Angiotensin II Type 2 Receptor Inhibits Epidermal Growth Factor Receptor Transactivation by Increasing Association of SHP-1 Tyrosine Phosphatase

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Abstract—Angiotensin (Ang) II has 2 major receptor isoforms, Ang type 1 (AT₁) and Ang type (AT₂). AT₁ transphosphorylates epidermal growth factor receptor (EGFR) to activate extracellular signal–regulated kinase (ERK). Although AT₂ was shown to inactivate ERK, the action of AT₂ on EGFR activation remains undefined. Using AT₁-overexpressing vascular smooth muscle cells from AT₂ transgenic mice, we studied these undefined actions of AT₂.

Maximal ERK activity induced by Ang II was increased 1.9- and 2.2-fold by AT₁ inhibition, which was abolished by orthovanadate but not okadaic acid or pertussis toxin. AT₁ inhibited AT₂-mediated EGFR tyrosine phosphorylation by 63%. The activity of SHP-1 tyrosine phosphatase was significantly upregulated 1 minute after AT₁ stimulation and association of SHP-1 with EGFR was increased, whereas AT₂ failed to tyrosine phosphorylate SHP-1. Stable overexpression of SHP-1–dominant negative mutant completely abolished AT₂-mediated inhibition of EGFR and ERK activation. AT₁-mediated c-fos mRNA accumulation was attenuated by 48% by AT₂ stimulation. Induction of fibronectin gene containing an AP-1 responsive element in its 5'-flanking region was decreased by 37% after AT₂ stimulation, corresponding to the results of gel mobility assay with the AP-1 sequence of fibronectin as a probe. These findings suggested that AT₂ inhibits ERK activity by inducing SHP-1 activity, leading to decreases in AP-1 activity and AP-1–regulated gene expression, in which EGFR dephosphorylation plays an important role via association of SHP-1.

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Key Words: angiotensin II ■ angiotensin II receptors ■ angiotensin II type 2 receptor ■ tyrosinephosphatase ■ SHP-1 ■ epidermal growth factor receptor

Angiotensin (Ang) II plays a critical role in regulation of the cardiovascular system via 2 main Ang II receptor subtypes, Ang type 1(At₁) and Ang type 2(At₂). Most of the Ang II–mediated vasoconstrictive actions are mediated by At₁, although little information is available regarding signal transduction by At₂. At₂ is abundantly and widely expressed in fetal tissues and is reexpressed in myocardial infarction and vascular injury. Although the level of expression of At₂ is low in large vessels, At₂ is present in high levels in vascular smooth muscle cells (VSMCs) of microvessels. At₂ antagonizes the in vivo effects of At₁ on blood pressure and renal natriuresis and mediates growth inhibition, differentiation, and/or apoptosis in VSMCs and in endothelial, neuronal, and fibroblast R3T3 cells.

These effects of At₂ are mediated mainly by the activation of protein tyrosine phosphatases (PTP), resulting in the inactivation of At₁-activated extracellular signal–regulated kinase (ERK). Recently, Ang II–induced ERK activation was shown to be mediated by epidermal growth factor receptor (EGFR) transactivated via At₁ in a Ca²⁺–dependent manner in cardiac fibroblasts, VSMCs, and liver epithelial cells. In contrast, activation of c-Jun NH₂-terminal kinase (JNK) by At₁ is dependent on Ca²⁺–sensitive tyrosine kinase Pyk2 activity. AT₂ was shown to inactivate ERK via mitogen-activated protein (MAP) kinase phosphatase-1 (MKP-1), src homology 2 domain–containing PTP (SHP-1), or serine-threonine phosphatase PP2A in NIE-115 and PC12W cells. Although At₁ has also been shown to inhibit tyrosine phosphorylation of signal transducers and activators of transcription and insulin receptor, it remained unclear whether At₂ signaling dephosphorylates EGFR to affect ERK activity.

We established AT₁-overexpressing VSMCs (AT₁-VSMCs) from VSMC-targeted AT₁ transgenic (TG) mice and attempted to define the AT₂ signaling in VSMCs of microvessels endogenously expressing AT₂. In the present

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study, we showed that (1) AT2 dephosphorylates EGFR by inducing SHP-1 activation and its association with EGFR, leading to ERK inactivation, and (2) inhibition of ERK activity causes a decrease in c-fos expression and AP-1 activity, leading to a decrease in the expression of AP-1-responsive genes such as fibronectin.

Methods

Antibodies were purchased from the following sources: Santa Cruz (c-fos, EGFR), Transduction Laboratories (SHP-1), New England BioLabs (phospho-specific ERK), and Upstate Biotechnology (4G10-HRP, ERK). AT1 antagonist RNH-6270 (active form of olmesartan) was provided by Sankyo Pharmaceutical Co Ltd (Tokyo, Japan).

Cell Culture

AT2-VSMCs isolated from the aorta of AT2 TG mice were stably transfected with dominant negative mutant of SHP-1 (SHP-1–DN1 and –DN2); AT1 and AT2 numbers (n=6) were determined by saturation binding through the use of membrane fractions and subsequent Scatchard analyses. SHP-1–DN cells and AT2-VSMCs were equally passaged (5 times) from the primary isolation. Bmax is expressed in fmoL/mg protein; Kd in nmol/L.

Assays for ERK and SHP-1 PTP Activities

ERK activities were determined by the immune-complex kinase assay with the use of anti-ERK antibodies as described previously.15,19 Immunoprecipitates were reacted with synthetic peptide (KRELVEPLTPAGEAPNGALLR) for ERK as a substrate. PTP activity in SHP-1 immunoprecipitates was determined by use of abl-tyrosine-phosphorylated myelin basic protein (MBP) as a substrate as described previously.13,20 The immunoblotting signals were visualized by chemiluminescence and quantified by densitometry.

Transfection of DNA, Northern Blotting, and Calcium Analysis

Because the efficiency for DNA transfection was low in VSMCs, we established stably transfected cell lines as reported previously.15,19 Briefly, AT2-VSMCs were cotransfected with wild-type mouse AT2 in pBC-SF (10 μg) and SHP-1 (C453/S) mutant in pcDNA3 (20 μg) using LipofectAMINE PLUS (GIBCO BRL). After selection with G418, 2 stable clones that expressed the highest levels of AT2 (determined by the binding assay) were selected (the Table). Total RNA was analyzed with c-fos or GAPDH cDNA probes.15,19 Ca2+ levels were measured with fura 2-AM as reported.

Gel Retardation Assay

The gel retardation assay using oligonucleotides corresponding to a DNA fragment containing an AP-1 sequence of rat fibronectin gene (between nt-438 and -473) as a probe was performed as previously reported.24 The core AP-1 sequence (TGGACGCA at -433→TGGATAA) was mutated by site-directed mutagenesis, and supershift assay was performed by adding anti-c-fos or anti-c-jun antibodies (1 μg each) to the samples, followed by incubation for 1 hour at room temperature.

Statistical Analysis

The results are expressed as mean±SE. Statistical analyses were performed by 1-way ANOVA followed by pairwise contrast (control versus condition) with Dunnett’s multiple comparison test. Data were considered statistically significant at P<0.05.

Figure 1. Effects of AT2 on activity and phosphorylation of ERK and tyrosine phosphatase activity. ERK activity and phosphorylation were measured by the immune-complex kinase assay and Western blots using anti-phospho-ERK antibody, respectively. A, VSMCs were challenged with Ang II (100 nmol/L) for the indicated periods. B, To test the effects of PD 123,319 (1 μmol/L) on ERK phosphorylation, AT2-VSMCs were pretreated with these compounds for 1 hour and then exposed to Ang II (100 nmol/L) for 7 minutes. PTX (1 μg/mL) was preincubated for 24 hours. Exposure to Ang II+PD 123,319 and Ang II+olmesartan caused selective activation of AT1 and AT2, respectively. C, Tyrosine-phosphatase activities in AT2-VSMCs exposed to Ang II+olmesartan (AT2 stimulation) were determined with abl-tyrosine-phosphorylated MBP as a substrate. Results shown are mean±SE (n=4). *P<0.01 vs time 0 control or unstimulated cells.
**Results**

AT$_2$ Induces ERK Inactivation and Increases PTP Activity

Addition of Ang II (100 nmol/L) to wild VSMCs stimulated ERK activity with a maximal increase (10.4-fold) at 7 minutes, whereas in AT$_2$-VSMCs isolated from AT$_2$ TG mice, the maximal increase in ERK was only 6.7-fold (Figure 1A). Ang II dose dependently activated ERK with a maximal peak at 100 nmol/L in both AT$_1$ and wild VSMCs (data not shown). AT$_1$ and AT$_2$ stimulation by addition of Ang II induced a moderate increase in ERK activation, which was further increased by 1.9-fold after AT$_2$ inhibition (Ang II + PD 123,319) (Figure 1B). ERK activation by Ang II was abolished by the specific AT$_1$ antagonist olmesartan. ERK levels were increased by nonselective tyrosine-phosphatase vanadate but not by the serine-threonine phosphatase inhibitor okadaic acid or inhibition of G$_i$ by pertussis toxin (PTX) (Figure 1B). These findings suggest that the mechanism for Ang II–mediated ERK activation is not sensitive to inhibition of serine-threonine phosphatases or inhibition of G$_i$ by PTX. Because the data with vanadate should be carefully interpreted, we next examined whether PTP activities are increased after AT$_2$ stimulation. PTP activities were measured with tyrosine-phosphorylated MBP as a substrate. PTP activities were increased significantly 1 minute after AT$_2$ stimulation and reached a maximum at $\approx$5 minutes (Figure 1C), similar to the time course of ERK activation.

AT$_2$ Inhibits Tyrosine Phosphorylation of EGFR

Because we reported that AT$_2$ transactivated EGFR to mediate EGFR-Ras-ERK signals, we next studied the effect of AT$_2$ on EGFR phosphorylation. Ang II moderately stimulated tyrosine phosphorylation of EGFR compared with unstimulated levels (Figure 2A). Inhibition of AT$_2$ function by addition of vanadate further increased Ang II–induced EGFR phosphorylation by 63% or 58% ($P<0.01$ versus Ang II alone), respectively. Although EGFR activation by AT$_2$ was reported to be mediated mainly by intracellular Ca$^{2+}$ level, PD 123,319 treatment did not affect Ang II–mediated Ca$^{2+}$ mobilization, and AT$_1$ inhibition by olmesartan abolished this Ca$^{2+}$ mobilization (Figure 2B), suggesting no involvement of AT$_2$ signals in the intracellular Ca$^{2+}$ level.

SHP-1 Is Involved in AT$_2$-Mediated Tyrosine Dephosphorylation of EGFR

SHP-1 was shown to mediate ERK inactivation by AT$_2$ in neuronal cells. SHP-1 activity with tyrosine-phosphorylated MBP as a substrate was significantly stimulated as rapidly as 1 minute after AT$_2$ stimulation (Ang II + olmesartan), reaching a maximum peak after 5 minutes (Figure 3A). AT$_2$ stimulation (Ang II + PD 123,319) did not induce any increase in SHP-1 activity (data not shown).

AT$_2$ stimulation (Ang II + olmesartan) but not AT$_1$ stimulation (Ang II + PD 123,319) increased the association of SHP-1 with EGFR (Figure 3B). In contrast, SHP-1 itself was not tyrosine phosphorylated by AT$_2$ or AT$_1$ stimulation, although fetal calf serum induced its tyrosine phosphorylation (Figure 3B).

We next tested the involvement of SHP-1 in EGFR and ERK activation using a dominant negative mutant (SHP-1–DN). Figure 3C shows that AT$_2$-VSMCs endogenously express SHP-1 and that stable clones (SHP-1–DN) overexpress SHP-1–DN. Furthermore, the Table indicates that the expression levels of AT$_1$ and AT$_2$ are similar between AT$_2$-VSMC and SHP-1–DN clones. We showed that PD 123,319 pretreatment significantly enhanced Ang II–mediated EGFR phosphorylation (Figure 2A) and ERK activation (Figure 1B), indicating the inhibitory effect of AT$_2$ on EGFR and ERK activity. Interestingly, in SHP-1–DN clones, EGFR and ERK activation by Ang II was not affected by PD 123,319 pretreatment (only SHP-1–DN1 data shown in Figure 3C), suggesting the involvement of SHP-1 in AT$_2$–mediated inactivation of EGFR and ERK.

AT$_2$-Mediated Inhibition of c-fos Expression

We have shown that induction of c-fos gene by Ang II is regulated mainly by EGFR-mediated signal. We therefore
tested whether AT\textsubscript{2} affects \textit{c-fos} gene expression. Ang II moderately induced \textit{c-fos} mRNA expression, whereas inhibition of AT\textsubscript{1} by PD 123,319 further increased Ang II–induced mRNA expression (\textit{c-fos}, 81% versus Ang II alone; \(P<0.01\)) (Figure 4). AT\textsubscript{1} inhibition by olmesartan abolished induction of \textit{c-fos} mRNA by Ang II.

**AT\textsubscript{2} Decreases Fibronectin Gene Expression by Inhibiting AP-1 Complex Formation**

Induction of fibronectin transcription by Ang II was shown to be regulated by binding of AP-1 complex to the AP-1 site in the 5\textsuperscript{'}-flanking region,\textsuperscript{23} suggesting that AT\textsubscript{2} may downregulate fibronectin gene expression. As shown in Figure 5A, induction of fibronectin mRNA by Ang II was further increased by inhibition of AT\textsubscript{2} (Ang II + PD 123,319, 48%; \(P<0.05\) versus Ang II alone) or orthovanadate treatment (51%, \(P<0.05\) versus Ang II alone), whereas okadaic acid did not affect the mRNA levels.

We also studied the effects of AT\textsubscript{2} on binding of AP-1 complex to the AP-1 site in the fibronectin gene (Figure 5B). Ang II stimulated the binding of nuclear extract to oligonucleotides containing the AP-1 sequence of the fibronectin gene (nt-438 to -473), whereas this binding was inhibited by an excess of cold probe but not by mutation of the AP-1 core sequence. Addition of anti–\textit{c-fos} antibody supershifted the binding of nuclear extract. Pretreatment with PD 123,319 significantly (\(P<0.05\)) enhanced Ang II–induced AP-1 activity by 44%, suggesting that AT\textsubscript{2} attenuates Ang II–induced fibronectin expression by reducing AP-1 complex formation.

**Discussion**

Ang II exerts positive or negative effects on cell growth and survival, depending on which subtype of receptor (AT\textsubscript{1} or AT\textsubscript{2}) is activated. ERK activation by Ang II was mediated mainly by downstream signals of EGFR transactivated by AT\textsubscript{1}-mediated Ca\textsuperscript{2+} signals in VSMCs\textsuperscript{16} or cardiac fibroblasts.\textsuperscript{15} Although recent evidence has suggested that ERK inactivation and pro-apoptotic action of AT\textsubscript{1} are mediated via SHP-1 or MKP-1 in neuronal cells such as NIE\textsuperscript{13} or PC12\textsuperscript{21,20} cells, it remained unknown whether AT\textsubscript{2} inhibits EGFR transactivation, leading to ERK inactivation. Furthermore, AT\textsubscript{2}-mediated association of SHP-1 with EGFR has not yet been defined. This study using VSMCs overexpressing AT\textsubscript{2} clearly demonstrated that (1) AT\textsubscript{2} dephosphorylates EGFR by inducing SHP-1 activity and its association with EGFR, leading to ERK inactivation, and (2) inhibition of ERK activity causes a decrease in AP-1 activity, leading to a decrease in the expression of AP-1–responsive genes such as fibronectin.

SHP-1 is a soluble PTP containing 2 SH2 domains that allow binding to phosphotyrosines and that participate in the negative regulation of receptor tyrosine kinase pathways.\textsuperscript{25} SHP-1 physically interacts with EGFR in a cell type–specific manner, whereas it appears to have little activity toward the
bound EGFR.\textsuperscript{26,27} When SHP-1 was transiently overexpressed or exogenous autophosphorylated EGFR was added, the SHP-1 could be activated to use the bound receptor as a substrate.\textsuperscript{26,28} In this study using VSMCs, we showed for the first time that AT\textsubscript{2} induced the association of SHP-1 with EGFR without affecting the phosphorylation level of SHP-1 and that overexpression of SHP-1–DN completely abolished AT\textsubscript{2}-mediated dephosphorylation of EGFR followed by ERK activation. The observation that AT\textsubscript{2} stimulation inhibited EGFR phosphorylation as rapidly as 1 minute after addition was in agreement with the time course of SHP-1 activation. AT\textsubscript{2} receptors have been shown to activate SHP-1 in neuronal cells such as NIE-115\textsuperscript{13} or PC12W cells,\textsuperscript{20} in which SHP-1 was shown to be involved in ERK inactivation or induction of apoptosis. However, the molecules interacting with SHP-1 were not defined in these earlier studies. Li et al\textsuperscript{29} reported that platelet thrombin receptor causes SHP-1 tyrosine phosphorylation in a PTX-dependent manner and suggested the role of tyrosine kinases linked to the thrombin receptor by Gi protein. However, the present study demonstrated that AT\textsubscript{2} inactivated ERK\textsuperscript{2} via a mechanism not sensitive to inhibition of Gi by PTX, consistent with the failure of AT\textsubscript{2} to induce SHP-1 phosphorylation. Recently, a structural model for SHP-1 was proposed\textsuperscript{30} in which SH2 domains of SHP-1 were shown to be capable of interacting with its C terminus in a phosphotyrosine-dependent manner and thereby drive the PTPase domain in an inactive conformation. It is possible that the conformational change of SHP-1 induced by AT\textsubscript{2} leads to the increased association of SHP-1 with EGFR and forms the basis for activation toward the receptor as observed in our study. Thus, AT\textsubscript{2} may have the capacity to disrupt this intramolecular interaction.

SHP-1 is predominantly expressed in hematopoietic cells and plays a key role in hematopoiesis.\textsuperscript{31} Although the role of SHP-1 in VSMCs has not been defined in detail, the present study suggested a novel function of SHP-1 in AT\textsubscript{2}-mediated EGFR inactivation followed by a growth inhibitory action. Tang et al\textsuperscript{32} reported that SHP-2 association with Ca\textsuperscript{2+}-sensitive tyrosine kinase Pyk2 negatively regulated AT\textsubscript{1}-mediated Pyk2 activation in endothelial cells. Chemokines stimulate the association of SHP-1 and SHP-2 with Pyk2 in T cells and positively affect Pyk2-mediating signals.\textsuperscript{33} AT\textsubscript{2} also inactivates insulin receptor by decreasing SHP-2 association with insulin receptors.\textsuperscript{22} Because SHP-1 was reported to interact with SHP-2,\textsuperscript{34} further studies are required to define the relationship between SHP-1 and SHP-2 in the mechanism of AT\textsubscript{2} activation.

Marrero et al\textsuperscript{15} reported that AT\textsubscript{1} stimulation in VSMC tyrosine phosphorylated Janus kinase-2 (JAK2) and that electroporation of neutralizing anti–SHP-1 antibody resulted in termination of JAK2 phosphorylation. Although we tested whether SHP-1 activity is increased after AT\textsubscript{1} stimulation, neither SHP-1 activity nor SHP-1 phosphorylation was induced after AT\textsubscript{1} stimulation, suggesting that SHP-1 activity is not modulated by AT\textsubscript{1} stimulation in VSMCs. Although our finding contrasts with the result of Marrero et al,\textsuperscript{15} the AT\textsubscript{1}-mediated increase in SHP-1 activity is too small to be detectable in our assay (MBP dephosphorylation), or the nonelectroperducible effect resulting from electroperoration of neutralizing anti–SHP-1 antibody\textsuperscript{15} might be involved in the discrepant result.

This study demonstrated that AT\textsubscript{1}-mediated c-fos expression was decreased by AT\textsubscript{2} stimulation. We reported that AT\textsubscript{2} downregulates c-fos expression by decreasing the binding activity of signal transducers and activators of transcription to the sis-inducible element of c-fos promoter.\textsuperscript{21} The c-fos expression is regulated by the net interaction with different transcriptional factors.\textsuperscript{36} Eguchi et al\textsuperscript{16} and we\textsuperscript{15} have shown that the inhibition of EGFR function completely abolished
AT1-mediated c-fos expression, suggesting a major role of ERK-activated serum response factor. Thus, the AT1-mediated decrease in AP-1 activity, followed by induction of AP-1-responsive genes such as fibronectin, is likely regulated by the inactivation of ERK.

Yamada et al.17 reported the involvement of MKP-1 but not PP2A in AT1-mediated ERK inactivation in PC12W cells, whereas Huang et al.14 showed the participation of PP2A in neurons, and Bedecs et al.13 reported no involvement of MKP-1 or PP2A. Although we could not define the involvement of MKP-1 in AT1-mediated effects because of low transfection efficiency of antisense oligonucleotide for MKP-1 into VSMCs, okadaic acid was found to have no significant effect on AT1 signals. These differences might be due to a variation of cell types or might reflect the complexity of the network involved in negative regulation of ERK activity. Thus, further dissection of the AT1 signaling pathway and identification of the cross-point with EGFR cascades may provide new perspectives for pharmacological targeting of proliferative diseases and a unique example of negative cross-talk in growth signals.

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The American Heart Association (AHA) published an expression of concern\(^1\text{-}^3\) simultaneously in Circulation, Circulation Research, and Hypertension, and we have now been notified by Kyoto Prefectural University of Medicine that certain data and figures in these 5 articles were falsified. Kyoto Prefectural University of Medicine sends their deepest apologies to the academic community at large.

The AHA is hereby retracting these articles:


References


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