Increased Angiotensin II–Mediated Src Signaling via Epidermal Growth Factor Receptor Transactivation Is Associated With Decreased C-Terminal Src Kinase Activity in Vascular Smooth Muscle Cells From Spontaneously Hypertensive Rats

Rhian M. Touyz, Xiao-Hua Wu, Gang He, Steven Salomon, Ernesto L. Schiffrin

Abstract—We investigated whether upregulation of Src by Ang II leads to increased extracellular signal–regulated kinase 1/2 (ERK1/2) phosphorylation in vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR) and whether these processes are associated with altered activation of C-terminal Src kinase (Csk), a negative regulator of Src. Furthermore, the role of epidermal growth factor receptor (EGFR) transactivation by angiotensin II (Ang II) was determined. Ang II–mediated c-Src phosphorylation was significantly greater (≈4-fold, \( P<0.01 \)) in SHR than in Wistar-Kyoto rats (WKY). Ang II increased Csk phosphorylation 2-to 3-fold in WKY but not in SHR. Treatment of the cells with AG1478, a selective EGFR tyrosine kinase inhibitor, decreased Ang II–mediated c-Src phosphorylation, particularly in SHR. Phosphorylation of cortactin and Pyk2/focal adhesion kinase, Src-specific substrates, was increased by Ang II \( (P<0.05) \). Ang II–induced ERK1/2 activation was significantly augmented \( (P<0.05) \) and sustained in VSMCs from SHR. PP2, a selective Src inhibitor, attenuated these effects and normalized the responses in SHR. Irbesartan, a selective Ang II type 1 receptor blocker, but not PD123319, a selective Ang II type 2 receptor blocker, inhibited Ang II actions. Our results demonstrate that c-Src phosphorylation and Src-dependent ERK1/2 signaling by Ang II are increased in VSMCs from SHR. These processes are associated with blunted Ang II–induced phosphorylation of Csk. EGFR transactivation contributes to Ang II–mediated Src-dependent ERK1/2 signaling. In conclusion, altered regulation of Ang II type 1 receptor–activated c-Src by Csk may be an important upstream modulator of abnormal ERK1/2 signaling in VSMCs from SHR. (Hypertension. 2002;39[part 2]:479-485.)

Key Words: angiotensin II ■ kinase ■ protein kinases ■ hyperplasia
The mechanisms contributing to enhanced ERK1/2 signaling by Ang II in hypertension remain unclear. Previous studies have suggested that changes in the regulation of MAP kinase phosphatase, which dephosphorylates MAP kinase, or increased sensitivity to intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) could play a role.\(^2,4,25\) It is also possible that alterations in regulatory tyrosine kinases (such as Src) that are upstream from ERK1/2 could be important. Src kinases are a family of nonreceptor tyrosine kinases, of which the prototype, c-Src, is the major isoform in the vasculature.\(^26,27\) Src is activated by autophosphorylation of Tyr416 and is inactivated by carboxy-terminal Src kinase (Csk), which induces the phosphorylation of Tyr527.\(^28\) We propose that the upregulation of Src by Ang II may be an important mediator of increased VSMC growth signaling in SHR. This is supported by studies demonstrating that Ang II activates Src and that Src regulates Ras, which activates MAP/ERK kinase, resulting in ERK1/2 phosphorylation, an essential growth signal in hypertension.\(^29\) Src also modulates [Ca\(^{2+}\)]\(_{i}\),\(^30\) which seems to be important in ERK1/2 hyperactivation in SHR.\(^25\) To our knowledge, there are no data regarding the role of Src (and specifically, c-Src) in Ang II–mediated growth signaling in hypertension. In the present study, we investigated whether the upregulation of Src by Ang II leads to increased ERK1/2 phosphorylation in VSMCs from SHR and whether these processes are associated with altered activation of Csk, which negatively regulates Src. We also evaluated whether Ang II transactivation of EGFR influences Src signaling in SHR. Findings from the present study suggest that augmented Src phosphorylation by AT\(_1\)-mediated EGFR transactivation may be an important early intracellular event mediating abnormal VSMC ERK1/2 signaling in SHR. The upregulation of Src by Ang II in SHR may be due, in part, to decreased activation of Csk.

**Methods**

**Cell Culture**

Adult male Wistar-Kyoto rats (WKY, \(n=28\)) and SHR (\(n=32\)) were studied. Systolic blood pressure was recorded in pretrained conscious rats by the tail-cuff method.\(^8,10,21\) Blood pressure was higher (\(P<0.001\)) in SHR (189±1.4 mm Hg) than in WKY (112±1.6 mm Hg). Rats were killed by decapitation. VSMCs derived from mesenteric arteries were isolated and characterized as described.\(^20,21\) Quiescent cells were stimulated with Ang II with or without PP2 (a specific Src inhibitor)\(^31\) or with AG1478 (a selective EGFR tyrosine kinase inhibitor)\(^32\) for 20 minutes. To determine the PP2 concentration that completely inhibits c-Src phosphorylation, cells were exposed to increasing concentrations of PP2. In our system, the IC\(_{50}\) for PP2 was 5×10\(^{-7}\) mol/L.\(^30\) At 10\(^{-5}\) mol/L, PP2 effectively blocked the agonist-stimulated c-Src phosphorylation. We used this concentration for further studies. For experiments with Ang II receptor antagonists, cells were preincubated with either irbesartan (10\(^{-5}\) mol/L) or PD123319 (10\(^{-3}\) mol/L) for 20 minutes. Protein was extracted from VSMCs as we described.\(^20,21\) Proteins (15 to 30 μg) were separated by electrophoresis on a 10% polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline solution with Tween for 1 hour at 24°C. Membranes were then incubated with phospho-specific antibodies (1:1000) overnight at 4°C; antibodies were as follows: anti-c-Src (pY416), which recognizes Tyr416 (the tyrosine residue that needs to be phosphorylated for c-Src activation), Upstate Biotechnology; anti-cortactin (pY421, BioSource Int Inc); anti-Pyk2 (pYPY579/580), which also recognizes focal adhesion kinase (FAK), BioSource Int; and anti-ERK1/2, Calbiochem. After incubation with secondary antibodies, signals were revealed with chemiluminescence, visualized by autoradiography, and quantified densitometrically.

**Immunoprecipitation and Immunoblot Analysis of Csk and EGFR**

VSMCs were lysed as described.\(^20,21\) Cell lysates were subjected to immunoprecipitation with antibodies to Csk and EGFR (2 μg). Immune complexes were recovered by the addition of protein A/G PLUS-Agarose (Santa Cruz Biotechnology) as described.\(^17,18\) Samples were centrifuged, and the beads were washed with lysis buffer, solubilized in sample buffer, and subjected to immunoblotting. Membranes were probed with anti-phosphotyrosine antibody PY20 (1:1000, Transduction Laboratory), and immunoreactive proteins were detected by chemiluminescence.

**Statistical Data**

Results are mean±SEM and compared by ANOVA or the Student t test. The Tukey-Kramer correction was used to compensate for multiple testing. A value of \(P<0.05\) was considered to be significant.

**Results**

**Ang II–Induced c-Src Phosphorylation Is Augmented in SHR**

c-Src phosphorylation was rapidly increased by Ang II in VSMCs from SHR (Figure 1). Maximal responses were obtained within 60 seconds and were sustained for up to 10 minutes after the addition of Ang II in SHR. Ang II increased c-Src phosphorylation 2-fold, and this was abrogated by irbesartan in WKY. In SHR, Ang II increased the phosphorylation of c-Src 4-fold and was also abrogated by irbesartan. PD123319 did not alter Ang II–elicited actions. PP2 attenuated the Ang II–induced phosphorylation of Src but did not completely inhibit the effects of Ang II (Figure 1). This may be related to the fact that PP2, a pyrazolo pyrimidine, inhibits Src activity by binding to the ATP-binding site within the tyrosine kinase domain of Src and may not directly influence phosphorylation. To confirm that Src activity was increased in SHR, the phosphorylation of 2 Src-specific substrates, cortactin and Pyk2 (calcium-dependent tyrosine kinase), was assessed. Protein levels of cortactin and Pyk2 were not different in WKY and SHR (data not shown). As demonstrated in Figure 2, top panel, the phosphorylation of cortactin, an 80/85-kDa cytoskeletal protein that facilitates the assembly of actin, was significantly increased by Ang II, with enhanced responses in cells from SHR compared with WKY. The phosphorylation of Pyk2 was also stimulated by Ang II, with greater effects in SHR cells compared with WKY cells (Figure 2, bottom panel). With the use of the anti-Pyk2 (pYPY570/580) phospho-specific antibody, Western blot analysis revealed 2 bands corresponding to Pyk2 and FAK (a Pyk2-related enzyme).

**Ang II Increases Csk Phosphorylation in WKY but Not in SHR**

Csk is a negative regulator of Src. Therefore, we examined whether altered Ang II–induced Src activation in SHR was
associated with changes in Csk activity. Csk immunoprecipitates from Ang II–stimulated cells were probed with an anti-phosphotyrosine antibody. Phosphorylation of Csk was little affected by Ang II in VSMCs from SHR but was potently and rapidly increased (3-fold) in cells from WKY (Figure 3).

**PP2 Decreases Ang II–Mediated ERK1/2 Activation in SHR**

ERK1/2 phosphorylation was augmented in cells from SHR compared with cells from WKY (Figure 4). Pretreatment of cells with PP2 decreased Ang II–induced ERK1/2 activation (Figure 4). PP2 effects were greater in VSMCs from SHR (change was 225±25% of control) than WKY (change was 38±8% of control). This suggests that Src modulates ERK1/2 and that Src dysregulation may underlie augmented ERK1/2 activity in SHR. ERK1/2 phosphorylation was not completely abolished by PP2, indicating that Src-independent pathways also regulate ERK1/2 activity.

**Role of EGFR Transactivation**

AG1478, a selective inhibitor of EGFR kinase, attenuated Ang II–induced phosphorylation of c-Src and ERK1/2 (Figure 5). These effects were greater in cells from SHR than in cells from WKY. Ang II–induced phosphorylation of EGFR was increased in VSMCs from SHR compared with those from WKY (Figure 6).

**Discussion**

Src plays an important role in the regulation of cell growth. However, the functional significance of this nonreceptor tyrosine kinase in VSMC growth signaling in hypertension remains unclear. Major findings from the present study demonstrate that (1) c-Src is rapidly and potently phosphorylated by Ang II; (2) Ang II actions are inhibited by the AT<sub>1</sub> receptor antagonist irbesartan but not by the Ang II type 2 receptor antagonist PD123319; (3) Ang II–stimulated phosphorylation of c-Src and phosphorylation of the Src substrates, cortactin and Pyk2/FAK, is augmented in SHR; (4) Csk phosphorylation is increased by Ang II in WKY but not in SHR; and (5) increased ERK1/2 phosphorylation by Ang II is mediated via Src-dependent pathways partly because of the transactivation of EGFR. Accordingly, our data
suggest that augmented Src signaling by AT<sub>1</sub>-mediated EGFR transactivation may be an important early event underlying abnormal VSMC ERK1/2 signal transduction in SHR. Blunted Csk activity may contribute to abnormal Src regulation in SHR.

Multiple members of the Src family protein tyrosine kinases have been identified, of which c-Src is the prototype. Ang II rapidly and robustly increased c-Src phosphorylation. These AT<sub>1</sub>-mediated c-Src effects were enhanced in VSMCs from SHR, suggesting an upregulation of Src-dependent signaling in genetically hypertensive rats. Ang II–induced phosphorylation of cortactin and Pyk2/FAK, downstream targets of Src, was also increased in SHR, further confirming the augmentation of Src activity in SHR. Mech-

Figure 3. Csk phosphorylation in response to Ang II. VSMCs were exposed to Ang II (10<sup>-7</sup> mol/L) for indicated times and harvested. Csk was immunoprecipitated from cell lysates with anti-Csk antibody. Immunoprecipitated proteins were subjected to SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody. IP indicates immunoprecipitation; IB, immunoblotting. Data points are mean±SEM of 3 experiments. *P<0.05 and **P<0.01 vs WKY counterpart.

Figure 4. Effects of PP2 on Ang II–induced ERK1/2 phosphorylation and expression in VSMCs from WKY and SHR. Top panels are representative Western blots. Line graph demonstrates time-dependent effects of Ang II (10<sup>-7</sup> mol/L) with or without PP2 (10<sup>-5</sup> mol/L). Results are mean±SEM of 4 to 6 experiments. **P<0.01 vs WKY counterpart; +P<0.05 and ++P<0.01 vs Ang II+PP2 counterpart.
\textbf{Figure 5.} Effects of EGFR kinase inhibitor \textit{AG1478 (AG)} on Ang II-induced phosphorylation of c-Src and ERK1/2 in VSMCs from WKY and SHR. Representative immunoblots demonstrate phosphorylated c-Src (top panels) and ERK1/2 (bottom panels) in control conditions (C) and after 3-minute and 5-minute stimulation with Ang II (10^{-7} \text{ mol/L}) in absence and presence of AG (10^{-5} \text{ mol/L}). Membranes were probed with phospho-specific antibodies. Data points are mean\pm SEM from 3 experiments. *P<0.05 and **P<0.01 vs Ang II counterpart.

Anisms contributing to increased c-Src activation in hypertension are unknown but are probably due to postreceptor phenomena and not to differences at the receptor level. This is supported by our previous findings that Ang II receptor density and AT\textsubscript{1} receptor mRNA and protein expression are not different in VSMCs from age-matched adult SHR and WKY.\textsuperscript{34,35} Furthermore, data from the present study demonstrate that Csk is phosphorylated by Ang II in WKY but not in SHR. Blunted activation of this kinase could lead to decreased inactivation of Src and consequent increased Src signaling, as observed in SHR in the present study. The link between AT\textsubscript{1} and Src is unclear, but interaction between G\textsubscript{\beta\gamma} subunits, their associated kinases, and kinase substrates (and possibly \(\beta\)-arrestin) may provide the signaling complex that binds c-Src.\textsuperscript{36,37}

We\textsuperscript{20,21} and others\textsuperscript{22–25} have previously demonstrated that Ang II dose-dependently increases vascular ERK1/2 phosphorylation and that responses are enhanced in hypertension. Altered MAP kinase phosphatase activity and increased [Ca\textsuperscript{2+}], have been implicated in these changes.\textsuperscript{24,25} In the present study, we demonstrate that in SHR, PP2 attenuates ERK1/2 activity, suggesting a role for Src in ERK1/2 hyper-activation in SHR. Because PP2 inhibits c-Src in addition to other Src members, we cannot exclude the possibility that many Src kinases are involved in ERK1/2 signaling by Ang II. However, because c-Src is the major isoform in the vasculature, the effects of PP2 are probably primarily through c-Src inhibition. PP2 did not completely inhibit ERK1/2 phosphorylation, indicating that Src-independent pathways also regulate VSMC ERK1/2 activity. Protein kinase C, particularly the \(\zeta\) subunit, and small molecular GTP-binding proteins, such as Rho, Rac, and Cdc42, have been identified as upstream modulators of ERK1/2\textsuperscript{36,39} and could potentially contribute to increased signaling in hypertension.
Increasing evidence suggests that Ang II signaling is mediated via transactivation of the EGFR, which serves as a scaffolding for preactivated c-Src and for downstream adapters in VSMCs. We investigated the possibility that EGFR activation by Ang II could contribute to the upregulation of Src-dependent growth signaling in VSMCs from SHR. The highly selective EGFR tyrosine kinase inhibitor AG1478 decreased Ang II–induced phosphorylation of c-Src and ERK1/2 in WKY and SHR. However, the effects were significantly greater in SHR, suggesting that Ang II transactivation of the EGFR contributes to vascular Src signaling in SHR. This was further supported by our findings that Ang II–mediated phosphorylation of EGFR is enhanced in VSMCs from SHR. AG1478 did not completely block Ang II–induced actions, indicating that EGFR-independent pathways also contribute to Src-mediated signaling in VSMCs. This may be due, in part, to Csk-dependent actions. However, from the present study, we cannot differentiate whether Csk is a downstream target of EGFR or whether it acts in parallel with EGFR activation. These aspects await further clarification.

In conclusion, Ang II–stimulated phosphorylation of c-Src is increased in VSMCs from SHR. This may be related to blunted Ang II–induced phosphorylation of Csk, which negatively regulates Src. Augmented Src-activated ERK1/2-dependent signaling is mediated, in part, via transactivation of the EGFR in SHR. Our data define a Src-dependent signal transduction cascade whereby Ang II regulates the signaling processes associated with VSMC growth and vascular remodeling in genetically hypertensive rats.

Acknowledgments
This study was supported by grants from the Canadian Institutes of Health Research (CIHR, grants 14080 and 13570 and a Group Grant to the Multidisciplinary Hypertension Group) and by a Medical School grant from Bristol-Myers Squibb/Sanoﬁ. Dr Touyz received a scholarship from the Canadian Hypertension Society/CIHR.

References


Increased Angiotensin II-Mediated Src Signaling via Epidermal Growth Factor Receptor Transactivation Is Associated With Decreased C-Terminal Src Kinase Activity in Vascular Smooth Muscle Cells From Spontaneously Hypertensive Rats
Rhian M. Touyz, Xiao-Hua Wu, Gang He, Steven Salomon and Ernesto L. Schiffrin

Hypertension. 2002;39:479-485
doi: 10.1161/01.HY.0000012883.00909.01

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/39/2/479

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/