Angiotensin II Activates Collagen I Gene Through a Mechanism Involving the MAP/ER Kinase Pathway

Pierre-Louis Tharaux, Christos Chatziantoniou, Fadi Fakhouri, Jean-Claude Dussaule

Abstract—Vascular remodeling and rearrangement of the extracellular matrix formation are among the major adaptive mechanisms to chronic increase in blood pressure. In previous studies we have found that angiotensin II (Ang II) participates in the hypertension-associated aortic and renal vascular fibrosis by stimulating collagen type I formation. The purpose of the present study was to gain insight into the molecular events that lead from the Ang II receptor to collagen I gene activation. To this end, we used a novel strain of transgenic mice harboring the luciferase gene under the control of the collagen I-α2 chain promoter [procolα2(I)]. Ang II produced an early (1 hour) 2- to 3-fold stimulation of procolα2(I) activity in freshly isolated aortas and renal cortical slices (P<0.01) followed by similar increase in procolα2(I) mRNA aortic levels. This effect of Ang II was inhibited by AT1-receptor antagonism (candesartan) and blockade of the MAPK/ERK cascade (PD98059); in contrast, inhibition of the P38 kinase pathway (SB202190) and blockade of the release of the transcription factor NFκB (PDTC) did not have any effect in the Ang II-induced activation of the collagen I gene. In addition, Ang II induced a rapid (5 minutes) increase of the MAPK/ERK activity that was accompanied by increased expression (3-fold) of the c-fos proto-oncogene. This increase of c-fos mRNA expression was blocked by PD98059; in addition, curcumin, a blocker of the transcriptional factor AP-1, canceled the effect of Ang II on the collagen I gene. Decorin, a scavenger of the active form of transforming growth factor-β (TGF-β), canceled the Ang II effect on collagen I gene, whereas inhibition of the MAPK/ERK pathway had no effect on the TGF-β–induced activation of procolα2(I). These data indicate that the cellular events after AT1 receptor stimulation and leading to activation of collagen I gene expression require activation of both the MAPK/ERK and TGF-β signaling pathways.

Key Words: collagen ■ angiotensin II ■ fibrosis ■ kinase ■ extracellular matrix ■ transforming growth factors

Hypertension is usually associated with the development of vascular and renal fibrosis.1 This pathophysiological process is characterized by structural changes in vasculature caused by increased synthesis and rearrangement of extracellular matrix proteins, such as the collagen type I.2 Several studies support a major role for the renin-angiotensin system in the development of fibrosis.3,4 Although the cellular mechanism(s) of the angiotensin II (Ang II)-induced vasocstriction have been extensively studied, it remains less known what is (are) the signaling pathway(s) involved in the fibrogenic action of Ang II.

In previous studies, we investigated the role of Ang II in the mechanisms of renal vascular fibrosis during hypertension by using a new strain of transgenic mice.5 These mice express the luciferase reporter gene under the control of the promoter of the α2 chain of collagen I gene [procolα2(I)].6 We have established that luciferase and collagen I gene expressions are closely correlated from the fetal development throughout the adult life under normal or pathophysiological conditions either in vivo or in freshly isolated tissues ex vivo.5–7 In particular, we investigated whether Ang II played a role in the mechanism(s) controlling the development of renal vascular and aortic fibrosis during hypertension. To this end, hypertension was induced in the procolα2(I) transgenic mouse by inhibiting nitric oxide synthesis, and the activation of collagen I gene was estimated in several vascular tissues such as afferent arterioles, glomeruli, heart, and aorta. We observed that pharmacological blockade of angiotensin receptors (AT1) completely suppressed the activation of collagen I gene and protected animals from the development of vascular fibrosis.5

The objective of the present study was to get insight into the cellular events after Ang II binding to its receptors and leading to collagen I gene activation. To this end, we tested in freshly isolated aortas and renal cortical slices the involvement of 3 pathways, MAPK/ERK, NFκB, and P38, thought to be among the major mediators of the mitogenic action of Ang II that use specific inhibitors or blockers of their enzymatic cascades. In addition, we investigated how transforming growth factor-β (TGF-β), another potent fibrogenic agent, participates in the Ang II–induced stimulation of procolα2(I).

Our data indicated that the MAPK/ERK cascade is an
important mediator in the signaling pathway leading from Ang II to collagen I gene activation. Moreover, we observed that although biological activity of TGF-β is required for collagen I gene activation, this action of TGF-β is insensitive to MEK blockade, the upstream kinase of MAPK/ERKs.

**Methods**

**Animal Treatment**

Male transgenic mice weighing 25 to 35 g (3 to 6 months old) at the time of the experiments were maintained on a normal salt diet. Animals had free access to chow and tap water. This transgenic line, named pGB 19.5/13.5, was generated in the laboratory of B. de Crombrugghe (University of Texas, Houston).6 These animals harbor a construction containing the sequences −19.5 to −13.5 kb and −350 to +54 bp of the promoter of the α2 chain of mouse collagen type I gene linked to 2 reporter genes, the firefly luciferase and the *Escherichia coli* β-galactosidase. The choice of these mice was based on previous studies showing that the expression pattern of the two reporter genes in embryos and adult animals closely correlates with cell and tissue distribution of collagen I.5,6 The protocol followed the INSERM guidelines for animal care and protection.

**Ex Vivo Addition of Ang II or TGF-β**

Experiments were performed on freshly isolated renal cortical slices and aortas incubated in RPMI 1640 medium (containing 10 mmol/L HEPES, 2 mmol/L t-glutamine, 100 U/mL penicillin, and 100 mg/L streptomycin) for up to 1 hour at room temperature. Ang II (100 nmol/L) was added either alone or in combination in tissues preincubated for 5 minutes with candesartan (100 nmol/L, AT1-receptor antagonist), PD 98059 (50 μmol/L, MEK inhibitor), AP-1 blocker), pyrrolidinedithiocarbamate (PDTC 100 μmol/L, specific inhibitor of NFκB inhibitor), or decorin (100 nmol/L, TGF-β scavenger). In separate experiments, TGF-β (0.8 nmol/L) was added with or without PD98059, curcumin, or decorin. In each experiment, segments of aorta or renal cortical slices from the same animal were used in a paired fashion to compare the effect of Ang II or TGF-β with or without the different inhibitors. After 1 hour of incubation, tissues were lysed, and luciferase or MAP kinase activity was measured as described below.

**Assays for Expression of Luciferase**

Luciferase activity was measured with a commercial reporter gene assay kit (Boehringer Mannheim) in homogenized tissues with the aid of a Lumat LB 9507 luminometer (EG & Berthold) as previously described.5,8 Results are expressed as luciferase light units per microgram of protein (LU/mg).

**Measurement of MAPK Activity**

MAP kinase activity was assayed with the p42/p44 MAP kinase enzyme assay system (Amersham), as described elsewhere.8 The enzymatic reaction was performed at 30°C for 30 minutes. ERK activity was normalized to the protein content of supernatants.

**Estimation of c-fos and α2 Collagen I mRNA by Reverse Transcription–Polymerase Chain Reaction**

One microgram of RNA extracted from aortas by use of the Trizol kit (Life Technologies LTD) was reverse-transcribed by means of the Superscript II protocol (Life Technologies LTD). One microliter of the reverse transcription (RT) reaction was incorporated in the polymerase chain reaction (PCR) (buffer: 2 mmol/L MgCl2, c-fos primers 15 pmol each, GAPDH primers 0.2 pmol each, 0.2 mmol/L dNTP, 1 U Taq DNA polymerase) and initially denatured for 5 minutes at 94°C.

We used oligonucleotides specific for c-fos (Stratagene); sense: GCT TTC CCC AAA CTT CGA CCA TG; antisense: CTG TCA CCG TGG GGA TAA AGT TGG; and oligonucleotides specific for GAPDH (Genset); sense: ACC ACA GTC CAT GCC ATC AC; antisense: TCC ACC ACC CTG TTG CTG TA.

The compatibility of primer pairs was verified with the use of Oligo4 software (Molecular Biology Insights). PCR products were sequenced (Genome Express) and compared with GenBank and EMBL genomic data bank with the BLAST algorithm to verify their identity with the theoretical targets.

The 28-cycle program performed with a Biometra-Trio-thermoblock thermocycler (Kontron Instruments) consisted of 45 seconds of denaturation at 94°C, 30 seconds of annealing at 60°C, and 1-minute extension at 72°C. After completion of the cycling program, samples were subjected to a 10-minute extension period at 72°C. Ten microliters of each PCR assay were run in a 2% agarose gel stained with ethidium bromide. After electrophoresis, gels were UV-transilluminated and digitalized. The optical densities of the bands corresponding to the coamplified c-fos (216-bp fragment) and GAPDH fragments (452-bp fragment) were calculated and compared by means of the NIH Image 1.61 software.

RT-PCR for the α2 chain of collagen I was performed with the same methodology as described above, except the number of cycles was 30. The primers used were previously reported.6

**Statistical Methods**

Statistical analyses were performed with the paired t test or ANOVA followed by protected least significance difference Fisher’s test of the Statview software package. Results with values of $P<0.05$ were considered statistically significant. All values are mean±SEM.

**Results**

**Transgenic Model**

The choice of these mice was based on previous studies showing that the expression pattern of luciferase in embryos and adult animals closely correlates with cell and tissue distribution of collagen I.5,6 For instance, tissues rich in collagen I (tail, skin) showed very high levels of luciferase activity (21 954±1 183 and 8245±8 15LU/mg for these tissues, respectively); tissues poor in collagen I (renal cortex, interlobular arteries, and afferent arterioles) showed little activity (184±23 and 214±25 LU/mg, for these tissues, respectively); and where collagen I is almost absent (glomeruli), there was negligible luciferase activity (19±2.2LU/mg).7 In these previous studies, we have also observed that the changes of luciferase activity in the renal cortex reflect mainly the changes of the luciferase activity of the renal cortical vascular tissue (interlobular arteries, afferent arterioles, glomeruli).

**Ang II–Induced Activation of Procol1α2(I) Gene**

First, we examined whether Ang II can induce collagen type I gene activation in freshly isolated aortas and renal cortical slices in vitro. As shown in Figure 1, Ang II produced a 2-fold increase in luciferase activity in aortas (1936±17 versus 3815±420LU/μg, $P<0.01$, in control and 1 hour after Ang II, respectively, Figure 1, top). A similar stimulatory effect was observed in renal cortical slices (178±17 versus 405±42LU/μg, $P<0.01$, in control and 1 hour after Ang II, respectively). This effect on procol1α2(I) promoter was mediated through an AT1 receptor activation, since it was completely canceled by candesartan (2127±224, Figure 1, and 207±23LU/μg, for aorta and renal cortex, respectively).
Effect of MAPK/ERK, NFκB, or P38 Kinase Pathway Inhibition on Ang II–Induced Procolα2(I) Gene Activation

As shown in Figure 1, bottom, PD98059, a specific MAPK/ERK-pathway inhibitor, blocked the stimulatory effect of Ang II on procolα2(I) gene in aorta (3760±267 versus 1812±213 LU/μg, P<0.01, in Ang II and Ang II+PD98059, respectively) and renal cortex (301±27 and 191±17 LU/μg, P<0.01). In contrast, addition of PDTC (NFκB inhibitor) or SB202190 (P38 inhibitor) did not alter the Ang II–induced increase of luciferase activity (PDTC: 3421±324 and 328±39, SB202190: 3506±371 and 292±34 LU/μg, for aorta and cortex, respectively).

Effect of MAPK/ERK Pathway Inhibition on Ang II–Induced Procolα2(I) mRNA Expression

To verify whether the above-mentioned changes of the procolα2(I) promoter were associated with changes of procolα2(I) mRNA expression, we estimated by RT-PCR the ratio of optical density of collagen I product over GAPDH product under control conditions and after administration of Ang II. As shown in Figure 2, top, Ang II induced a 2-fold increase of procolα2(I) mRNA expression in aorta (1.67±0.05 versus 0.85±0.03 ratio of optical density of α2 col I RT-PCR product on GAPDH product, P<0.001, in Ang II and control, respectively). As was the case with the procolα2(I) promoter activation, PD98059 blunted the stimulatory effect of Ang II on procolα2(I) mRNA expression (1.09±0.05, P<0.01 versus Ang II alone, Figure 2).

Ang II–Induced Activation of MAPK/ERK Activity

To verify whether Ang II could activate the MAPK/ERK cascade, measurements of MAPK/ERK activity were per-
formed in aortas of the transgenic mice. In agreement with the literature, Ang II produced an early increase (150% increase at 5 minutes) of MAP kinase activity that plateaued after 30 minutes (340% of baseline, Figure 2, bottom). The Ang II–induced increase of MAPK/ERK activity was completely inhibited by the AT1-receptor antagonist candesartan (103% of baseline).

**Effect of AP-1 Inhibition on Ang II–Induced Procollagenα(I) Gene Activation**

Ang II produced an early increase (peak at 15 minutes) of c-fos mRNA expression in aortas of transgenic mice (Figure 3A). Concomitant addition of PD98059 canceled the Ang II–induced increase of c-fos mRNA expression, suggesting involvement of MAPK/ERK in the Ang II–induced c-fos synthesis. To investigate if the AP-1 transcriptional complex was implicated in the activation of the procollagenα(I) gene, experiments were performed in which luciferase activity was measured in aortas of transgenic mice. As shown in Figure 3B, curcumin completely canceled the Ang II–induced increase of luciferase activity in aortas of transgenic mice. Similar results were obtained with renal cortical slices (315±27 versus 153±13 LU/µg, P<0.03, for Ang II and Ang II+curcumin, respectively).

**TGF-β–Induced Procollagenα(I) Gene Activation**

In these experiments, we tested whether TGF-β interacted with Ang II to activate the collagen I gene. Exogenous TGF-β1 increased 2-fold the luciferase activity in aortas (1786±203 versus 2689±384 LU/µg, P<0.05, for control and TGF-β1, respectively, Figure 4, top). This increase was completely blocked by decorin, a TGF-β scavenger (1656±183, P<0.05), but not by PD98509 or curcumin (2935±648 and 2612±384 LU/µg, for these 2 agents, respectively, Figure 4). Interestingly, decorin inhibited the Ang II–induced increase of luciferase activity (2923±315 versus 1945±224 LU/µg, P<0.05, for Ang II and Ang II+decorin, respectively, Figure 4, bottom).

**Discussion**

In the present study, a strain of transgenic mice harboring the luciferase reporter gene under the control of the collagen I promoter permitted us to investigate mechanism(s) that control the activation of collagen type I gene in aorta and renal cortex. Specifically, we found that Ang II can rapidly activate the procollagenα(I) gene in freshly isolated aortic and renal cortical tissues. Using specific inhibitors of several intracellular signaling pathways, we observed that MAPK/ERK and AP-1 played a major role in the Ang II–mediated activation of collagen I gene. In addition, we confirmed the involvement of TGF-β in this action of Ang II. Interestingly, the mechanism by which TGF-β induced collagen I gene activation was insensitive to blockade of the MAPK/ERK-AP-1 pathway.

Several recent studies point to a leading role of the renin-angiotensin system in the development of renal and cardiac fibrosis. For instance, Ang II stimulated collagen protein synthesis in cultured cardiac fibroblasts in vitro and increased collagen I mRNA expression in rat hearts in vivo.11,12 In the L-NAME model of hypertension, we observed that AT1 antagonism prevented activation of collagen I gene in the renal and aortic vasculature and reduced the development of renal vascular fibrosis.5 Similarly, ACE inhibition markedly reduced the development of renal and cardiac fibrosis and improved survival of rats during chronic inhibition of nitric oxide.13 Our present data clearly indicate that Ang II produced an AT1 receptor–mediated increase of collagen I gene expression in freshly isolated renal and aortic vascular tissues. The magnitude of the procollagenα(I) increase (2- to 3-fold versus baseline) and the kinetics were similar to those previously observed when Ang II was administered in the same strain of mice in vivo.5 Evaluation of mRNA expression of the α2 chain of collagen I by RT-PCR confirmed the Ang II–induced activation of the collagen I gene, at least up to the mRNA level (Figure 2).

It is well established that the mitogenic action of Ang II in the vascular smooth muscle cells is mediated mainly by the MAPK/ERK enzymatic cascade. It is also known that exogenous administration of Ang II is usually accompanied by increased MAPK/ERK activity in vascular tissues. For instance, acute in vivo or ex vivo administration of Ang II produced a marked increase of ERK activity in rat aortas.8,14
In addition, the glomerular ERK activity was 2- to 3-fold increased during prolonged infusion of Ang II. In contrast, it is less clear what the contribution of this pathway is in the vascular fibrosis induced by Ang II. Our results show that Ang II increased procolα2(I) expression through the AT1 receptor (Figure 1) and that this increase was accompanied by a rapid stimulation of MAPK/ERK activity (Figure 2). These two phenomena were associated because specific inhibition of MEK completely blocked the Ang II effect on collagen I gene activation evaluated by luciferase activity and RT-PCR. To our knowledge, this is the first study that establishes a link between Ang II, AT1 receptor, MAPK/ERK activity, and procolα2(I) activation in freshly isolated vascular and renal tissues.

Ang II can also induce oxidative stress-dependent effects in vascular smooth muscle cells, leading to hypertrophy and proliferation.16 These events are usually associated with rapid release of NFκB and/or P38 MAPK activation in vitro.17 However, the activation of these two pathways by Ang II may not be directly linked with fibrogenesis. In this regard, chronic administration of an ACE inhibitor reduced the arterial expression of NFκB and of proinflammatory chemokines without impeding the increased levels of mRNA expression and protein content of collagen I in a model of atherosclerosis in rabbits.18 In our experiments, neither the NFκB nor the P38 inhibitor altered the increased procolα2(I) expression in aorta and renal cortex. Thus, contrary to MAPK/ERK cascade, these redox-sensitive signaling pathways play a negligible role in the acute, Ang II–induced activation of collagen I gene.

A major downstream signal after MAPK/ERK activation is the phosphorylation of Elk-1/TCF transcription factors, leading to the induction of c-fos proto-oncogene expression to form the AP-1 transcriptional complex.19 The AP-1 complex is usually an heterodimer formed by c-Fos and c-Jun. Contrary to the c-Fos expression that is inducible, c-Jun can be present constitutively or activated through the Jun kinase pathway. Our data showing that Ang II increased c-fos mRNA expression and that this increase was blocked in the presence of an MEK inhibitor (Figure 3) suggest that c-fos is involved in the fibrotic effect of Ang II. It is also possible that Ang II activated the Jun kinase pathway. In this regard, Ang II stimulated PDGF mRNA expression and induced cell proliferation in vascular smooth muscle cells in vitro by activating both ERK and JNK pathways.20 The use of curcumin does not allow us to exclude the concomitant activation of both pathways because it prevents the c-Fos/c-Jun complex from binding to the AP-1 motif of DNA.10 Whatever the case is, the promoter of the α2 chain of collagen I contains several sites of AP-1 recognition,21,22 thus supporting our hypothesis of an Ang II–MAPK/ERK–AP-1 interaction.

Activation of the MAPK/ERK–AP-1 signaling pathway has been proposed to mediate the suppressor, antifibrotic action of estradiol in cultured mesangial cells.23 However, this hypothesis contrasts with the findings of another study in which the antimitogenic, antifibrotic effects of estrogens were attributed to the inhibition of the MAPK/ERK cascade activity in human aortic smooth muscle cells.24 Moreover, estrogens reversed the Ang II–induced increase of MAPK activity in human vascular smooth muscle and endothelial cells.25

TGF-β is another known activator of collagen I gene expression, and several studies associate the fibrogenic action of Ang II to TGF-β.26 In addition, the TGF-β promoter contains AP-1–dependent transcriptional regulatory domains.27 For these reasons, we tested next whether TGF-β could be involved in the Ang II–MAPK/ERK–procolα2(I) interaction by using decorin, a scavenger of its active form. Interestingly, neither MAPK/ERK inhibition nor AP-1 blockade altered the TGF-β–induced stimulation of procolα2(I) activity (Figure 4), whereas decorin effectively canceled the

**Figure 3.** A, Effect of MAPK/ERK antagonism on Ang II–induced stimulation of c-fos mRNA; B, luciferase activity in aortas of transgenic mice under control conditions and in presence of Ang II with or without the AP-1 complex inhibitor curcumin (20 μmol/L). Values are mean±SEM of 8 experiments. *P<0.05 vs control; #P<0.05 vs Ang II.
Ang II–induced collagen I gene activation. This finding leads us to propose the following alternatives: one hypothesis is that Ang II increased procollagen I expression by a MAPK/ERK–AP-1–dependent induction of TGF-β activation, as was the case in mesangial or aortic smooth muscle cells.28–30 Another alternative is that Ang II activated in parallel 2 signaling pathways, MAPK/ERK–AP-1 and TGF-β–Smads and that both cooperate and are necessary to induce procollagen I expression. In this regard, it was recently reported that MAPK/ERK and TGF-β–Smad signaling pathways may converge at the AP-1 binding promoter sites.31,32 A third mechanism, proposed for fibronectin production in human mesangial cells in vitro, is that TGF-β–Smad signaling pathways may contribute in this case, because TGF-β–Smad stabilizing mRNA.35 This kind of interaction seems unlikely to our case, because TGF-β increased the activity of collagen I promoter (Figure 4).

In conclusion, we investigated mechanisms leading to the increased expression of collagen I gene in aortic and renal vascular tissues by using a new model of transgenic mouse harboring the luciferase reporter gene under the control of collagen I promoter. Our data indicate that the fibrogenic effects of Ang II are mediated through the AT1 receptor; furthermore, the cellular events after AT1 receptor stimulation and leading to activation of collagen I gene expression require TGF-β and a MAPK/ERK-mediated formation of the AP-1 transcriptional complex. The mechanisms of the interaction between these two pathways will be pursued in future studies.

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References


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