Adrenomedullin inhibits angiotensin II-induced oxidative stress via Csk-mediated inhibition of Src activity

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Liu J, Shimosawa T, Matsui H, Meng F, Supowit SC, DiPette DJ, Ando K, Fujita T. Adrenomedullin inhibits angiotensin II-induced oxidative stress via Csk-mediated inhibition of Src activity. Am J Physiol Heart Circ Physiol 292: H1714–H1721, 2007. First published October 27, 2006; doi:10.1152/ajpheart.00486.2006.—We have demonstrated that adrenomedullin (AM) protects against angiotensin II (ANG II)-induced cardiovascular damage through the attenuation of increased oxidative stress observed in AM-deficient mice. However, the mechanism(s) that underlie this activity remain unclear. To address this question, we investigated the effect of AM on ANG II-stimulated reactive oxygen species (ROS) production in cultured rat aortic vascular smooth muscle cells (VSMCs). ANG II markedly increased ROS production through activation of NADPH oxidase. This effect was significantly attenuated by AM in a concentration-dependent manner. This effect was mimicked by dibutyl-cAMP and blocked by pretreatment with N-[2-(p-bromocinnamylamino)ethyl]-5-isouquinolinesulfonamide hydrochloride (H-89), a protein kinase A inhibitor, and CGRP8–37, an AM/CGRP receptor antagonist. This inhibitory effect of AM was also lost following the expression of a constitutively active Src. Moreover, AM intersected ANG II signaling by inducing COOH-terminal Src kinase (Csk) activation that, in turn, inhibits Src activation. These data, for the first time, demonstrate that AM attenuates the ANG II-induced increase in ROS in VSMCs via activation of Csk, thereby inhibiting Src activity.

reactive oxygen species; antioxidant; lucigenin; carboxy-terminal Src kinase

ENHANCED PRODUCTION of reactive oxygen species (ROS) and activation of redox-dependent signaling cascades by vasoactive agents are critical processes underlying a number of cardiovascular and metabolic diseases (37, 43). Virtually all types of vascular cells produce ROS, such as O2− and H2O2. The major source of oxygen intermediates in the vascular wall is nonphagocytic NADPH oxidase (6, 11, 20, 22, 34). The activation of redox-dependent signaling cascades by vasoactive agents are critical processes underlying a number of cardiovascular and metabolic diseases (37, 43). Virtually all types of vascular cells produce ROS, such as O2− and H2O2. The major source of oxygen intermediates in the vascular wall is nonphagocytic NADPH oxidase (6, 11, 20, 22, 34). Accumulating evidence indicates that NADPH oxidase-derived ROS are essential participants in signaling events in vascular cells (7, 28).

Angiotensin II (ANG II) is an important mediator of vascular smooth muscle cell (VSMC) function, and a number of the effects of ANG II are mediated through the generation of ROS (4, 9, 42). In particular, ANG II-induced hypertrophy is inhibited by overexpression of catalase, which hydrolyzes H2O2, or by inhibition of vascular NADPH oxidases (3, 8, 42).

Adrenomedullin (AM), a potent vasodilator peptide that acts primarily as a protective autocrine/paracrine factor in the cardiovascular system, has antiproliferative and antimigrative effects (32). We previously reported that AM gene transfer prevents the development of cuff-induced vascular injury (31). AM-deficient heterozygous knockout mice also exhibited significant damage in multiple organ systems that was mediated by enhanced oxidative stress in models of ANG II/salt-induced hypertension (30), aging (44), hypoxia (21), and cuff injury (15). In addition, AM expression was stimulated by ROS in vascular endothelial cells (24). These data are consistent with the hypothesis that AM is an endogenous antioxidant that plays a protective role against increased oxidative stress. However, the underlying cellular mechanism(s) and identification of the critical AM-regulated signal transduction pathways remained to be clarified.

Several signaling molecules, such as phospholipase D, protein kinase C, phospholipase A2, and phosphatidylinositol 3-kinase (PI3-kinase) are implicated in ANG II-evoked NADPH oxidase activation (10, 38–40). Whether these pathways function in parallel or in series to stimulate this enzyme is unclear. However, multiple lines of evidence strongly indicate that Src, which influences all of these signaling molecules, is a common upstream mediator (29). Src kinases are a family of nonreceptor tyrosine kinases, of which the prototype c-Src, is the major isoform in the vasculature (23, 35). Src can be switched from an inactive to an activated state through control of phosphorylation at two major sites, Tyr416 and Tyr527. Phosphorylation of Tyr416 activates Src, whereas phosphorylation of Tyr527 inactivates this tyrosine kinase. COOH-terminal Src kinase (Csk) is a key enzyme that phosphorylates Src at Tyr527 (19). In VSMCs, c-Src is rapidly activated by ANG II and plays a key role in growth signaling (12). Furthermore, it has been shown that PKA intersects Src signaling in mammalian cells (1). Since cAMP-PKA is the primary postreceptor signal transduction pathway of AM and PKA upregulates Csk activity through phosphorylation, c-Src inactivation via Csk is a likely candidate through which AM intersects ANG II-stimulated ROS signaling.

Therefore, in the present study, we examined whether AM directly inhibits intracellular ROS generation stimulated by ANG II in VSMCs and assessed the role(s) that Src and Csk play in the regulation of the cellular signaling pathway(s) responsible for its antioxidant effect.

MATERIALS AND METHODS

Materials. Rat AM and human CGRP8–37 were purchased from the Peptide Institute (Osaka, Japan). Fetal bovine serum (FBS) was from...
Trace Scientific (Melbourne, Australia). Anti-Src and anti-phosphotyrosine MAb (4G10) antibody, a dominant-negative mutant of Src (DN-Src), and Csk-small-interfering (si)RNA kit came from Upstate Biotechnology (Lake Placid, NY). Lipofectin was from Invitrogen (Palo Alto, CA), and Mirus reagent was from Mirusbio (Madison, WI). Anti-Src phospho-specific antibody Tyr416 was from Biosource International (Camarillo, CA), and anti-Src phospho-specific antibody Tyr527 was purchased from Cell Signaling Technology (Beverly, MA). Anti-Csk antibody and protein A/G PLUS-Agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Dulbecco’s modified Eagle’s medium (DMEM), ANG II, rotenone, allopurinol, N-[2-(p-bromocinnamylamino)ethyl]-5-isouquinolinesulfonamide hydrochloride (H-89) and dibutyl-cAMP, TNF-α, and calcium ionophore A-23187 were from Sigma (St. Louis, MO).

Cell culture. All animal studies were approved by the Institutional Animal Care and Use Committee. Rat aortic VSMCs were prepared from the thoracic aorta of 8-wk-old male Sprague-Dawley rats using the explant method (33) and cultured in DMEM containing 10% FBS at 37°C in a humidified atmosphere of 95% air-5% CO2. Subcultured cells (passages 3–12) were deprived of serum for 48 h before experimental use.

Transient transfection. Either a dominant-negative mutant of Src or a constitutive-active mutant of Src was overexpressed in VSMCs by transient transfection. All Src constructs were in the pUSEamp (+) expression vector. Transient transfection was performed with 2 μg of total DNA per 35-mm dish and Lipofectin according to the manufacturer’s instructions (DNA:LipofectAMINE, 1:3 ratio). The transfection efficiency was confirmed by immunoblotting for total protein. Pilot studies have documented a transfection efficiency of 15–25% in VSMCs using this approach. Cells transfected with an expression vector containing the same promoter, but without the subcloned transgene cassette (empty expression vector), served as a control. All Csk siRNAs were synthesized and annealed at Dharmaco. VSMCs were plated 2 days before transfections and grown to 60–80% confluence in 12-well plates. SMARTpool Csk or control siRNA (200 nM) was transfected into VSMCs with Mirus reagent (Mirusbio) according to the manufacturer’s protocol.

Measurement of intracellular ROS levels. We measured ROS both in living cells and in total VSMC homogenates. ROS production in living cells was measured by the lucigenin method (11) with minor modifications. Briefly, cells were pretreated with or without test compounds for the indicated time periods and then stimulated with or without ANG II (100 nmol/l) for 10 min and AM for 5 min. In some experiments, CGRP8–37 or H-89 was added 30 min before, dibutyl-cAMP 1 h before, TNF-α 2 h before, and the calcium ionophore A-23187 3 h before AM treatment. The cells were then trypsinized and collected by centrifugation, and the pellet was washed in modified Krebs buffer with 1 mg/ml BSA, and the cell concentration was adjusted to 1 × 10^6 in 900 μl buffer. To measure ROS production, the cell suspension was placed in a luminometer (LB 9507; Berthold, Wildbad, Germany). Measurements were started by an injection of 100 μl lucigenin (final concentration, 5 × 10^−4 M). Photon emission was counted every 1 min for up to 10 min. Modified Krebs buffer was used as control (blank). For total cell homogenate, cells were treated the same as in living cells and were scraped into ice-cold Hanks’ balanced salt solution supplemented with MgCl2 (0.8 mmol/l) and CaCl2 (1.8 mmol/l). Cells were disrupted by rapid freezing in liquid nitrogen followed by sonication. O2 production was measured in the presence of NADPH (100 μmol/l) for 10 min and was expressed in mean arbitrary light units per min.

Western blot analysis for phosphorylated Src. Western blot analysis was performed as described previously (17). Briefly, 30 μg of the cell lysates were subject to 10% SDS-PAGE, and proteins were transferred to nitrocellulose membranes (Genetics, Tokyo, Japan). The membranes were blocked for 1 h at room temperature with 5% skimmed milk. The blots were then incubated overnight at 4°C with anti-Src and anti-Src phosphospecific antibodies, followed by incubation for 1 h with a secondary peroxide-conjugated antibody (Amersham Biosciences UK), and the proteins were detected by enhanced chemiluminescence (ECL) Western blotting detection kit.
In figures showing Western blots, the blots shown are representative of at least three independent experiments.

Immunoprecipitation and immunoblot analysis of Csk. Cells were lysed and centrifuged as described elsewhere (36). The supernatant was mixed with the antibody to Csk (2 µg) and rocked at 4°C for 2–16 h, and protein A/G-Agarose was then added for an additional 2 h to overnight. Immunoprecipitates were washed, fractioned by electrophoresis, and transferred to nitrocellulose membranes (Genetics). After being blocked with 5% milk, the membrane was treated with an anti-phosphotyrosine (4G10) antibody conjugated with horseradish peroxidase (Upstate Biotechnology). Immunoreactive proteins were detected by ECL (Amersham Pharmacia Biotech).

Statistical analysis. Data are expressed as means ± SE. Differences between groups were examined for statistical significance using either ANOVA followed by the Dunnett’s test or unpaired t-test, where they were appropriate. P values < 0.05 were considered statistically significant.

RESULTS

AM attenuates intracellular ROS production stimulated by ANG II. A 10-min treatment of VSMCs with ANG II evoked a significant increase (~3- to 7-fold from the lowest to the highest concentration of ANG II) in ROS production (Fig. 1A), which was attenuated by AM in a concentration-dependent manner (10⁻⁸–10⁻⁶ mol/l, Fig. 1B). The highest concentration of AM (10⁻⁶ mol/l) inhibited ROS generation to almost the same degree as the NADPH-specific oxidase inhibitor apocynin (Fig. 1B), and neither inhibition of mitochondrial electron transport by rotenone (10 µmol/l) nor inhibition of xanthine oxidase by allopurinol (10 µmol/l) significantly altered ANG II-induced ROS production (Fig. 1C). Likewise, AM treatment had no effect on the Ca²⁺ ionophore A-23187 or TNF-α-induced ROS production (Fig. 1D).

AM inhibits intracellular ROS generation via an AM receptor-mediated and cAMP-PKA-dependent pathway. To determine whether the antioxidant effect of AM is mediated via the AM receptor/cAMP-PKA-dependent pathway, we tested the effects of an AM receptor antagonist and cAMP-related compounds both in living cells (Fig. 2A) and cell homogenates (Fig. 2B). Pretreatment with 3 × 10⁻⁶ mol/l CGRP8–37, an AM/CGRP receptor antagonist (2), completely abolished the AM-induced inhibition of intracellular ROS generation stimulated by ANG II. Since the cAMP-PKA pathway is the most well-documented post-receptor signal transduction pathway of AM (5, 46), we examined the involvement of this pathway on the inhibitory mechanism of AM on ROS generation. Dibutyryl-cAMP (10⁻³ mol/l) significantly inhibited ROS generation by ANG II to a similar extent as AM (10⁻⁷ mol/l). In contrast, the inhibitory effect of AM on ROS generation was completely abolished by H-89 (10⁻⁵ mol/l), a PKA inhibitor. Treatment with AM,
CGRP₈₋₃₇, dibutyl-cAMP, or H-89 alone did not alter intracellular ROS levels in intact cells (Fig. 2A). Similar results were obtained with cell homogenates (Fig. 2B).

AM inhibits phosphorylation of Src (Tyr416). It has been reported that c-Src regulates ANG II-mediated NADPH oxidase-derived O₂⁻ in rat VSMCs (29). Based on these data, experiments were performed to determine whether AM inhibits ANG II-induced ROS production specifically through inhibition of Src (Tyr416) phosphorylation. As shown in Fig. 3A, phosphorylation of Src (Tyr416) was significantly increased in VSMCs by ANG II (10⁻⁷ mol/l) stimulation, which was, in turn, inhibited by AM in a concentration-dependent manner (10⁻⁸–10⁻⁶ mol/l). Figure 3B shows the effects of dibutyl-cAMP (10⁻³ mol/l), CGRP₈₋₃₇, and H-89 (10⁻⁵ mol/l) treatment on phosphorylation. Dibutyl-cAMP significantly (P < 0.05) inhibited the phosphorylation of Src (Tyr416) by ANG II, which was comparable with that of AM (10⁻⁷ mol/l). As expected, H-89 and CGRP₈₋₃₇ completely abolished the inhibitory effect of AM on Src (Tyr416) phosphorylation. In the absence of ANG II stimulation, treatment with AM, CGRP₈₋₃₇, dibutyl-cAMP, or H-89 alone did not significantly change the state of Src phosphorylation.

Effects of DN-Src and CA-Src on the antioxidant activity of AM. To confirm that the attenuation of Src activity is directly attributable to the inhibitory effect of AM on ANG II-induced ROS generation, we undertook experiments to transfect VSMCs with DN- or CA-Src constructs. The transfection efficiency was confirmed by immunoblotting for total Src protein. Figure 4A shows that both of the mutant Src constructs were effectively overexpressed in VSMCs. Using the live transfected cells, we then assessed the effect of AM on ANG II-induced ROS production by the lucigenin chemiluminescence method. As seen in Fig. 4B, the DN-Src-transfected cells that serve as the negative controls for this set of experiments display basal levels of ROS generation that were unchanged following treatment with ANG II alone or ANG II and AM. In contrast, in CA-Src overexpressing cells, ROS generation was significantly enhanced and was not increased further by ANG II treatment (Fig. 4C). Consistent with these results, AM

Fig. 3. AM inhibits Src (Tyr416) phosphorylation stimulated by ANG II via a receptor-mediated and cAMP-PKA-dependent pathway in VSMCs. A: concentration-dependent response of Src (Tyr416) phosphorylation by ANG II. VSMCs were pre-treated with various concentrations of AM (10⁻⁸–10⁻⁶ mol/l) for 5 min and then stimulated with ANG II (10⁻⁷ mol/l) for 10 min. Top: representative immunoblot demonstrating phosphorylation of Src (Tyr416). Bottom: represents averaged data from 3 independent experiments quantified by densitometry of immunoblots and expressed as fold increases in phosphorylation compared with unstimulated cells. B: effect of dibutyl-cAMP, CGRP₈₋₃₇, and H-89 on phosphorylation of Src (Tyr416). VSMCs treated with or without AM (10⁻⁷ mol/l), dibutyl-cAMP (10⁻³ mol/l), H-89 (10⁻⁵ mol/l), or CGRP₈₋₃₇ (3 × 10⁻⁵ mol/l) for 0.5–1 h were stimulated with or without ANG II (10⁻⁷ mol/l) for 10 min. In some experiments, CGRP₈₋₃₇ or H-89 was applied 30 min before the AM pretreatment. Phosphorylation of Src (Tyr416) was evaluated by Western blot analysis. Top: a representative immunoblot demonstrating phosphorylation of Src (Tyr416). Bottom: represents averaged data from 3 independent experiments quantified by densitometry of immunoblots and expressed as fold increases compared with unstimulated cells. p-Src, phospho-Src. *P < 0.05 vs. CTR; †P < 0.05 vs. ANG II.
treatment failed to inhibit the elevated ROS generation (Fig. 4C).

**AM- and ANG II-enhanced Csk phosphorylation increases Src (Tyr527) phosphorylation, and the inhibitory effect of AM on Src activation is lost in Csk knockdown VSMCs.** Inactivation of Src kinase involves phosphorylation of Tyr527 by Csk (19). Therefore, we examined whether AM inhibition of ANG II-induced Src activation was associated with changes in Csk activity. Csk immunoprecipitates were probed with an anti-phosphotyrosine antibody. Phosphorylation of Csk was significantly increased approximately twofold by ANG II but not by treatment with AM alone. A much more robust increase (approximately fourfold) was observed when the VSMCs were treated with the combinations of ANG II and either AM (10^{-7} mol/l) or dibutyl-cAMP (10^{-3} mol/l) (Fig. 5A). In contrast, treatment of the cells with ANG II did not increase the phosphorylation of Src (Tyr527) as shown in Fig. 5C. Treatment with AM produced an increase Src (Tyr527) phosphorylation, but it did not achieve statistical significance (data not shown). However, the combination of ANG II and AM evoked a significant increase in Src (Tyr527) phosphorylation, again in the absence of any changes in total Src protein (Fig. 5C). In addition, Csk knockdown studies using siRNA also reversed the inhibitory effects of AM on Src activation (Fig. 5C).

**DISCUSSION**

Accumulating evidence strongly supports the hypothesis that AM possesses significant protective properties against end-organ damage, produced by a number of pathophysiological conditions, through inhibition of oxidative stress (15, 21, 30, 44). The mechanisms underlying this activity vary depending on cell types and experimental conditions. In mesangial cells, AM suppresses ROS production via the cAMP-PKA pathway (5), whereas, in the rat ventricle, increased oxidative stress caused by ischemia-reperfusion injury can be attenuated by AM-mediated inhibition of NADPH oxidase via the nitric oxide-cGMP signaling pathway (14). In contrast, enhanced hypoxia-induced generation of ROS in human alveolar epithelial cells is attenuated by a marked AM-stimulated increase in the potent ROS scavenger glutathione (16).

In light of the aforementioned reports, the significant findings of this study are as follows. First, in using rat VSMCs in conjunction with a lucigenin chemiluminescence methodology, we have confirmed that NADPH oxidase is the source of the marked increase in ROS following treatment with ANG II and that the inhibitory effects of AM in this setting are mediated through the cAMP/PKA signal transduction pathway (46). Second, the data presented herein clearly support the hypothesis that, in VSMCs, Src is the primary upstream signaling molecule that mediates the ANG II increase in NADPH-generated ROS (29). Third, we have shown for the first time that the attenuation of Src activity in ANG II-treated VSMCs is regulated by the phosphorylation and subsequent activation of Csk, which is dependent on AM via the cAMP-PKA signaling pathway, and may also require ANG II, which could be playing an autoregulatory role by initiating a potential negative feedback loop.

Before we initiated the series of experiments on the roles that Src and Csk play in AM-mediated attenuation of ANG II-evoked ROS production, it was necessary to demonstrate that ANG II treatment produced, in a concentration-dependent manner, a rapid and robust enhancement in VSMC ROS production that was significantly inhibited to near-basal levels by AM, again in a concentration-dependent fashion. The results presented herein are consistent with those reported by Yoshimoto et al. in both rat aortic VSMCs (46) and endothelial cells (47). Intracellular ROS generation stimulated by ANG II in these cell types has been shown to be mainly derived from the activation of NADPH oxidase (11, 22). This is clearly supported by our findings that inhibitors of xanthine oxidase

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Fig. 4. Effects of AM on ANG II-induced ROS production in dominant-negative (DN)- or constitutively active (CA)-Src-transfected cells. A: transfection efficiency for these experiments was confirmed by immunoblotting for total Src protein. ROS production was examined by lucigenin-chemiluminescence method, both in DN-Src- (B) and CA-Src-transfected living cells (C). CTRs are DN-Src- and CA-Src-transfected cells in the absence of stimulation. Values are representative of 3 independent experiments, each performed in triplicate.
(allopurinol) and mitochondrial-derived ROS (rotenone) have no significant effect on ANG II-stimulated ROS production. In contrast, treatment of the VSMCs with apocynin, a specific NADPH oxidase inhibitor, reduced ANG II-stimulated ROS generation to levels observed in the untreated control cells, which were similar to those observed following AM treatment. However, apocynin was somewhat more potent than AM for reasons that remain to be clarified. Furthermore, AM was without effect on Ca²⁺ ionophore A-23187 or TNF-α-induced ROS production, indicating that its ability to attenuate ANG II-stimulated ROS production in cultured VSMCs is regulated by specific signaling pathway(s) that inhibit, primarily, the activity of NADPH oxidase rather than acting as a general antioxidant.

Although the cAMP-PKA pathway is the primary signal transduction pathway stimulated by AM, this peptide can also trigger other signaling cascades, including NF-κB (27), tyrosine kinase (13), protein phosphatase 2A (26), and PI3K/Akt...
(25, 45), depending on the cell type or experimental conditions. To confirm that the antioxidant effects of AM observed in this study were in fact mediated primarily by the AM receptor-mediated upregulation of the cAMP-PKA signal transduction pathway, we demonstrated that the inhibitory effect of AM on ANG II-stimulated ROS generation was completely abolished by pretreatment of the VSMCs with CGRF8–37, an AM receptor antagonist. As expected, dibutyl-cAMP significantly inhibited the ANG II-evoked increase in ROS generation to a similar extent as AM. Likewise, the AM-mediated inhibition of ANG II-evoked ROS production was abolished by H-89, a PKA inhibitor. These data, therefore, provide additional evidence that AM inhibits ANG II-stimulated NADPH oxidase-dependent ROS production through the AM receptor-mediated activation of the cAMP-PKA pathway.

A central role for Src in the regulation of the redox status of ANG II-treated VSCMs was indicated by the observation that phosphorylation of Src (Tyr416), a key step in the activation of this protein kinase, was markedly increased following 10 min of treatment with ANG II. The link between the ANG II type 1 receptor and Src, however, is not clear. It has been reported that in VSCMs, ANG II signaling is regulated, in part, by the transactivation of the epidermal growth factor receptor, which serves as a scaffolding for preactivated c-Src and downstream adaptors. Indeed, interactions between Gβγ subunits, their associated kinases, and kinase substrates may form the signaling complex that binds c-Src (18, 48). This phosphorylation of Src was significantly attenuated in a concentration-dependent manner by AM in the absence of any change in total Src protein. Moreover, this attenuation of Src phosphorylation by AM was consistent with the concentration-dependent inhibitory effect of AM on ANG II-enhanced ROS generation. Likewise, the degree of Src Tyr416 phosphorylation, after normalization to total Src protein, following treatment with agents that activated or blocked the AM receptor/cAMP-PKA pathway, was in line with the ability of these agents to inhibit ANG II-evoked ROS production. Indeed, ANG II was unable to stimulate ROS production above baseline levels in DN-Src-transfected VSCMs, and the inhibitory effect of AM on ANG II-induced ROS generation was lost in CA-Src transfected cells, indicating that c-Src is the target molecule through which AM inhibits ROS generation induced by ANG II in VSCMs.

As described previously, c-Src is activated by autophosphorylation of Tyr416 and inactivated by Csk that phosphorylates Tyr527 (1, 19). Indeed, we observed that the knockdown of Csk by siRNA blocked the phosphorylation of Src527, thereby indicating a direct relation between Csk and Src inactivation. It was, however, surprising that treatment of the cells with ANG II under the same conditions that enhance ROS generation produced a significant increase in Csk phosphorylation, one mechanism by which this tyrosine kinase is activated, whereas AM alone did not. However, addition of ANG II in combination with either AM or dibutyl-cAMP resulted in a marked increase in Csk phosphorylation without a change in total Csk protein. In contrast, when Src Tyr527 phosphorylation was assessed, neither ANG II nor AM alone was able to significantly stimulate phosphorylation at this site, whereas the combination of the two did result in a statistically significant upregulation of Try527 phosphorylation.

Although we do not have any direct evidence to support this hypothesis, it is tempting to speculate that to maintain a balance between active and inactive Src, which clearly participate in the modulation of the redox status of this cell type, ANG II treatment simultaneously activates Src and also sets the stage for the downregulation of this protein by phosphorylation of Csk. This change in the activation status of Csk is such that it cannot deactivate Src via Tyr527 phosphorylation, except in the presence of a negative regulatory agent like AM that is able to trigger Csk up to a level of activity that is sufficient to downregulate Src, thereby reestablishing homeostasis of the redox state of the cell. Indeed, a report from Touyz et al. (41) provides indirect evidence for this argument. In this study they demonstrated that ANG II-stimulated VSMC growth and remodeling was mediated by the activation of c-Src and that this effect was significantly greater in VSMCs from spontaneously hypertensive rats (SHR) compared with those from normotensive Wistar-Kyoto (WKY) rats. ANG II-mediated c-Src phosphorylation (Tyr527) was approximately fourfold greater in SHR than WKY rats. However, ANG II increased Csk phosphorylation two- to threefold in WKY but not in SHR. From these data it was concluded that c-Src phosphorylation and Src-dependent cell growth following ANG II treatment are increased in VSMCs from SHR and that these processes are associated with blunted ANG II-induced phosphorylation of Csk. Since the VSMCs used in our study came from normal rats, our tentative conclusion regarding the mechanism underlying the Csk-mediated inhibition of Src activity and ROS production is consistent with the data reported by Touyz et al. (41). We are, however, well aware that multiple mechanisms for Src and Csk regulation exist, and it is still unclear as to the exact contribution of the different regulatory mechanisms in a given physiological setting (36).


