Angiotensin II Stimulates Collagen Synthesis in Human Vascular Smooth Muscle Cells
Involvement of the AT\textsubscript{1} Receptor, Transforming Growth Factor-\textbeta, and Tyrosine Phosphorylation

Carol M. Ford, Shaohua Li, J. Geoffrey Pickering

Abstract—Angiotensin II is an established regulator of vascular tone and smooth muscle cell (SMC) growth. However, there are little data about its effect on collagen synthesis by SMCs and none regarding the mechanism of such an effect. We studied the effect of angiotensin II on collagen production by human arterial SMCs, using uptake of [\textsuperscript{3}H]proline into collagenase-digestible proteins, and by ribonuclease protection assay for mRNA encoding the pro\textalpha\textsubscript{1}(I) collagen mRNA abundance, with the half-maximal effect at 1.7 nmol/L. Angiotensin II–stimulated collagen expression was associated with a 6-fold increase in transforming growth factor-\textbeta (TGF-\textbeta) production and was inhibited by a neutralizing antibody to TGF-\textbeta. Both collagen production and TGF-\textbeta release were inhibited by the AT\textsubscript{1}-specific antagonist, losartan, but not by the AT\textsubscript{2} receptor antagonist, PD123319. To determine if tyrosine phosphorylation was functionally linked to collagen synthesis, we studied the effect of 2 mechanistically distinct inhibitors of tyrosine kinase, genistein, and tyrphostin A25. These inhibitors abrogated angiotensin II–mediated procollagen mRNA expression and angiotensin II–mediated TGF-\textbeta production, whereas the inactive homolog tyrphostin A1 had no effect. We conclude that angiotensin II stimulates collagen production in human arterial SMCs via the AT\textsubscript{1} receptor and an autocrine loop of TGF-\textbeta, induction of which requires tyrosine phosphorylation. (Arterioscler Thromb Vasc Biol. 1999;19:1843-1851.)

Key Words: angiotensin \hspace{1mm} smooth muscle cell \hspace{1mm} collagen \hspace{1mm} tyrosine kinase \hspace{1mm} transforming growth factor

Accumulation of interstitial collagen is a structural hallmark of vascular disease. Collagen can occupy most of the volume of occlusive atherosclerotic plaque\textsuperscript{1} and is a major constituent of human restenosis lesions that develop after angioplasty.\textsuperscript{2} Increased vascular collagen is also found in hypertension and probably contributes to the increased arterial stiffness of this condition.\textsuperscript{3,4}

The production of collagen in the vessel wall is likely regulated by a combination of mechanical forces and soluble growth factors. Of the latter, transforming growth factor-\textbeta (TGF-\textbeta) is known to stimulate synthesis of collagen in arteries of growing rats\textsuperscript{22} and in spontaneously hypertensive rats.\textsuperscript{23} Recently, infusion of angiotensin II into rats was shown to induce aortic fibrosis.\textsuperscript{24} These studies thus highlight a relationship between angiotensin II and collagen metabolism, although the mechanism is poorly defined.

Angiotensin II, long recognized as a regulator of vascular tone, has also garnered interest because of its capacity to act as a vascular cell growth factor. For example, SMCs respond to angiotensin II by increasing protein synthesis,\textsuperscript{8-10} by stimulating DNA synthesis with or without cell proliferation,\textsuperscript{11-13} and by expressing c-fos, c-jun, and c-myc.\textsuperscript{14-16} Although less well studied, angiotensin II may also regulate vascular collagen metabolism. Angiotensin II was found to stimulate collagen synthesis in mesangial cells,\textsuperscript{17,18} which are functionally similar to SMCs, and angiotensin II has been reported to increase collagen production in rat, chick embryo, and porcine arterial SMCs.\textsuperscript{19-21} The concentration of angiotensin II required for an effect in some of these studies was high,\textsuperscript{19,20} and possibly not physiological; however, animal data lend support to a potential role for angiotensin II on vascular collagen accumulation. Inhibition of angiotensin-converting enzyme activity suppressed the accumulation of collagen in arteries of growing rats\textsuperscript{22} and in spontaneously hypertensive rats.\textsuperscript{23} The concentration of angiotensin II required for an effect in some of these studies was high,\textsuperscript{19,20} and possibly not physiological; however, animal data lend support to a potential role for angiotensin II on vascular collagen accumulation. Inhibition of angiotensin-converting enzyme activity suppressed the accumulation of collagen in arteries of growing rats\textsuperscript{22} and in spontaneously hypertensive rats.\textsuperscript{23} Recently, infusion of angiotensin II into rats was shown to induce aortic fibrosis.\textsuperscript{24} These studies thus highlight a relationship between angiotensin II and collagen metabolism, although the mechanism is poorly defined.

Angiotensin II interacts with 2 major subtypes of receptors, designated AT\textsubscript{1} and AT\textsubscript{2}, which belong to the superfamily of receptors with 7 transmembrane domains.\textsuperscript{25-27} The best-characterized signal transduction pathway is the activation, by the AT\textsubscript{1} receptor, of guanine nucleotide protein (G protein)-coupled phospholipase C-\textbeta, which in turn liberates...
inositol trisphosphate and calcium. Recently, angiotensin II has also been found to initiate signaling cascades that are similar to those activated by receptor tyrosine kinases. For instance, angiotensin II activates the mitogen-activated protein (MAP) kinase cascadeto and can stimulate tyrosine phosphorylation of phospholipase C-γ2 signaling transducers and activators of transcription (STATs) and paxillin. Evidence is emerging that the contractile and hypertrophic actions of angiotensin II are at least partly mediated by tyrosine kinase-dependent signaling pathways. This participation of tyrosine phosphorylation-mediated signaling events downstream to a G protein–coupled receptor both highlights the diversity of signaling cascades that can be initiated by angiotensin II and brings new opportunities for therapeutic control of vascular remodeling.

The biochemical pathway by which angiotensin II controls collagen production in SMCs is unknown. The transmembrane signal transduction pathway is obscure and it is likewise not known if the effect of angiotensin II on collagen production is direct or via the release of other fibrogenic factors. Accordingly, the purpose of this study was (1) to determine if angiotensin II stimulates the elaboration of collagen by human vascular SMCs; (2) to clarify the membrane receptor subtype that mediates angiotensin II–stimulated collagen expression; (3) to determine whether TGF-β is involved in the fibrogenic response to angiotensin II; and (4) to investigate the role of tyrosine kinases in angiotensin II–mediated collagen production. Our findings provide novel evidence that angiotensin II initiates a tyrosine kinase–mediated fibrogenic cascade in human SMCs.

Methods

Reagents

All culture media and supplements, as well as genistein were purchased from Life Technologies. Human angiotensin II, sodium ascorbate, and bacterial collagenase were purchased from Sigma Chemical Co. Typhostin A25 and tyrophostin A1 were purchased from Calbiochem. Anti-smooth muscle α-actin (clone 1A4) was obtained from Dako. Neutralizing monoclonal antibody to TGF-β1 and TGF-β2 was obtained from Genzyme. Human fibronectin was isolated from human plasma by gelatin-Sepharose chromatography. Anti-phosphorylated α-actin (clone 14A) was obtained from Promega. Specific activity of the probe was >10^8 cpm/µg. Labeled riboprobe (120 µg) was added to 2.0 to 3.0 µg of total RNA from each experimental sample. Hybridization was performed at 63°C for 16 hours in the presence of 80% formamide, 40 mmol/L HEPES, pH 6.7, 0.4 mol/L NaCl, 1 mmol/L EDTA, and 0.24 µg/µl transfer RNA. The samples were then incubated with RNase A (50 µg/µl) and RNase T1 (34 µg/µl) for 1 hour at 34°C to degrade single-stranded RNA. The remaining double-stranded RNA was precipitated with 50 µg of herring sperm DNA and cold 20% trichloroacetic acid, filtered through glass-fiber filters, and counted in scintillation cocktail. The amount of probe (I) collagen mRNA per sample was determined from a standard curve, generated from hybridization reactions by using known concentrations of a probe(I) collagen mRNA 300-base fragment. The latter was derived by transcribing the cDNA template, using SP6 RNA polymerase, and quantifying the transcript by UV spectrophotometry. The result was multiplied by 17.7 ([cpm (5800+4800)/2×300]), recognizing that the full-length probe(I) collagen mRNA consists of 2 transcripts of roughly equal abundance, of size 5.8 and 4.8 kb. Expression of human gliceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA (pHeGAP, American Type Culture Collection (ATCC)) was also quantified by RNase protection assay, and abundance of probe(I) collagen mRNA was expressed relative to that of GAPDH.

Collagen Synthesis Assay

Human arterial SMCs at near confluence were rendered quiescent in media supplemented with 1% FBS for 24 hours and subsequently stimulated with the designated concentration of angiotensin II and sodium ascorbate (10 µg/ml) for 48 hours. Control cultures were incubated with ascorbate plus vehicle. SMCs were pulsed for the last 20 hours with 15 µCi/ml of [3H]proline (100 mCi/mmol) in proline-free DMEM. During this period the media was supplemented with 1% dialyzed FBS, fresh sodium ascorbate (10 µg/ml), and β-aminopropionitrile (80 µg/ml).

The relative rate of collagen synthesis was measured according to Kolpakov et al and Kulik and Alvarado. In brief, medium was mixed with buffer containing 0.65 mol/L NaCl, 100 mmol/L Tris-Cl, pH 7.4, 4.7 mmol/L CaCl2, 1.25 mg/ml n-ethyl maleimide, and 50 µg/ml BSA. Samples were split into 2 equal portions and highly specific collagenase (Sigma type VII, 10 U/ml) was added to 1 portion. The samples were incubated for 90 minutes at 37°C and undigested proteins precipitated on ice with 10% trichloroacetic acid. Washed pellets were air-dried, dissolved in 0.1 N NaOH, and radioactivity incorporated into trichloroacetic acid–precipitable counts was measured by liquid scintillation counting. The rate of collagen synthesis, relative to that of all proline-containing proteins, was calculated assuming that the number of proline residues in collagen is 5.4-fold higher than that in noncollagen proteins. The relative rate of collagen synthesis was thus determined as:

\[
\frac{(\text{cpm}_{\text{protein precipitate from sample not collagenase-treated}})}{(\text{cpm}_{\text{protein precipitate from collagenase-treated sample}})} \times 5.4
\]

RNA Isolation and Ribonuclease Protection Assay for probe(I) Collagen mRNA

SMCs were stimulated with various concentrations of angiotensin II, in the presence or absence of angiotensin II receptor antagonists, anti-TGF-β antibody, and tyrosine kinase inhibitors. After incubation of SMCs under the designated conditions, cells were lysed in a solution of 4 mol/L guanidinium isothiocyanate, 25 mmol/L sodium citrate, pH 7.0, 0.1 mol/L 2-mercaptoethanol and 0.5% Sarkosyl. Total RNA was isolated by using the acid-phenol technique.

Absence of probe(I) collagen mRNA was quantified by ribonuclease (RNase) protection assay. A cDNA fragment of human probe(I) collagen (pSP3, gift of Dr C. Farrell, Amgen Inc, Thousand Oaks, California) served as the template from which a 300-base radiolabeled riboprobe was synthesized. The template was linearized with XhoI and 0.5 µg of template was incubated with T7 RNA polymerase and [α-35P]UTP (800 Ci/mmol), as per the manufacturer’s instructions (Promega). Specific activity of the probe was >10^8 cpm/µg. Labeled riboprobe (120 µg) was added to 2.0 to 3.0 µg of total RNA from each experimental sample. Hybridization was allowed to proceed at 63°C for 16 hours in the presence of 80% formamide, 40 mmol/L HEPES, pH 6.7, 0.4 mol/L NaCl, 1 mmol/L EDTA, and 0.24 µg/µl transfer RNA. The samples were then incubated with RNase A (50 µg/µl) and RNase T1 (34 µg/µl) for 1 hour at 34°C to degrade single-stranded RNA. The remaining double-stranded RNA was precipitated with 50 µg of herring sperm DNA and cold 20% trichloroacetic acid, filtered through glass-fiber filters, and counted in scintillation cocktail. The amount of probe(I) collagen mRNA per sample was determined from a standard curve, generated from hybridization reactions by using known concentrations of a probe(I) collagen mRNA 300-base fragment. The latter was derived by transcribing the cDNA template, using SP6 RNA polymerase, and quantifying the transcript by UV spectrophotometry. The result was multiplied by 17.7 ([cpm (5800+4800)/2×300]), recognizing that the full-length probe(I) collagen mRNA consists of 2 transcripts of roughly equal abundance, of size 5.8 and 4.8 kb. Expression of human gliceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA (pHeGAP, American Type Culture Collection (ATCC)) was also quantified by RNase protection assay, and abundance of probe(I) collagen mRNA was expressed relative to that of GAPDH.

Quantification of TGF-β1

Quiescent SMCs (1% FBS) were stimulated with angiotensin II for 48 hours in the presence or absence of protein tyrosine kinase inhibitors or their inactive homologs. Angiotensin II at both 10^-5 and 10^-7 mol/L were used for these studies, as these concentrations were...
Angiotensin II Stimulates Collagen Synthesis in Human SMCs

We examined the effect of angiotensin II on the synthesis and secretion of collagen by human SMCs by metabolically labeling cells with [3H]proline and measuring collagenase-digestible and collagenase-indigestible proteins. As illustrated in Figure 1, the relative rate of collagen synthesis was increased by angiotensin II in a dose-dependent fashion, with a statistically significant increase at 10^{-8} and 10^{-7} mol/L angiotensin II. Time course analysis indicated no detectable increase in collagen production 5 or 10 hours after stimulation with angiotensin II, an intermediate effect at 24 hours, and plateau levels by 48 hours (data not shown).

Angiotensin II Stimulates Expression of procoll(1) Collagen mRNA in Human SMCs

To assess the effect of angiotensin II on type 1 procollagen gene expression, RNA was harvested after 48 hours of stimulation with angiotensin II. To accurately quantify procollagen mRNA abundance, we established an RNase protection assay for procoll(1) collagen mRNA. A standard curve was generated from hybridization reactions by using known amounts of procoll(1) collagen mRNA, and was used to quantify absolute concentrations of procoll(1) collagen mRNA and to confirm that the experimental results fell within the linear range of the assay. As shown in Figure 2A, stimulation with angiotensin II yielded a potent, dose-dependent increase in procoll(1) collagen mRNA with a half-maximal effect at 1.7 nmol/L. GAPDH levels were unaffected by angiotensin II.

Angiotensin II Stimulates Collagen Expression via the AT1 Receptor

The increase in procoll(1) collagen mRNA abundance was found to reflect that for relative collagen synthesis rate. Because of this, and in view of the sensitive quantification afforded by the RNase protection assay, we used procollagen mRNA abundance, relative to that of GAPDH mRNA, as an end point to characterize the relevant transmembrane signaling events. To establish the cell surface receptor mediating the response, SMCs were incubated with losartan, which acts as a competitive inhibitor of the AT1 receptor, or the AT2 receptor antagonist PD123319, each added 30 minutes before addition of 10^{-8} mol/L angiotensin II. As shown in Figure 2B, neither losartan (10^{-7} mol/L) nor PD123319 (10^{-7} mol/L) affected basal expression of procoll(1) collagen mRNA. However, angiotensin II–stimulated procollagen mRNA expression was inhibited by the 10-fold molar excess of losartan but not by the same concentration of PD123319; 100-fold excess of the respective inhibitors showed similar results (data not shown).

Angiotensin II–Stimulated Collagen Expression Is Mediated by Autocrine TGF-β

Angiotensin II may influence cell function by inducing the expression of growth factors, which then act on the cell in an autocrine fashion. Extrapolating from observations in other experimental systems, we considered that angiotensin II might stimulate collagen indirectly, specifically by inducing the expression of TGF-β. To determine if stimulation of SMCs with angiotensin II resulted in elaboration of TGF-β1, we used a rat mink lung epithelial cell bioassay. As shown in Figure 3, 48 hours of treatment with either 10^{-7} mol/L or 10^{-6} mol/L angiotensin II increased total TGF-β (latent plus active) over basal levels by ~6-fold (P<0.001). The proportion of active TGF-β in the culture media was 3.4% and 2.6% of total TGF-β in control and angiotensin-treated cultures, respectively. Incubation of SMCs with angiotensin II increased the amount of active TGF-β in the culture media by ~4-fold (P<0.001). As with the expression of procoll(1) collagen mRNA, production of TGF-β by angiotensin II was inhibited by losartan, but not by PD123319, implicating the AT1 receptor in this response (Figure 3B).

We next determined if angiotensin II–stimulated production of TGF-β mediated the observed increase in collagen expression in SMCs. This was performed by incubating cells with angiotensin II together with a monoclonal anti-TGF-β antibody that neutralizes the biological activity of TGF-β1 and TGF-β2 (1D11.16, Genzyme). Procoll(1) collagen mRNA was quantified by RNase protection assay. Figure 4

Statistics

Data are expressed as mean±SEM values. Comparisons were made by t test or ANOVA with Scheffe’s post hoc test. Statistical significance was set at P<0.05.

Results

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We next determined if angiotensin II–stimulated production of TGF-β mediated the observed increase in collagen expression in SMCs. This was performed by incubating cells with angiotensin II together with a monoclonal anti-TGF-β antibody that neutralizes the biological activity of TGF-β1 and TGF-β2 (1D11.16, Genzyme). Procoll(1) collagen mRNA was quantified by RNase protection assay. Figure 4
shows that the anti-TGF-\(\beta\) antibody (30 \(\mu\)g/mL) had no significant effect on basal collagen expression; however, it significantly inhibited the angiotensin II–stimulated increase in procollagen mRNA. In contrast, control IgG (P3, 30 \(\mu\)g/mL; ATCC) had no effect on either basal or angiotensin II–stimulated procollagen expression.

### Angiotensin II–Stimulated Collagen Expression Is Mediated by Tyrosine Kinase Action

To explore the possibility that tyrosine kinases may be involved in upregulation of type I collagen by angiotensin II, we used 2 mechanistically distinct inhibitors of tyrosine phosphorylation. Genistein inhibits ATP binding to tyrosine kinases and thus its ability to donate phosphate.\(^5^0\) Tyrphostins inhibit tyrosine kinases by competitively binding to the catalytic site of the enzyme.\(^5^1\) By using RNase protection assay, we first established the effect of a 48-hour exposure to various concentrations of genistein and tyrphostin A25, in the absence of angiotensin II, on expression of procollagen mRNA. This allowed us to define an upper dose for each tyrosine kinase inhibitor that would not impair basal human SMC function vis-à-vis collagen expression; this proved to be 10 \(\mu\)mol/L for genistein and 50 \(\mu\)mol/L for tyrphostin A25.

Subsequently, quiescent SMCs were treated with these doses of inhibitor for 40 minutes followed by stimulation with angiotensin II in the presence of inhibitor for a further 48 hours. As illustrated in Figure 5, genistein significantly inhibited angiotensin II–mediated procollagen mRNA expression. In similar manner, tyrphostin A25, but not the inactive homolog tyrphostin A1, blocked angiotensin II–stimulated procollagen expression.

### Tyrosine Kinase Action During Angiotensin II–Mediated Collagen Production Is Localized Upstream of TGF-\(\beta\) Expression

Given that production of collagen by angiotensin II was mediated by an autocrine loop of TGF-\(\beta\), 2 potential transmembrane pathways must be considered with respect to localizing the tyrosine kinase–dependent events. We speculated that the proximal arm, ie, stimulation of TGF-\(\beta\) expression, was tyrosine kinase dependent. To test this, SMCs were incubated for 48 hours with angiotensin II and either genistein, tyrphostin A25, or the inactive homolog tyrphostin A1, and conditioned medium was assayed for TGF-\(\beta\) activity. As shown in Figure 6, 10 \(\mu\)mol/L genistein as well as 50 \(\mu\)mol/L tyrphostin A25 signifi-

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**Figure 2.** A, Dose–response curve of effect of angiotensin II on procollagen mRNA abundance, as determined by RNase protection assay. SMCs were incubated with angiotensin II for 48 hours. B, Effect of 10-fold molar excess of losartan and PD123319 on 10\(^{-8}\) mol/L angiotensin II (Ang II)–induced procollagen mRNA expression. Procollagen mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase mRNA abundance and expressed as a percentage of vehicle-treated controls. *\(P<0.05\) versus control SMCs without angiotensin II. †\(P<0.05\) versus SMCs treated with angiotensin II alone.
cantly inhibited angiotensin II–stimulated TGF-β production, whereas 50 μmol/L tyrphostin A1 had no effect. Direct addition of either tyrosine kinase inhibitor to epithelial cell cultures showed no effect on DNA synthesis rates of the epithelial cells (data not shown).

Figure 3. A, Effect of 10⁻⁸ mol/L angiotensin II on TGF-β production by human SMCs, measured by using mink lung epithelial cell TGF-β bioassay. Top, Total TGF-β in the media, measured after transient acidification to activate latent TGF-β. Bottom, Values obtained before acidification of culture medium and reflecting TGF-β in its activated form. *P<0.01 versus unstimulated SMCs. B, Effect of losartan (10⁻⁷ mol/L) and PD123319 (10⁻⁷ mol/L) on angiotensin II (Ang II) (10⁻⁷ mol/L)–mediated total TGF-β production. *P<0.01 versus control SMCs; †P<0.01 versus SMCs treated with angiotensin II.

Figure 4. Role of TGF-β in angiotensin II (Ang II)–stimulated collagen expression by human SMCs. Neutralizing antibody to TGF-β (50 μg/mL) but not a control IgG (50 μg/mL) abrogated the increase in procα(I) collagen mRNA expression (normalized to changes in glyceraldehyde 3-phosphate dehydrogenase mRNA levels) induced by 10⁻⁸ mol/L angiotensin II. *P<0.05 versus control IgG; †P<0.05 versus angiotensin II and control IgG.

Figure 5. Effect of tyrosine kinase inhibitors on angiotensin II (Ang II)–mediated procα(I) collagen mRNA levels (relative to glyceraldehyde 3-phosphate dehydrogenase mRNA) by human SMCs. Basal and angiotensin II (10⁻⁸ mol/L)–treated SMCs were incubated with genistein (10 μmol/L), tyrphostin A25 (50 μmol/L), or tyrphostin A1 (50 μmol/L). *P<0.05 versus control SMCs; †P<0.01 versus SMCs treated with angiotensin II in the absence of tyrosine kinase inhibitor.

Discussion

Although the mechanism of angiotensin II–mediated SMC contraction has been intensively studied, much less is known about the chronic trophic changes induced by angiotensin II. Vascular SMC collagen synthesis is critical to vascular remodeling2,52,53 and previous studies have suggested that angiotensin II may stimulate SMC collagen production.19–21 The mechanism of such an effect, however, has been unknown. In these studies, we have demonstrated that angiotensin II stimulates collagen synthesis in human arterial SMCs. The response is associated with expression of mRNA encoding the procα(I) chain of type I collagen, the major collagen species found in the normal and diseased artery wall. We further found that the AT₁ receptor was responsible for
and human SMC responses to angiotensin II must be consid-
ered in view of the discrepant findings of pharmacological
disruption of the renin–angiotensin axis on vascular remodel-
ing in rats and humans (for review, see Pratt and Dzau60).

It has been increasingly recognized that angiotensin II may
exert at least some of its growth factor-like effects by
inducing the secretion of growth factors from SMCs, which
can then act in an autocrine fashion. In rat aortic SMCs,
angiotensin II has been reported to increase expression of
TGF-β1,12,39 platelet-derived growth factor A-chain,16 and
fibroblast growth factor-2.39 Because TGF-β is a potent
stimulator of collagen synthesis in SMCs5 and probably
involved in the genesis of several fibrotic disorders, we
considered that the angiotensin II–mediated production of
collagen by human SMCs may be mediated by TGF-β. Our
data established that angiotensin II stimulated the release of
a substantial amount of TGF-β from human SMCs, and that
TGF-β in the activated form increased in the media by
≈400%. The absolute amount of active TGF-β found in the
media (≈0.5 to 0.6 ng/mL) was within the concentration
range previously shown to stimulate collagen synthesis in
human SMCs,3 and within the range that we have observed
leads to increased expression of proα1(I) collagen mRNA
(data not shown). Furthermore, angiotensin II–mediated col-
lagen expression was blocked by a neutralizing antibody to
TGF-β1/TGF-β2, establishing that the production of bioac-
tive TGF-β was a mechanistic pathway for the increase in
collagen expression. In addition to mediating angiotensin
II–induced collagen production in SMCs, autocrine TGF-β
has also been suggested to modulate the proliferative and
migratory response of SMCs to angiotensin II.12,61 Release of
TGF-β because of activation of the AT1 receptor may thus be
an important means by which angiotensin II acts as a
multifunctional growth factor to control vascular structure.

The AT1 receptor lacks intrinsic tyrosine kinase activity.
Nevertheless, there is now substantial evidence that, when
activated, this receptor induces tyrosine phosphorylation of
cytoplasmic second-messenger proteins. Angiotensin II has
been shown to directly activate the protein tyrosine kinase
pp60^src and several substrates of pp60^src are phosphorylat-
ed on tyrosine after angiotensin II stimulation, including
phospholipase C-γ1, pp120, pp125^FAK, and paxillin.34,35,63
Tyrosine phosphorylation and activation of the JAK–STAT
signaling cascade have also been shown to be a direct
response to AT1 receptor stimulation by angiotensin II.33,64
Recently, angiotensin II has also been demonstrated to
activate the Ras pathway, via the actions of the tyrosine
kinase pp60^src.65

Despite major progress in understanding the tyrosine phos-
phorylation cascades induced by angiotensin II, relatively
little is known about the functional consequences of these
actions. We used 2 different inhibitors of tyrosine kinase to
study the role of protein tyrosine phosphorylation on angio-
tensin II–stimulated collagen synthesis. Genistein inhibits
the binding of ATP to tyrosine kinases and its use has previously
suggested that tyrosine kinase pathways modulate angioten-
sin II–induced calcium transients in SMCs.66 Tyrophostins
bind the substrate binding site of the protein tyrosine kinase
domain, and may be more specific for tyrosine kinases than
genistein. These agents have been used recently to demon-
strate that angiotensin II–mediated SMC growth requires
tyrosine phosphorylation.37,38 Our data revealed that both
genistein and tyrphostin A25 inhibited angiotensin II–mediated expression of proc1(I) collagen by human SMCs at concentrations that did not impair basal collagen expression. The observation that under conditions of stable basal collagen production, these biochemically distinct inhibitors produced a similar effect strongly supports a role for tyrosine phosphorylation in angiotensin II–mediated collagen production by arterial SMCs.

In view of our observation that angiotensin II stimulates collagen expression indirectly, ie, via elaboration of TGF-β, there are 2 broad pathways that potentially could involve tyrosine phosphorylation, angiotensin II–induced production of TGF-β or TGF-β–induced production of collagen (or both). We established that the initial limb is a tyrosine kinase–dependent process, because both genistein and tyrphostin A1, but not the inactive homolog tyrphostin A1, blocked the production of TGF-β by angiotensin II. We speculate that this proximal arm of the fibrogenic cascade is unique in its requirement for tyrosine kinase action. Based on current understanding of TGF-β signaling, there is little support for the involvement of protein tyrosine phosphorylation in TGF-β–induced collagen production. The transmembrane receptor events in this include interaction of primary and transducing receptor subtypes, both of which are serine–threonine kinases. Subsequent propagation of signal is mediated by members of the Smad protein family, which become phosphorylated on serine residues.67 In contrast to the signaling cascade initiated by TGF-β stimulation, there is supportive evidence that regulation of TGF-β expression may require tyrosine kinase activity. Recently, expression of TGF-β after arterial injury was shown to be inhibited by genistein.68 As well, TGF-β1 gene transcription in neuronal cells was found to be activated by ligands of protein tyrosine kinase receptors and this response was mediated by p21ras.69 The involvement of Ras in TGF-β gene expression is particularly noteworthy in view of recent observations that angiotensin II can activate the Ras–Raf–MAP kinase pathway in SMCs and does so in a Src tyrosine kinase–dependent fashion.65 Thus, the site of action of the tyrosine kinase inhibitors in the present study might be localized between the AT1 receptor and Ras. A potential pathway depicting the process of angiotensin II–mediated collagen production in SMCs is shown in Figure 7.

In summary, angiotensin II stimulates collagen synthesis in human arterial SMCs. The response is seen at angiotensin II concentrations that are likely relevant to human vascular disease and is consistent with its role as a multifunctional growth factor for SMCs. The effect is mediated by the AT1 receptor, suggesting that pharmacological therapy with AT1 receptor antagonists may modulate collagen accumulation during vascular disease. Furthermore, our data suggest a novel mechanism for this angiotensin II–initiated fibrogenic cascade, whereby tyrosine kinase activity mediates the stimulated production of TGF-β.

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References

Figure 7. Potential pathway for angiotensin II–induced collagen production by human vascular SMCs. Data from the current study establish that the AT1 receptor, tyrosine phosphorylation, and TGF-β are sequential components in the fibrogenic pathway. AT1 receptor–mediated activation of the tyrosine kinase pp60–src and downstream activation of the mitogen-activated protein kinase (MAPK) pathway are also proposed to be involved, based on recent studies.66 G indicates G protein; MEK, MAPK/extracellular signal-related kinase kinase.


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