAMP with angiotensin II signaling. The same results were obtained when the cells were incubated with the cell-permeable cAMP analogue cBIMPs, which specifically activates protein kinase A (Figure 5E).

**Discussion**

The 2K,1C model of renovascular hypertension is characterized by an activated renin-angiotensin system. The elevated levels of CTGF observed in the hypertrophic left ventricles of hypertensive mice suggested a connection between angiotensin II and CTGF but could not exclude the induction of TGF-β or other mediators that subsequently might induce CTGF. Cell culture studies were thus necessary to prove angiotensin II to be a direct inducer of CTGF expression by activation of endogenous AT1 receptors. Our data are in line with a recent publication that showed induction of CTGF in a rat fibroblast cell line that overexpressed the AT1 receptor. CTGF protein was upregulated within 1 hour. The rapid induction of CTGF mRNA within 30 to 45 minutes and the superinduction in the presence of cycloheximide were consistent with CTGF’s representing an immediate-early response gene. This excluded the involvement of another newly synthesized protein, eg, TGF-β, as a mediator of angiotensin II–mediated CTGF mRNA induction. In different models of renal or cardiac fibrosis, modulation of the synthesis of extracellular matrix proteins by angiotensin II was shown to be mediated by TGF-β, which itself was induced by angiotensin II (summarized in Williams et al.). Our data do not exclude TGF-β as a mediator of CTGF induction at later time points in vivo but indicate that angiotensin II might also directly affect the synthesis of CTGF.

Analysis of the signaling pathways involved in the regulation of CTGF expression revealed a novel interaction between the p42/44 MAP kinase pathway and Rho protein–mediated gene transcription. Activation of p42/44 MAP kinase proved to be an essential part of the signaling pathway leading to the induction of CTGF by angiotensin II, because interference by the MEK inhibitor inhibited angiotensin II–mediated expression of CTGF (Figure 6). However, isolated activation of MAP kinase signaling was not sufficient for CTGF induction: complete downregulation of all Rho proteins by simvastatin or toxin B prevented stimulation by angiotensin II, even in the presence of MAP kinase activation. The importance of the Rho pathway was also evident when the same cells were stimulated by lysophosphatidic acid (LPA). In contrast to angiotensin II, LPA activates p42/44 MAP kinase and RhoA activity, which was essential for CTGF induction, whereas inhibition of MAP kinase activation had no effect on LPA-mediated CTGF induction. These data indicate that a strong activation of RhoA signaling is sufficient for the induction of CTGF, whereas RhoA signaling and MAP kinase signaling interact when both systems are submaximally stimulated. This interaction might become significant in vivo, when stimulatory signals of different bioactive factors are being integrated within a given cell. Cross-talk between MAP kinase signaling and SMAD-dependent induction of CTGF has been reported with TGF-β used as a stimulus. This suggests an even higher level of signaling complexity, because TGF-β–mediated induction of CTGF is also subject to inhibition by statins and thus, sensitive to changes in Rho proteins.

Simvastatin effectively blocked CTGF in the low micromolar concentration range, consistent with previous data obtained in LPA-stimulated fibroblasts or mesangial cells and data regarding other cellular effects, such as interference with the induction of matrix proteins or inhibition of cellular proliferation. Prevention of the inhibitory effects of simvastatin by GGPP indicated an involvement of Rho proteins, as confirmed by toxin B and Y27632. Comparison of the complete downregulation of CTGF by simvastatin or toxin B and the partial reduction by Y27632, which selectively targets RhoA–associated kinases, hints to a role for other Rho proteins besides RhoA in the regulation of CTGF.

Statins inhibit the expression of AT1 receptors in vascular smooth muscle cells. Therefore, the expression of AT1 receptors was investigated at the mRNA and protein levels. Even 10 times higher concentrations of simvastatin than those needed for complete downregulation of CTGF did not significantly reduce AT1 receptor expression, as determined by semiquantitative RT-PCR or Western blot analysis. The contradictory results might be attributed to the different cell types used, because consistent with our findings, also in rat vein endothelial cells, AT1 receptor expression was not modulated by simvastatin.

Elevation of cAMP seems to be one of the major signals to interfere with the expression of CTGF: cAMP interferes with...
at least 2 regulatory steps in CTGF activation: (1) activation of p42/44 MAP kinase (this study and Stratton et al.26) and (2) RhoA-mediated changes in cell architecture.16 This implies that activators of adenylate cyclase or protein kinase A might be envisaged as potential antagonists of CTGF expression, as shown in the case of iloprost or follicle-stimulating hormone.27,28 Direct induction of CTGF might thus contribute to the profibrotic, hemodynamic-independent effects of angiotensin II. This study focused on the contribution of p42/44 MAP kinase, RhoA, and cAMP to the regulation of CTGF expression. The pronounced interference with the geranylgeranyl modification of signaling proteins by statins was indicative of additional cross-talk between regulatory pathways, which remain to be analyzed in detail. Based on the in vitro results, activation of protein kinase A or interference with geranylgeranylation seems to be a promising strategy to prevent excess synthesis of CTGF. As a model system to study these aspects of angiotensin-mediated end-organ damage, the angiotensin II–dependent 2K,1C model might be useful, as it showed strong upregulation of CTGF in the left ventricle of all animals tested.

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