Endothelin-1 expression in vascular adventitial fibroblasts

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An, Sheng Jun, Ryan Boyd, Ying Wang, Xiaofan Qiu, and Hui Di Wang. Endothelin-1 expression in vascular adventitial fibroblasts. Am J Physiol Heart Circ Physiol 290: H700–H708, 2006. First published August 19, 2005; doi:10.1152/ajpheart.00326.2005.—Endothelial cells are a major source of endothelin (ET)-1, but the possibility that vascular adventitial fibroblasts generate ET-1 has not been explored. We hypothesized that aortic adventitial fibroblasts have the ability to produce ET-1, which may contribute to extracellular matrix synthesis. Vascular adventitial fibroblasts were isolated from mouse aorta and incubated with various concentrations of angiotensin II (ANG II), mRNA levels of preproET-1 and type I procollagen were detected with relative RT-PCR. ET-1 levels in culture medium were measured with ELISA. Protein levels of procollagen were detected with Western blotting. ANG II (10 and 100 nM, 1 μM) induced a time- and concentration-dependent increase in preproET-1 mRNA levels (P < 0.05). Induction of preproET-1 mRNA was accompanied by release of immunoreactive peptide ET-1 (P < 0.05). ANG II-evoked increases in preproET-1 mRNA expression and ET-1 release were blocked by losartan (100 μM), an AT1 receptor antagonist, but not PD-123319 (100 μM), an AT2 receptor antagonist. To further confirm our findings, we cloned and then sequenced vascular fibroblast preproET-1 bidirectionally with T7 and T3 reverse sequencing primers. Their nucleotide sequences were identical to preproET-1 cDNA from mouse vascular endothelial cells (accession no. AB081657). Moreover, ANG II-induced type I procollagen mRNA and protein expression were inhibited by BQ-123 (10 μM), an ETα receptor inhibitor, but not BQ-788 (10 μM), an ETβ receptor inhibitor, suggesting a significant role of adventitial ET-1 in regulation of extracellular matrix synthesis. The results demonstrate that vascular adventitial fibroblasts are able to synthesize and release ET-1 in response to ANG II.

IN CONTRAST TO THE ACCEPTED ROLE of the vascular endothelium in the regulation of vascular smooth muscle function, the role of the adventitia in the regulation of vascular smooth muscle function is largely unexplored. Recently, the contribution of adventitial fibroblasts to neointima formation and vascular remodeling has received considerable attention (24, 34, 36, 39, 49). Moreover, we and others have demonstrated that the adventitia is the primary site of superoxide anion generation in the mouse (45, 46), rat (28, 47, 48), and rabbit aorta (28). Indeed, investigators in our group discovered that generation of superoxide anion from NADPH oxidase contributes to angiotensin II (ANG II) evoked hypertension and that adventitial NADPH oxidase appears to contribute to this effect of ANG II (45, 46).

The role of ANG II in hypertension is well established. Certain types of hypertension are associated with elevated circulating levels of ANG II. On the other hand, local production of ANG II in the vessel wall may have autocrine and paracrine effects, even though the circulating peptide level is normal or low (23). By activating ANG II type 1 (AT1) receptors, ANG II may exert its effects on the vasculature either directly, through activation of phospholipase C, or indirectly, through the endothelin system. Indeed, several studies indicate that adventhel (ET)-1 may contribute to the vascular actions of ANG II (3, 7, 8, 12, 21, 26). In addition, overexpression of ET-1 has been found in some models of hypertension, including the ANG II-infused hypertensive rat (11, 30).

ET-1 is a 21-amino acid peptide containing two disulfide bridges. It is the most potent endogenous vasoconstrictor (2, 35), as well as a potent mitogenic agent (17, 22). The most abundant source of ET-1 in vivo under physiological conditions is vascular endothelium (19, 51). The effects of ET-1 are mediated by endothelin type A (ETα) and endothelin type B (ETβ) receptors (33). ET-1 synthesis also has been reported in nonendothelial cells, including vascular smooth muscle cells (16, 18, 41), cardiomyocytes (1, 32), and cardiac fibroblasts (9, 10, 15). However, the possibility that the adventitia of the vasculature might generate ET-1 has not been studied.

Both ANG II (14, 43) and ET-1 (42) have been reported to stimulate vascular collagen synthesis. Accumulation of interstitial collagen is frequently associated with vascular disease (20, 29). Although vascular collagen production is regulated by many factors, studies of renal vascular fibrosis have suggested that ET-1 also could contribute to ANG II-evoked collagen expression in the renal vasculature (6). However, the possibility that the ANG II-evoked collagen expression may occur at the level of the vascular adventitia has not been reported.

In view of these considerations, we sought to determine whether the adventitia of the vasculature is capable of generating ET-1 in response to ANG II and to determine whether such an event is biologically relevant. In the present study, we demonstrated that ANG II is able to induce ET-1 release from vascular adventitial fibroblasts. Using reverse transcriptase-polymerase change reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA), we compared ET-1 synthesis and release from cultured aortic adventitial fibroblasts in the presence and absence of ANG II. We further demonstrated that this induction of ET-1 is mediated by AT1 receptors. Finally, we determined whether ET-1 release from adventitial fibroblasts exerts a biologically meaningful effect by examining its role in ANG II-induced collagen expression.

MATERIALS AND METHODS

Cell culture. Male C57BL/6J mice, 16–18 wk of age, were obtained from Jackson Laboratory (Bar Harbor, ME). The mice were harvested and the aorta was obtained, flushed, and digested to obtain a cell suspension. The cells were plated in T25 flasks and cultured under standard conditions (5% CO2, 95% air, 37°C). The cells were passaged and used between passages 3 and 5.

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Table 1. Primers for vWF, myosin heavy chain, desmin, CD8, and β-actin

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<thead>
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<th>Primers</th>
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<td>Sense primer</td>
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<td></td>
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<td>TCT CAG CTC TCG TGG TGA AG</td>
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vWF, von Willebrand factor.

Table 2. Primers for preproET-1, ETA and ETB receptors, and type I procollagen α1

<table>
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<tr>
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<td>PreproET-1</td>
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<td></td>
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<td>GAA GCC ATG TTT TGG TAC C</td>
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<tr>
<td>ETβ receptor</td>
<td>Sense primer</td>
<td>TCT GCT ATT ATG TGC</td>
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<tr>
<td></td>
<td>Antisense primer</td>
<td>TCT CAG CTC TCG TGG TGA AG</td>
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ETα and ETβ, endothelin type A and B receptors, respectively.
The PCR-amplified open reading frame-containing mouse preproET-1 cDNA was purified and ligated into pCR2.1 cloning vector by using a TA cloning kit (Invitrogen). The construct was transformed and propagated in INtO’ competent cells (Invitrogen) with white/blue selection using ampicillin-selective Luria-Bertani medium. Candidate colonies were selected for confirmation with EcoRI restriction endonuclease (3.89 and 0.64 kb). To further determine the orientation of the inserted preproET-1 cDNA clones, we used ApaI restriction endonuclease to pick up preproET-1/pCR2.1F clones that were reverse-complementary to the strand containing T7 promoter (4.25 and 0.64 kb). To further determine the orientation of the inserted preproET-1 cDNA clones, we used EcoRI restriction endonuclease homologous with M13 reverse sequencing primers with an automatic DNA sequencer (ABI 373A; Applied Biosystems) for further confirmation. The primer sequences for mouse preproET-1, ETA/ETB receptor, and type I procollagen were similar to the procedures described above. The primer sequences for mouse preproET-1, ETA/ETB receptor, and type I procollagen α1 are listed in Table 2. For quantification of expression of preproET-1 transcript before and after ANG II treatment, a QuantumRNA β-actin internal control was used for relative RT-PCR (Ambion). Briefly, the RT-PCR protocol was similar to the qualitative RT-PCR except for a 2:8 ratio of β-actin primers. The competimers mix was added to PCR reactions. The amplification was carried out for 30 cycles (94°C, 30 s; 55°C, 1 min; and 68°C, 1 min). The PCR product was analyzed by electrophoresis with a 1.2% agarose gel and visualized by ethidium bromide staining.

Measurement of ET-1 release by ELISA. The method has been described previously (50). The concentration of ET-1 in the culture medium was determined using a commercial ELISA kit (ALPCO, Windham, NH). The anti-ET-1 antibody that was used in the kit showed 100% specificity for ET-1 and ET-2, with <1% cross-reactivity to ET-3 and big ET-1. The cellular protein concentration was determined with Bradford reagent (Bio-Rad). The ET-1 release was expressed as femtomoles of ET-1 per milligram of protein.

**RESULTS**

Characterization of vascular adventitial fibroblasts. Under the microscope, adventitial fibroblasts derived from the explants displayed a smooth cell border and spindlelike bipolar and tripolar morphology. Each pole presented a small process. Plants displayed a smooth cell border and spindlelike bipolar morphology. Each pole presented a small process. After confluence, these cells displayed a multilayer phenotype. Immunocytochemical analysis of cultured cells derived from the adventitia showed no staining for human vWF (Fig. 1A), Dil-Ac-LDL (Fig. 1B), and desmin (Fig. 1C). In contrast, all cells had strong positive staining for vimentin (Fig. 1D). These results indicate that our cell culture contained primarily fibroblasts with little or no contamination from endothelial and smooth muscle cells.

![Characterization of cells cultured from mouse aortic adventitia by immunofluorescence staining](image)

Fig. 1. Characterization of cells cultured from mouse aortic adventitia by immunofluorescence staining. Immunofluorescence stains of mouse aortic adventitial cells are shown for von Willebrand factor (vWF), a marker for endothelial cells (A), Dil-Ac-LDL (LDL), a marker for endothelial cells (B), antibody of desmin, a marker for differentiated vascular smooth muscle cells (C), and antibody of vimentin, a nonspecific marker for many cell types (D). There is no dense staining for vWF, LDL, and desmin but strong staining for vimentin in mouse aortic adventitial cells. Each photograph is a representative example of 3 experiments from 3 mice.
Characterization of mRNA of various cell markers in cultured vascular adventitial cells by RT-PCR is shown in Fig. 2. Cultured cells derived from the adventitia of the aorta showed no expression for vWF, CD8, myosin heavy chain (vascular smooth muscle cell marker), and desmin (vascular smooth muscle cell marker). In contrast, the aorta had strong positive staining for vWF, CD8, myosin heavy chain, and desmin. Both cultured fibroblasts and aorta tissue expressed β-actin.

Cloning and sequencing of mouse ET-1 cDNA. Sequencing of cloned mouse fibroblast preproET-1 cDNA showed that the sequence was 100% identical to a mouse cDNA that was previously found in the GenBank database (accession no. NM_010104). Because this preproET-1 cDNA clone represented a new clone from a new tissue type of mouse that was not reported previously, the sequence of the clone was deposited into the DDBJ/EMBL/GenBank database (accession no. AB081657).

Effect of ANG II on preproET-1 mRNA expression. The effect of incubation time with ANG II on the expression of preproET-1 mRNA in cultured aortic adventitial fibroblasts is presented in Fig. 3. The expression of preproET-1 mRNA increased within the first 30 min of incubation with ANG II (100 nM) and continued to increase further, reaching a maximum at the 1.5 h mark (P < 0.05). Thereafter, preproET-1 mRNA expression decreased progressively. After 12 h, expression of preproET-1 mRNA was similar to that observed in the control group (P > 0.05).

The effects of various concentrations of ANG II on expression of preproET-1 mRNA in cultured aortic adventitial fibroblasts are presented in Fig. 4. The cells were incubated in the presence and absence of ANG II (10 nM, 100 nM, and 1 μM) for 90 min. In the absence of ANG II, preproET-1 mRNA expression was detectable but low. In contrast, addition of ANG II evoked a concentration-dependent increase in preproET-1 mRNA expression.

Figure 5 presents the effects of ANG II receptor antagonists on the increase in preproET-1 mRNA expression evoked by ANG II. Pretreatment with the AT1 receptor antagonist losartan (100 μM), but not the AT2 receptor antagonist PD-123319 (100 μM), blocked the elevations in preproET-1 mRNA evoked by ANG II. Neither antagonist affected preproET-1 mRNA expression in the absence of ANG II. These data suggest that the induction was mediated by AT1 receptors.

Effects of ANG II on ET-1 release. The effects of various concentrations of ANG II on ET-1 peptide levels in culture medium are presented in Fig. 6. In the absence of ANG II, ET-1 levels in the medium were modest. The addition of ANG II evoked concentration-dependent increases in ET-1. The maximum increase evoked by ANG II was three- to fourfold higher than basal levels. Figure 7 presents the effects of ANG II receptor antagonists on ET-1 release. The ANG II-evoked increases in ET-1 release were blocked by the AT1 receptor antagonist losartan (100 μM) but not by the AT2 receptor antagonist PD-123319 (100 μM). Neither antagonist affected ET-1 levels in the absence of ANG II.

Fig. 2. Expression of various cell markers and β-actin mRNA in cultured vascular adventitial fibroblasts and aorta tissues as determined by RT-PCR. Lane 1, DNA marker; lanes 2 and 3, vWF (endothelial cell marker) and β-actin mRNA expression in the cultured vascular fibroblasts and aorta tissue, correspondingly; lanes 4 and 5, CD8 (leukocyte marker) and β-actin mRNA expression in the cultured vascular fibroblasts and aorta tissue, correspondingly; lanes 6 and 7, myosin heavy chain (MHC, smooth muscle cell marker) and β-actin mRNA expression in the cultured vascular fibroblasts and aorta tissue, correspondingly; and lanes 8 and 9, desmin (smooth muscle cell marker) and β-actin mRNA expression in the cultured vascular fibroblasts and aorta tissue, correspondingly. The photograph is a representative example of 3 experiments from 3 mice.

Fig. 3. Effect of different time courses on expression of preproET-1 induced by ANG II in cultured aortic adventitial fibroblasts. The cells were incubated with ANG II (100 nM) for various times. A: a representative blot of preproET-1 mRNA levels assessed by relative RT-PCR. B: preproET-1 gene expression compared with that of control cells (in the absence of ANG II). Maximal stimulation was detected after 60–90 min of incubation. Results are means ± SE of 3 experiments. *P < 0.05 compared with control cells.
receptor antagonist BQ-788. Moreover, the increase in type I procollagen mRNA levels evoked by ANG II was inhibited by AT1 receptor antagonist losartan (100 \( \mu \)M) but not by the AT2 receptor antagonist PD-123319 (100 \( \mu \)M).

The protein levels of type I procollagen \( \alpha \)1 in aortic adventitial fibroblasts are presented in Fig. 9. The responses of the type I collagen protein parallel the results for procollagen mRNA.

Expression \( \text{ETA and ETB receptors in adventitial fibroblasts.} \)

To test whether ET-1 receptors are present in adventitial fibroblasts, we tested the mRNA of \( \text{ETA} \) and \( \text{ETB} \) receptors using RT-PCR. The results show that both \( \text{ET}_{\text{A}} \) and \( \text{ET}_{\text{B}} \) receptors are expressed in aortic adventitial fibroblasts (Fig. 10).

**DISCUSSION**

In this study, we have demonstrated for the first time that adventitial fibroblasts synthesize and release ET-1 after stimulation by ANG II (10 nM to 1 \( \mu \)M) and that the released ET-1 is biologically active. Five lines of evidence support this conclusion. First, we discovered that ANG II evokes a concentration- and time-dependent increase in preproET-1 mRNA. Thus adventitial cells express the message for ET-1 synthesis, and the expression is responsive to an external stimulus. Second, we also showed for the first time that adventitial fibroblasts release ET-1, the peptide product. Thus we have provided evidence at both the gene expression level and the end-product level for the synthesis and release of ET-1 in response to stimulation by a hormone involved in blood pressure regulation, namely, ANG II. Increases in preproET-1 mRNA were evident within 30 min after ANG II treatment and reached a peak within 1.5 h. Because of the sensitivity of the assay for ET-1, it was not possible to measure the time course of ET-1 release in the medium; rather, it proved necessary to measure the amount of ET-1 accumulated over the 24-h incubation period. This approach seems reasonable, because one
would expect the synthesis of the peptide to lag the expression of the message. Third, we cloned and sequenced the proproET-1 cDNA from mouse adventitial fibroblasts. We found that the nucleotide sequence of adventitial proproET-1 cDNA is identical to the cDNA of endothelial cells. Fourth, we demonstrated a potential role of vascular adventitial fibroblast-released ET-1 in modulating ANG II-induced extracellular matrix production. We found that the ANG II-stimulated type I procollagen αI mRNA expression and protein synthesis were inhibited by the ETA receptor inhibitor BQ-123 but not by the ETB antagonist BQ-788. Fifth, we demonstrated the existence of ETA and ETB receptors in vascular adventitial fibroblasts. These findings are consistent with the notion that ET-1 may contribute to collagen synthesis by stimulating ETA receptors. Collagen is a major component of the extracellular framework of blood vessels, comprising up to 60% of the total protein content (4, 38). Collagen is produced primarily by fibroblasts, although smooth muscle and endothelial cells may participate. Of the many different types of collagen, type I is the predominant type of collagen found in artery walls. Altogether, the work reported in this article provides strong evidence demonstrating the synthesis and release of ET-1 in response to ANG II, a hormone implicated in some models of hypertension. Moreover, the effects of ANG II-evoked ET-1 release on collagen synthesis suggest a potentially biologically meaningful role for this interaction at the functional level.

The ANG II-evoked increases in ET-1 synthesis and release appear to be mediated by the AT1 subtype. The AT1 antagonist losartan, but not the AT2 antagonist PD-123319, blocked the increase in ET-1 evoked by ANG II. The signal transduction pathways mediating release of ET-1 are complex. The topic has been reviewed by Russell and Davenport (31). In endothelial cells, there appear to be two distinct secretory pathways, a constitutive pathway involving continuous release and a regulated pathway involving stimulated release. The constitutive pathway is modulated at the level of mRNA transcription, whereas the regulated pathway appears to involve release from storage granules known as Weibel-Palade bodies. Both reactive oxygen species and Ras-Raf-ERK pathway are required for ET-1-induced ET-1 gene expression in rat cardiac fibroblasts (9, 10). Cheng et al. (9) reported that antioxidant suppressed ET-1-induced ET-1 gene expression. Inhibition of ERK could inhibit transcription of the ET-1 gene. Dominant negative mutant of Ras, Raf, and MEK1 also decreased ET-1 transcription. It is not known whether secretory pathways and Ras-Raf-ERK pathway in adventitial cells parallel those in endothelial cells or rat cardiac fibroblasts. Such studies would be complex and are beyond the scope of the current study. Notwithstanding, the data in this article demonstrate that an AT1 antagonist blocks both ET-1 release and the increases in preproET-1 mRNA and type I procollagen expression. The blockade of ANG II-evoked procollagen synthesis by both the AT1 receptor antagonist and the ETA antagonist is consistent with the notion that ANG II evokes release of ET-1 and that this release is biologically meaningful.

Our data suggest that ET-1 contributes to the biological effects of ANG II, and this is consistent with previous findings. ANG II-induced ET-1 expression has been reported in both rat cardiac fibroblasts (10) and aortic smooth muscle cells (18).
In this article, we report a novel function of oxidative stress associated with the ANG II-infused hypertensive model. In this model, ANG II-evoked ET-1 release from the adventitia could conceivably contribute to other functions. ANG II may be released from perivascular fat tissues. Perivascular fat tissues have been shown to be a rich source of angiotensinogen (13). ANG II has been shown to generate reactive oxygen species, especially superoxide anion, from the adventitia. Alternatively, ANG II may promote the production of ET-1, which in turn would contribute to either the contraction of the medial smooth muscle cells or the release of reactive oxygen species (25).

Adventitial ET-1 could also play a significant role in attracting white cell infiltration. It has been reported that hypertension is in part an inflammatory disorder. C-reactive protein level, a marker of inflammation, is increased in humans with hypertension (37). C-reactive protein level is also a good predictor of subsequent development of hypertension (5). ET-1 has been shown to increase inflammatory cell infiltration, which caused renal tissue damage in aldosterone-dependent hypertension (44). In addition, ET-1 has been reported to account for polymorphonuclear leukocyte infiltration in ischemia-reperfusion-induced mucosal dysfunction (27). Thus, although our data suggest a role for ANG II-evoked ET-1 release in the regulation of the extracellular matrix, other roles for adventitial ET-1 are potentially important.

In conclusion, the results reported in this article demonstrate for the first time that the adventitial fibroblasts, like their neighboring endothelial and smooth muscle cells, synthesize and release ET-1 in response to the stimulation of ANG II. We also have shown that ANG II-evoked ET-1 release contributes to type I procollagen expression through activation of adventitial fibroblasts, namely, the synthesis and release of ET-1.

In addition to the contribution of adventitial ET-1 in regulation of collagen I release, ANG II-evoked ET-1 release from the adventitia could conceivably contribute to other functions. ANG II may be released from perivascular fat tissues. Perivascular fat tissues have been shown to be a rich source of angiotensinogen (13). ANG II has been shown to generate reactive oxygen species, especially superoxide anion, from the adventitia. Alternatively, ANG II may promote the production of ET-1, which in turn would contribute to either the contraction of the medial smooth muscle cells or the release of reactive oxygen species (25).

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ET\textsubscript{A} receptors, suggesting a functional role for ANG II-evoked ET-1 release in the regulation of the extracellular matrix. Finally, the results imply that under certain circumstances when the ANG II system is activated (such as in ANG II-dependent hypertension), ANG II may enhance ET-1 synthesis and release from the vascular adventitia, which conceivably may play an important role in the regulation of vascular function in either a paracrine or autocrine fashion.

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GRANTS

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