Angiotensin-(1-7) Counterregulates Angiotensin II Signaling in Human Endothelial Cells

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Abstract—Angiotensin (Ang)-(1-7), acting through the Mas receptor, opposes the actions of Ang II. Molecular mechanisms for this are unclear. Here we sought to determine whether Ang-(1-7) influences Ang II signaling in human endothelial cells, focusing specifically on Src homology 2-containing inositol phosphatase 2 (SHP-2) and its interaction with c-Src. Ang II–induced phosphorylation of c-Src, extracellular signal regulated kinase (ERK)1/2, and SHP-2 and activation of NAD(P)H oxidase were assessed in the absence and presence of Ang-(1-7) (10^{-7} mol/L, 15 minutes) by immunoblotting and lucigenin-enhanced chemiluminescence, respectively. (D-Ala7)-Ang I/II (1-7) (Ang fragment 1-7 receptor antagonist) was used to block Ang-(1-7) effects. Association between SHP-2 and c-Src was assessed by immunoprecipitation/immunoblotting studies. Ang II significantly increased activation of c-Src, ERK1/2, and NAD(P)H oxidase and reduced phosphorylation of SHP-2 (P<0.05) in human endothelial cells. These effects were abrogated in cells pre-exposed to Ang-(1-7). Ang fragment 1-7 receptor antagonist pretreatment blocked the negative modulatory actions of Ang-(1-7) on Ang II–induced signaling. Ang-(1-7) alone did not significantly alter phosphorylation of c-Src, ERK1/2, and SHP-2 and had no effect on basal activity of NAD(P)H oxidase. SHP-2 and c-Src were physically associated in the basal state. This association was increased by Ang-(1-7) and blocked by Ang fragment 1-7 receptor antagonist. Our findings demonstrate that, in human endothelial cells, Ang-(1-7) negatively modulates Ang II/Ang II type 1 receptor–activated c-Src and its downstream targets ERK1/2 and NAD(P)H oxidase. We also show that SHP-2–c-Src interaction is enhanced by Ang-(1-7). These phenomena may represent a protective mechanism in the endothelium whereby potentially deleterious effects of Ang II are counterregulated by Ang-(1-7). (Hypertension. 2007;50:1093-1098.)

Key Words: Ang-(1-7) ■ Ang II signaling ■ Src ■ ERK1/2 ■ human endothelial cells ■ NO

Angiotensin (Ang) II binding to the Ang II type 1 receptor (AT1R) activates diverse signaling cascades in the vasculature leading to contraction, growth, migration, endothelial dysfunction, expression of proinflammatory mediators, and modification of extracellular matrix protein deposition. These effects are induced through modulation of tyrosine kinases, mitogen-activated protein (MAP) kinases, protein tyrosine phosphatases, nicotinamide adenine dinucleotide (phosphate) [NAD(P)H] oxidase, and Akt-mediated regulation of NO synthase.

Growth-promoting and proinflammatory properties of Ang II involve c-Src–regulated activation of NAD(P)H oxidase, generation of reactive oxygen species, and phosphorylation of the mitogen-activated protein (MAP) kinases extracellular signal regulated kinase (ERK)1/2, p38MAP kinase, and jun N-terminal kinase. Src homology 2-containing inositol phosphatase 2 (SHP-2), a redox-sensitive protein tyrosine phosphatase, negatively influences downstream signaling molecules, such as MAP kinases and Akt, through dephosphorylation, thereby inhibiting growth and inflammatory signaling by Ang II. 

Ang-(1-7), acting through the G protein–coupled receptor Mas, opposes many of the actions of Ang II. Exact molecular mechanisms responsible for the counterregulatory effects are unknown. Most studies demonstrated indirect actions, where Ang-(1-7) stimulates NO and prostaglandin release, causing vasodilation and inhibition of cell proliferation, thereby opposing Ang II–induced vascular contraction and growth. Whether Ang-(1-7)/Mas interaction directly modulates signaling molecules that would interfere with Ang II signal transduction are unclear, but this may be a possibility, because it has been suggested that the AT1R dimerizes with the Mas receptor. In renal proximal tubular cells, Ang-(1-7) reversibly inhibited Ang II–induced activation of ERK1/2, p38MAP kinase, and jun N-terminal kinase and partially attenuated Ang II–stimulated production of transforming growth factor-β1.
In the present study we sought to determine, in human endothelial cells, whether Ang-(1-7) negatively modulates Ang II–stimulated activation of NAD(P)H oxidase and phosphorylation of ERK1/2 and questioned whether the upstream regulators c-Src and SHP-2 may be targets of Ang-(1-7). Human endothelial cells were studied, because in vivo studies indicate that Ang-(1-7) may be important in the regulation of endothelial function.

Materials and Methods

Reagents
The following antibodies were used: anti-phospho-ERK1/2 MAP kinase (1:1000, Cell Signaling), anti-c-Src (pY416; 1:1500, Bio-source), anti-c-Src, clone GD11 (1:1500, Upstate Biotechnology), anti-phospho-SHP2 (Cell Signaling), and anti-β-actin (1:10000, Sigma). Ang-(1-7) was purchased from Sigma Chemical Co. (D-Ala³)-angiotensin I/II¹⁷ (angiotensin fragment 1-7 receptor antagonist; A-779) was from Bachem.

Cell Culture
Human aortic endothelial cells (HAECs; Cascade Biologics), between passages 4 and 6, were cultured in Medium 200 (Cascade Biologics) supplemented with FBS, 2% v/v; hydrocortisone, 1 µg/mL; human epidermal growth factor, 10 ng/mL; basic fibroblast growth factor, 3 ng/mL; and heparin, 10 µg/mL. Cells were serum deprived for 24 hours before experimentation to render them quiescent.

Western Blotting
Cell stimulation was carried out at 37°C in serum-free medium. In some experiments cells were pre-exposed to A-779 (10⁻⁵ mol/L, 15 minutes). Immunoblotting was performed as described previously.²³ Briefly, cell lysates were prepared in lysis buffer, and protein concentration was measured. Solubilized protein was separated by electrophoresis and transferred to nitrocellulose membranes. Non-specific binding was blocked by incubation in 5% milk in Tris-buffered saline Tween 20. Membranes were probed with the specific antibodies (anti-phospho-ERK1/2, 1:1000; anti-c-Src [pY416], 1:1500; anti-c-Src, clone GD11, 1:1500; anti-phospho-SHP2, 1:1000; and anti-β-actin, 1:10000), followed by incubation with horseradish peroxidase–conjugated secondary antibodies. Immunoreactive bands were visualized by enhanced chemiluminescence and quantified by densitometry. Protein loading was visualized by probing the stripped membrane with anti–β-actin antibody (1:10 000).

Immunoprecipitation
To verify whether c-Src interacts with SHP-2 and whether Ang-(1-7) modulates this interaction, lysates of cells stimulated with Ang-(1-7) in the presence or absence of A-779 were incubated with anti-SHP-2 antibody and then probed with anti-Src antibody. Cells were lysed as described previously.²³,²⁷,²⁸ The cell lysate was incubated in lysis buffer (400 µg/0.5 mL) and anti-SHP-2 antibody (2 µg/mL) added and incubated at 4°C overnight. The immunocomplex was captured by adding protein G agarose beads and incubating at 4°C for 2 hours. After centrifugation (13 000 rpm, 4°C, 30 seconds), beads were collected, washed, resuspended in Laemmli sample buffer, and boiled for 5 minutes. After centrifugation (13 000 rpm, 4°C, 30 seconds), the supernatant was aspirated and loaded onto SDS-PAGE.

After SDS-PAGE separation of proteins, samples were transferred to polyvinylidene difluoride membranes. Membranes were incubated with polyclonal anti-phospho-c-Src antibody (diluted 1:500, Cell Signal Technology), monoclonal anti-c-Src antibody (diluted 1:1000, Upstate), and monoclonal anti-SHP-2 antibody (diluted 1:2000, Santa Cruz Biotechnology). Washed membranes were incubated with specific peroxidase-conjugated secondary antibodies. Immunoreactive proteins were detected by chemiluminescence. Blots were analyzed densitometrically (Image-Quant software, Molecular Dynamics).

Measurement of NAD(P)H Oxidase Activity
Confluent HAECs preincubated with Ang-(1-7) (10⁻⁶ mol/L, 15 minutes) were stimulated with Ang II (10⁻⁴ mol/L, 10 minutes). In some experiments, cells were preincubated with the AT₁R antagonist irbesartan (10⁻³ mol/L, 10 minutes). After stimulation, cells were washed with cold PBS and homogenized in lysis buffer (20 mmol/L of KH₂PO₄, 1 mmol/L of EGTA, 1 µg/mL of aprotinin, 1 µg/mL of leupeptin, 1 µg/mL of pepstatin, and 1 mmol/L of PMSF). Activity of NAD(P)H oxidase was measured by lucigenin-derived chemiluminescence in a plate luminometer (AutoLumat LB 953, Berthold) as described previously. The enzymatic activity was expressed as nanomoles of O₂⁻ per minute per milligram of protein.

RNA Interference and Cell Transfection
High-performance purity grade (>90% pure) small-interfering RNAs (siRNAs) were generated against human SHP-2 (QIAGEN Inc). Cells were exposed to transfecant alone (control cells) or transfected with siRNA as described previously.²⁹ A nonsilencing siRNA oligonucleotide sequence that does not recognize any known homology to mammalian genes was also generated as a negative control. Cells were seeded at a density of 60% to 70% confluence in 60-mm plates. Cells were transfected with siRNA using HiPerfect Transfection Reagent (QIagen Inc) according to the manufacturer’s instructions. Briefly, siRNA was diluted in 100 µL of culture medium without serum to give a final concentration of 20 nmol/L. HiPerfect reagent was added to the diluted siRNA, and the mix was added dropwise onto the cells. Twenty-four hours after transfection, cells were stimulated with Ang-(1-7) (10⁻⁶ mol/L). Phospho-c-Src was monitored at the protein levels by Western blotting as described above. Control cells were exposed to the transfecant in the absence of siRNA.

Statistical Analysis
Experiments were repeated 3 to 5 times in duplicate. Results are presented as mean±SEM and compared by ANOVA. A value of P<0.05 was considered significant.

Results

Ang-(1-7) Modulates Ang II–Stimulated ERK1/2 Phosphorylation
Figure 1 shows effects of Ang-(1-7) on Ang II–induced ERK1/2 phosphorylation. HAECs were preincubated with Ang-(1-7) and then stimulated with Ang II. Whereas Ang II stimulation significantly increased phosphorylation of ERK1/2, Ang-(1-7) alone had no effect. However, when cells were preincubated with Ang-(1-7), the ERK1/2 effects of Ang II were abrogated.

Ang-(1-7) Inhibits Ang II–Induced c-Src Activation
As shown in Figure 2, Ang II stimulated phosphorylation of c-Src. This effect was significantly attenuated (P<0.01) when cells were pre-exposed to Ang-(1-7). Incubation of cells with Ang-(1-7) alone did not influence activation of c-Src.

Ang-(1-7) Reduces Activation of Ang II–Stimulated NAD(P)H Oxidase in HAECs
To evaluate whether inhibition of c-Src phosphorylation induced by Ang-(1-7) is paralleled by changes in NAD(P)H oxidase activity, HAECs preincubated with Ang-(1-7) (10⁻⁶ mol/L, 15 minutes) were stimulated with Ang II (10⁻⁴ mol/L, 10 minutes). As demonstrated in Figure 3, Ang-(1-7) signif-
significantly decreased Ang II–stimulated activation of NAD(P)H oxidase. AT$_1$R blockade with Irbesartan inhibited Ang II–mediated actions.

**Ang-(1-7) Stimulates SHP-2 Phosphorylation**

Protein tyrosine phosphatases regulate protein status by dephosphorylating and, in general, inactivating proteins. Of the many protein tyrosine phosphatases, SHP-2 has been implicated to be important in vascular smooth muscle cells. Accordingly, we tested the hypothesis that Ang-(1-7) inhibitory effects may be related to activation of SHP-2. HAECs pre-exposed to Ang-(1-7) were stimulated with Ang II. Some experiments were performed in the presence of Irbesartan. As shown in Figure 4, Ang-(1-7) alone significantly increased phosphorylation of SHP-2, whereas Ang II induced dephosphorylation. In cells preincubated with Ang-(1-7), Ang II–mediated SHP-2 effects were normalized. Irbesartan blocked Ang II–induced actions.

**A-779 Inhibits Ang-(1-7) Effects on Ang II–Induced Activation of c-Src**

To confirm that Ang-(1-7) effects on Ang II signaling are specific, we evaluated effects of Ang-(1-7) on Ang II–mediated c-Src activation in the presence of A-779, an Ang-(1-7) receptor antagonist. As shown in Figure 5, Ang-(1-7) alone or in combination with A-779 had no effect on c-Src activation. In cells pre-exposed to Ang-(1-7) and A-779
(15 minutes), Ang II–induced phosphorylation of c-Src at 5 minutes was significantly increased (P<0.01).

c-Src Interacts With SHP-2 in Human Endothelial Cells: Modulation by Ang-(1-7)

To investigate in greater detail whether Ang-(1-7) influences c-Src signaling through SHP-2–related processes, we questioned whether c-Src physically associates with SHP-2 by immunoprecipitating SHP-2 and then probing for c-Src. As shown in Figure 6, in the basal state c-Src associates with SHP-2. Ang-(1-7) induced a significant increase in SHP-2–Src interaction. This effect was blocked by A-779.

Downregulation of SHP-2 Is Associated With Increased c-Src Phosphorylation in Response to Ang-(1-7)

To demonstrate that SHP-2 negatively regulates c-Src in response to Ang-(1-7), we examined c-Src status in endothelial cells in which SHP-2 was downregulated using siRNA. As shown in Figure 7, siRNA transfection markedly reduced SHP-2 protein content. In SHP-2 knockdown cells, c-Src phosphorylation in resting and Ang-(1-7)–stimulated cells was significantly increased (P<0.05 versus non-siRNA cells; Figure 7).

Discussion

Major findings from the present study demonstrate that Ang-(1-7) counterregulates Ang II signaling in human endothelial cells. This is evidenced by the following: (1) Ang-(1-7) blunts phosphorylation of c-Src and ERK1/2; (2) activation of NAD(P)H oxidase by Ang II is attenuated by Ang-(1-7); (3) Ang-(1-7) stimulates phosphorylation of SHP-2 and prevents Ang II–induced SHP-2 dephosphorylation; and (4) Ang-(1-7) promotes interaction between SHP-2 and c-Src. We also show that SHP-2 is critical for negative regulation of c-Src by Ang-(1-7). A-779, an Ang-(1-7) receptor antagonist, inhibited Ang-(1-7) actions, indicating that effects are receptor mediated, probably through receptor Mas, as we demonstrated previously, and not because of nonspecific actions.

In keeping with the modulatory concept, it has been shown that Ang-(1-7) counterregulates vasoconstrictor effects of...
Ang II by causing vasodilatation. This has been demonstrated in many vascular beds, including piglet pial arterioles, aorta and coronary arteries, rabbit afferent arterioles, isolated precapillary resistance vessels, and a human mammary artery. NO1,4–17,19 and prostaglandin release, as well as bradykinin potentiation, have been implicated in Ang-(1-7)–mediated vasodilation.

Other than its NO-elicited vasodilatory effects, it has been suggested that there is a direct interplay between signaling pathways activated by Ang-(1-7) and Ang II. Ang II is a potent stimulator of p38MAP kinase, ERK1/2, and jun N-terminal kinase. These kinases are crucial for Ang II signaling and vascular function, including cell growth, survival, migration, inflammation, and fibrosis. Ang-(1-7) antagonizes Ang II–induced activation of protein kinase C and ERK1/2 in vascular smooth muscle cells. Similar results were obtained in rat aortic cells, where Ang-(1-7) inhibited Ang II–stimulated MAP kinase phosphorylation through prostacyclin-mediated production of cAMP and activation of cAMP-dependent protein kinase. Transfection of cultured myocytes with an antisense oligonucleotide to the Ang-(1-7) receptor Mas blocked Ang-(1-7)–mediated inhibition of serum-stimulated MAP kinase activation. This negative modulation on Ang II–stimulated ERK1/2 activity was confirmed recently in proximal renal tubular cells. In this model, Ang-(1-7) also reduced Ang II–induced phosphorylation of p38 MAP kinase and jun N-terminal kinase. These data in experimental animals are in agreement with our finding in human endothelial cells where Ang-(1-7) inhibits Ang II–mediated ERK1/2 activation while having no effect alone on ERK1/2 phosphorylation. These findings are particularly relevant considering that ERK1/2 is a key regulator of signaling cascades involved in endothelial permeability, apoptosis, and inflammatory responses.

It has become increasingly evident that NAD(P)H oxidase represents one of the most important sources of reactive oxygen species in both endothelial and vascular smooth muscle cells. Ang II potently activates NAD(P)H oxidase and O2− generation. This activation depends, in large part, on nonreceptor tyrosine kinase c-Src activation, which, in turn, induces p47phox NAD(P)H oxidase subunit phosphorylation and translocation, critical for NAD(P)H oxidase assembly and activation. We found that Ang-(1-7) abolished Ang II–induced c-Src phosphorylation. Considering that c-Src is upstream of NAD(P)H oxidase, it is not surprising that Ang-(1-7) attenuates Ang II–induced NADPH oxidase–driven generation of reactive oxygen species in endothelial cells.

To further investigate signaling pathways whereby Ang-(1-7) could negatively modulate Ang II actions, we explored the role of Ang-(1-7) on SHP-2, which is generally thought to function as the inactivating component of signaling pathways, by reversing the stimulatory phosphorylation events. In this regard, the results presented in our study show that Ang-(1-7) stimulation potently phosphorylated SHP-2, which could act as a negative proximal regulator of Ang II–induced MAP kinase kinase and c-Src signaling. We also show by immunoprecipitation/immunoblotting studies that SHP-2 physically interacts with c-Src and that Ang-(1-7) modulates this interaction. Previous studies demonstrated that SHP-2 associates with Src and inhibits its catalytic activity by dephosphorylating a positive regulatory tyrosine 418 within the Src kinase domain. In the context of our findings, Ang-(1-7) increased association of SHP-2 and c-Src, which probably contributes to dephosphorylation of c-Src and reduced activity. Although the magnitude of the increase was modest, the functional consequence was significant, as evidenced by the marked inhibitory effects of Ang-(1-7) on Ang II–induced c-Src activation. This may reflect the situation where even a small change in proximal signaling events has profound downstream consequences. It may also be possible that processes independent of c-Src interaction with SHP-2 underlie Ang-(1-7) effects on Ang II signaling. It should be emphasized that SHP-2 has many cellular effects and that it both negatively and positively modulates growth/apoptosis signaling pathways. Hence, the exact overall consequence of SHP-2 phosphorylation by Ang-(1-7) still remains to be elucidated.

To further examine the relationship between SHP-2 and c-Src in response to Ang-(1-7) stimulation, we examined c-Src status in endothelial cells, in which SHP-2 was downregulated using siRNA. In the absence of functional SHP-2, basal and Ang-(1-7)–induced phosphorylation of c-Src were increased. These findings confirm the negative modulatory role of SHP-2 on c-Src and highlight the critical role of SHP-2 in Ang-(1-7) signaling. Exact mechanisms whereby SHP-2 controls c-Src have not been fully elucidated but may relate to C-terminal Src kinase, a negative regulator of Src family kinases, which itself interacts with SHP-2. We showed previously that C-terminal Src kinase inhibits c-Src activation in vascular smooth muscle cells. Whether Ang-(1-7) modulates this process and whether SHP-2 is involved awaits further clarification.

In summary, findings from our study demonstrate that, in human endothelial cells, Ang-(1-7) negatively modulates c-Src, ERK1/2, and NAD(P)H oxidase–driven generation of reactive oxygen species by Ang II. This phenomenon may represent a protective mechanism in the endothelium whereby potentially deleterious effects of Ang II are counterbalanced by Ang-(1-7). These actions probably involve cross-talk between Ang-(1-7) and Ang II signaling pathways. SHP-2 activation by Ang-(1-7) may be a key signaling molecule in this process.

Perspectives

Emerging evidence indicates that Ang-(1-7) opposes actions of Ang II/AT1R-mediated actions, thereby protecting against damaging effects of Ang II in the cardiovascular system. Molecular mechanisms whereby Ang-(1-7) and Ang II interact are elusive. Previous studies demonstrated that Ang-(1-7) induces effects through prostacyclin-mediated production of cAMP and activation of cAMP-dependent protein kinase, by attenuation of MAP kinase activation, and by activation of NO synthase to generate NO. Here we further explored signaling pathways through which Ang-(1-7) regulates endothelial cell function focusing specifically on the role of SHP-2 and its downstream targets c-Src, ERK1/2, and NAD(P)H oxidase. Our data demonstrate that Ang-(1-7) negatively
controls Ang II–induced activation of c-Src and its downstream targets, ERK1/2 and NAD(P)H oxidase. These effects are mediated via SHP-2, which is critical for negative modulation of c-Src by Ang-(1-7). Our findings identify a novel signaling pathway in endothelial cells, highlighting the importance of SHP2/c-Src in response to Ang-(1-7).

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Disclosures

None.

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