Angiotensin II via Activation of Type 1 Receptor
Upregulates Expression of Endoglin in Human Coronary Artery Endothelial Cells

Dayuan Li, Hongjiang Chen, Jawahar L. Mehta

Abstract—Transforming growth factor-β1 and its subtype receptor endoglin are key components in angiogenesis. We explored the role of angiotensin (Ang) II in the expression of endoglin and the underlying intracellular signaling mechanism in human coronary artery endothelial cells. Incubation of cells with Ang II upregulated endoglin expression in a concentration- and time-dependent manner (maximal effect with 10⁻⁶ mol/L Ang II at 24 hours). The Ang II type 1 receptor blocker losartan, but not the type 2 receptor blocker PD 123,319, completely blocked the effect of Ang II. In parallel experiments, the mitogen-activated protein kinase inhibitor PD 098,059 fully inhibited the effect of Ang II on the expression of endoglin. Incubation of endothelial cells with Ang II also increased the expression of transforming growth factor-β1 and -β2 receptors and simultaneously decreased the levels of transforming growth factor-β1. These effects of Ang II were also attenuated by losartan. We propose that Ang II via its type 1 receptor activation modulates the expression of transforming growth factor-β1 receptors in human coronary endothelial cells. The activation of mitogen-activated protein kinase plays an important role in this process. These observations provide a new clue regarding the regulatory effect of Ang II on vascular remodeling after injury. (Hypertension. 2001;38:1062-1067.)

Key Words: angiotensin II ■ endothelium ■ transforming growth factors ■ protein kinases

Increasing evidence shows that the transforming growth factor (TGF) superfamily, especially TGF-β1, plays a critical role in angiogenesis, wound healing, and inflammation. The TGF-β superfamily of structurally related peptides includes the TGF-β isoforms (β1, β2, β3, and β3), the activins, and the bone morphogenetic proteins.1,2 TGF-βs exert their effects by binding to specific receptors, including receptors type 1, type 2, β-glycan, and endoglin.3,4 The molecular mechanism of the receptor 1/receptor 2 interplay in response to TGF-β has been well elucidated.5,6 β-Glycan has been shown to potentiate the binding of the ligand to cells.7 Endoglin has been shown to modulate the signals transmitted by the receptor complex.8

Endoglin (CD105) is a homodimeric integral membrane glycoprotein composed of disulfide-linked subunits of 90 to 95 kDa. In humans, it is expressed at high levels on vascular endothelial cells.9 Endoglin plays a key role in angiogenesis, inasmuch as inhibition of its expression markedly attenuates angiogenesis in vitro.10 Another study11 showed that mice lacking endoglin die from defective vascular development, whereas mice lacking TGF-β have normal vascular growth. Angiotensin (Ang) II has been known to be a key factor in hypertension, atherosclerosis, and coronary heart disease. Ang II induces apoptosis of endothelial cells12 and enhances the proliferation of smooth muscle cells.13 Experimental and clinical studies14,15 show that Ang II plays a critical role in the remodeling of blood vessels and myocardium after acute myocardial infarction. The biological function of Ang II is mainly mediated by its type 1 (AT₁) and type 2 (AT₂) receptors. The activation of Ang II receptors causes activation of intracellular signals, such as protein kinase C, mitogen-activated protein kinase (MAPK), and nuclear factor (NF)-κB, a transcription factor.

Previous studies16–19 have suggested an important interaction between Ang II and TGF-β1. Ang II increases TGF-β1 synthesis in fibroblasts, smooth muscle cells, and renal mesangial cells. The present study was designed to observe the effect of Ang II on the expression of endoglin (mRNA and protein) and the signal transduction pathway in this process in human coronary artery endothelial cells (HCAECs). We also examined the Ang II–mediated regulation of other TGF-β1 receptors and immunoreactive TGF-β1 in HCAECs.

Methods

Cell Culture and Study Design

The culture of HCAECs was described earlier by us.20 HCAECs were incubated with Ang II (10⁻⁶ to 10⁻¹ mol/L) for 6, 12, and 24 hours to determine the expression of endoglin. In other experiments, HCAECs were pretreated with the specific AT₁ receptor inhibitor
Angiotensin II and Endoglin

Losartan (10⁻⁶ mol/L) or the specific AT₂ receptor inhibitor PD 123,319 (10⁻⁶ mol/L) for 30 minutes, and then the cells were exposed to Ang II to determine the receptor specificity of the effect of Ang II. HCAECs were also pretreated with the MAPK inhibitor PD 098,059 (10⁻⁵ mol/L) and then exposed to Ang II to explore the signaling pathway of the effect of Ang II on endoglin expression. The concentration of all reagents and the duration of incubation were decided on the basis of previous studies. To observe the regulation of TGF-β1 type 1 and 2 receptors, HCAECs were incubated with Ang II (10⁻⁸ to 10⁻⁴ mol/L) for 24 hours. HCAECs were pretreated with losartan or PD 123,319 (10⁻⁶ mol/L) for 30 minutes, and then the cells were exposed to Ang II to determine the receptor specificity of Ang II action in the expression of TGF-β1 type 1 and 2 receptors. Immunoreactive TGF-β1 was also measured in these experiments.

Semiquantitative Reverse Transcription–PCR for TGF-β1 Receptor mRNA

Total RNA (1 μg) extracted from cultured HCAECs was reverse-transcribed with oligo(dT) (Promega) and M-MLV reverse transcriptase (Promega) at 37°C for 1 hour. Amplification of TGF-β1 type 1 and type 2 receptors and endoglin was achieved by using specific primers (see the Table). The products of polymerase chain reaction (PCR) were visualized on 1.5% agarose gels with the use of ethidium bromide. mRNA bands were normalized with β-actin mRNA bands. The relative intensity of the band of interest was analyzed by UN-SCAN-IT gel software (Silk Scientific) and expressed as the ratio of the band of interest to the β-actin mRNA band.

Western Analysis for TGF-β1 Receptor Protein

HCAEC lysates were separated and transferred to nitrocellulose membranes. After they were blocked, the membranes were incubated with 1:2000 dilution second antibody, and the membranes were probed with immunoreactive TGF-β1 type 1 and 2 receptors (Santa Cruz Biotechnology) were used in 1:1000 dilution.

Measurement of TGF-β1 Activity

TGF-β1 activity in culture medium was measured with an ELISA kit (Promega). The technique for ELISA was the same as that recommended by the manufacturer, with absorbance determined at 450 nm. TGF-β1 activity was expressed as picograms per milligram protein.

Determination of MAPK Phosphorylation

HCAEC lysates were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. After they were blocked, the membranes were incubated with 1:1000 dilution phospho-specific MAPK antibodies (Calbiochem) that detect p42MAPK and p44MAPK. Thereafter, the membrane was stripped and reprobed with MAPK antibody.

Data Analysis

All data (mean±SD) represent the mean of 6 independent experiments. Statistical significance was determined in multiple comparisons among groups of data in which ANOVA and the F test indicated the presence of significant differences. A value of P≤0.05 was considered significant.

Results

Ang II and Expression of Endoglin

Incubation of HCAECs with Ang II induced the expression of endoglin mRNA in a concentration (10⁻⁸ to 10⁻⁴ mol/L)–dependent manner. Ang II also induced the expression of endoglin protein in a concentration-dependent manner. The maximal effect of Ang II was observed with 10⁻⁴ mol/L concentration. Compared with the lower Ang II concentration (10⁻⁶ mol/L), the highest concentration of Ang II (10⁻⁵ mol/L) caused less effect on the expression of endoglin, which may be related to the toxicity of a high concentration of Ang II (Figure 1).

Incubation of HCAECs with Ang II induced the expression of endoglin in a time-dependent manner. The maximal effect of Ang II was observed at 24 hours (Figure 2).

Ang II Receptor Subtypes and Endoglin Expression

To determine the role of Ang II receptors in endoglin expression, HCAECs were pretreated with the specific AT₁ receptor blocker losartan (10⁻⁶ mol/L) or the AT₂ receptor blocker PD 123,319 (10⁻⁶ mol/L) for 30 minutes, and then the cells were exposed to Ang II (10⁻⁶ mol/L) for 24 hours. We found that the AT₁ receptor blocker losartan completely blocked the effect of Ang II on endoglin mRNA and protein expression, whereas the AT₂ receptor blocker PD 123,319 had no effect on Ang II–induced endoglin expression (Figure 3).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers 5’→3’ (Sense/Antisense)</th>
<th>Annealing Temperature, °C</th>
<th>Cycles, n</th>
<th>Product Length, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>CGTGCCTGACATCTATGCAAT/</td>
<td>61</td>
<td>25</td>
<td>250</td>
</tr>
<tr>
<td>R1</td>
<td>AGCTGGTCCATTTGAGCATC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-β1</td>
<td>ATCCGCCACCTTTTACAGAC/</td>
<td>61</td>
<td>25</td>
<td>250</td>
</tr>
<tr>
<td>R2</td>
<td>CATCCAAAGGAGGGTCTTTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endoglin</td>
<td>GCTGGAAGGCGGAGAGGTC</td>
<td>58</td>
<td>30</td>
<td>805</td>
</tr>
<tr>
<td></td>
<td>CCTGCCCTAGAGAGCTGAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GTCAAAATGGACTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>CGAATTCTGGAGAAGGCTATGA</td>
<td>55</td>
<td>25</td>
<td>201</td>
</tr>
<tr>
<td></td>
<td>GCTGCCCG/TGGATCCGAGCCTGCCCCAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CAGACGAGCAGCTGTGTTG</td>
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</tbody>
</table>

R1 indicates receptor type 1; R2, receptor type 2.

Primer Sequences, Amplification Conditions, and Product Length of Analyzed Genes
Intracellular Signal Transduction
To determine intracellular signal in the effect of Ang II on endoglin expression, we examined the activation of intracellular MAPK. Incubation of HCAECs with Ang II had no effect on MAPK protein, but it caused an increase in activated MAPK (determined by phosphorylation of MAPK) compared with control (P<0.01). Most important, losartan inhibited Ang II–induced activation of MAPK, whereas PD 123,319 had no effect. Pretreatment of HCAECs with a specific MAPK inhibitor, PD 098,059, fully blocked Ang II–induced activation of MAPK (Figure 4).

Finally, we found that pretreatment of cells with PD 098,059, the specific MAPK inhibitor, inhibited the expression of Ang II–induced endoglin mRNA and protein (Figure 3).

Ang II and Expression of TGF-β1 Type 1 and 2 Receptors
Incubation of HCAECs with Ang II also induced the expression of TGF-β1 receptor type 1 and 2 mRNA and protein in a concentration (10⁻⁸ to 10⁻⁶ mol/L)-dependent manner. The maximal effect of Ang II was observed with 10⁻⁶ mol/L concentration. The effect of Ang II was mediated by activation of the AT1 receptor but not the AT2 receptor, inasmuch as losartan, but not PD 123,319, blocked the Ang II–induced upregulation of TGF-β1 type 1 and 2 receptor expression (Figure 5).

Ang II and TGF-β1 Levels
Incubation of HCAECs with Ang II decreased the expression of TGF-β1 levels in a concentration (10⁻⁸ to 10⁻⁶ mol/L)-dependent manner. Again, pretreatment of HCAECs with losartan, but not PD 123,319, prevented the Ang II–induced decrease in TGF-β1 levels (Figure 6).

Discussion
The present study shows that Ang II markedly upregulates the expression of endoglin. This effect of Ang II is mediated by activation of its AT1 receptor. The activation of MAPK elicited by Ang II appears to be a critical signaling pathway, because PD 098,059, the specific MAPK inhibitor, blocked the upregulation of endoglin expression in response to Ang II. The present study also shows that Ang II via AT1 activation increases the expression of TGF-β1 type 1 and 2 receptors. We also found that as receptor expression increased in response to Ang II, TGF-β1 release by HCAECs decreased.

Interaction Between Ang II and TGF-β1
Both Ang II and TGF-β1 are pleiotropic factors in the cardiovascular system. Physiological levels of Ang II play an important role in the maintenance of blood pressure. High levels of Ang II induce endothelial dysfunction,12 vasoconstriction, and proliferation of smooth muscle cells.13 These effects of Ang II, mediated predominantly by AT1 receptor activation, contribute to the development of hypertension and atherosclerosis. The role of TGF-β1 in vascular growth has gained increasing attention in recent years. TGF-β1 plays an important role in the maintenance of blood vessel integrity.23,24 Recent studies from our laboratory have demonstrated that TGF-β1 inhibits apoptosis of cardiac myocytes induced by hypoxia/reoxygenation25 and modulates myocardial injury after a brief period of ischemia/reperfusion.26 On the other hand, other studies have shown that TGF-β1 promotes the
development of atherosclerosis\(^2^7\) and restenosis after angioplasty.\(^2^8\) Recent studies\(^1^6–^1^9\) indicate that there is an important interaction between Ang II and TGF-\(\beta\).\(^1^\) Kupfahl et al\(^1^6\) have demonstrated that Ang II directly increases the expression of TGF-\(\beta\) in the human heart. Motajima et al\(^1^8\) have shown that TGF-\(\beta\) upregulates Ang II–induced plasminogen activator inhibitor mRNA. These facilitative interactions between Ang II and TGF-\(\beta\) lead to the development of atherosclerosis and vascular injury. In contrast to the increase in TGF-\(\beta\) synthesis in smooth muscle cells in response to Ang II,\(^2^9\) we observed that Ang II decreases TGF-\(\beta\) levels in HCAECs. This unique effect of Ang II in endothelial cells may reflect the binding of TGF-\(\beta\) to upregulated TGF-\(\beta\) receptors. Another potential mechanism for decrease in immunoreactive levels of TGF-\(\beta\) is the downregulation of its synthesis, inasmuch as the expression of TGF-\(\beta\) receptors increases dramatically when endothelial cells are exposed to Ang II. Nonetheless, the contrasting effects of Ang II relative to the release of TGF-\(\beta\) in endothelial cells and smooth muscle cells must await further work.

**Ang II and Its Receptors and Expression of TGF-\(\beta\) Receptors**

Recent molecular genetic studies in humans and mice have shown that TGF-\(\beta\) is involved in vasculogenesis and the maintenance of blood vessel integrity.\(^2^5,^2^4\) There is significant alternation in the expression of TGF-\(\beta\) type 1 and 2 receptors in atherosclerotic tissues.\(^3^0,^3^1\) Experimental studies\(^1^0,^1^1\) have shown endoglin to be a critical factor in angio-

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**Figure 3.** The receptor specificity of effects of Ang II on endoglin expression. The effects of Ang II on endoglin expression were blocked by the AT\(_1\) receptor blocker losartan, whereas the AT\(_2\) receptor blocker PD 123,319 had no effect. The gels represent results of 6 independently performed experiments. Normalized data (bar graphs) are presented as mean±SD.

**Figure 4.** Role of MAPK pathway in Ang II–induced endoglin expression. Incubation of HCAECs with Ang II markedly activated MAPK determined by phosphorylation (p-MAPK). Ang II did not affect levels of MAPK protein in HCAECs. The effect of Ang II on MAPK activation was blocked by the AT\(_1\) receptor blocker losartan but not by the AT\(_2\) receptor blocker PD 123,319. To further examine the role of MAPK in this process, HCAECs were pretreated with a specific inhibitor of MAPK, PD 098,059, to observe the effect of Ang II on endoglin expression. We found that the MAPK inhibitor PD 098,059 not only inhibited Ang II–induced MAPK activation but also prevented the Ang II–induced expression of endoglin mRNA and protein (Figure 3). The gels represent results of 6 independently performed experiments. Normalized data (bar graphs) are presented as mean±SD. Numbers on abscissa correspond to gels 1 through 5 (from left to right).
the blood vessels and cardiac tissues. These observations gain support from findings indicating that AT1 receptor blockers, such as losartan, markedly attenuate the development of atherosclerosis and the remodeling of infarcted myocardium.34,35

**Signal Transduction Mechanism of Ang II–Induced Endoglin Upregulation**

Ang II exerts its biological effects via activation of different signal transduction pathways, such as protein kinase C12 and MAPK.36 The MAPK cascade is a signal transduction pathway that mediates many changes in cell biology. Activation of MAPK causes the activation of transcription factors, such as NF-κB and activator protein-1.37,38 In the present study, we confirmed the activation of MAPK by Ang II in HCAECs. This effect of Ang II was mediated by the AT1 receptor, because losartan fully blocked the activation of MAPK. Most important, we found that the specific MAPK inhibitor PD 098,059 not only inhibited the activation of MAPK but also inhibited the endoglin expression induced by Ang II, indicating a critical role of MAPK activation as a signaling mechanism.

**Summary**

The present study provides clear evidence that Ang II induces the expression of endoglin by activation of the AT1 receptor in HCAECs. In this process, the activation of MAPK plays an important role in signal transduction. Ang II via AT1 activation also increases the expression of TGF-β1 type 1 and type 2 receptors. These observations provide a novel insight into the role of Ang II in the pathobiology of vascular remodeling. These studies in human coronary endothelial cells may be relevant to atherogenesis and vascular growth in humans.

**Acknowledgments**

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**References**


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