c-Src–Dependent Nongenomic Signaling Responses to Aldosterone Are Increased in Vascular Myocytes From Spontaneously Hypertensive Rats

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Abstract—Aldosterone plays an important role in the pathogenesis of hypertension. We previously demonstrated that nongenomic signaling by aldosterone in vascular smooth muscle cells occurs through c-Src–dependent pathways. Here we tested the hypothesis that upregulation of c-Src by aldosterone plays a role in increased mitogen-activated protein (MAP) kinase activation, [3H]proline incorporation, and NADPH-driven generation of reactive oxygen species, thereby inducing cell growth, collagen production, and inflammation, respectively, in vascular smooth muscle cells from spontaneously hypertensive rats. The time course of c-Src phosphorylation by aldosterone was shifted to the left in vascular myocytes from hypertensive animals. Aldosterone rapidly increased phosphorylation of p38 MAP kinase and extracellular signal–regulated kinase with significantly greater effects in cells from spontaneously hypertensive rats versus control cells (P<0.05). Aldosterone increased NADPH oxidase activity with significantly greater responses in vascular smooth muscle cells from hypertensive animals (P<0.05). These events were associated with enhanced [3H]proline incorporation (index of collagen synthesis) in cells from spontaneously hypertensive rats (P<0.05). The NADPH oxidase activity increase, collagen synthesis, c-Src, and MAP kinase phosphorylation induced by aldosterone were significantly reduced by eplerenone (selective mineralocorticoid receptor blocker) and PP2 (selective c-Src inhibitor). In conclusion, nongenomic signaling by exogenous aldosterone, mediated through c-Src, is increased in vascular smooth muscle cells from spontaneously hypertensive rats. Upregulation of c-Src signaling may be important in the profibrotic and proinflammatory actions of aldosterone in this genetic model of hypertension. (Hypertension. 2005;46[part 2]:1032-1038.)

Key Words: aldosterone ■ signal transduction ■ rats, inbred SHR ■ oxidative stress ■ collagen

The potential role of aldosterone in hypertension and cardiovascular disease has been highlighted. Several studies have shown vascular and target-organ protective effects of aldosterone receptor antagonism.1–4 In aldosterone-infused rats, spironolactone or the more specific antagonist eplerenone prevents inflammation and fibrosis in the heart, blood vessels, and kidney; improves endothelial function; and reduces activation of NAD(P)H oxidase.5–9 In animal models of hypertension, such as stroke-prone spontaneously hypertensive rats, aldosterone receptor antagonism also reduced vascular remodeling and renal damage, particularly in the presence of high salt.10–13 In addition, actions that are usually attributed to the direct effects of angiotensin II, such as vascular remodeling, endothelial dysfunction, increased oxidative stress, and inflammation of the vascular wall and heart, may be mediated, at least in part, by aldosterone.14–16 These studies are consistent with a major role for endogenous mineralocorticoids as mediators of cardiovascular injury.

Aldosterone effects extend beyond the classic genomic action of mineralocorticoid receptors that involve transcription and protein synthesis. Aldosterone induces rapid cellular responses by modulating intracellular calcium (Ca2+) and cAMP levels, Na+/H+ exchanger activity, and phosphorylation of signaling molecules, including protein kinase C, epidermal growth factor receptor, and mitogen-activated protein kinases (MAPks), including c-Jun NH2-terminal kinase, and extracellular signal–regulated kinases (ERks) 1/2.17–28 We recently demonstrated that aldosterone rapidly increases activation of p38 MAPK and NAD(P)H oxidase through c-Src–dependent pathways in vascular smooth muscle cells (VSMCs). In addition, the profibrotic action of aldosterone was dependent on c-Src–regulated p38 MAPK.29

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Activation of these pathways is known to be critically involved in VSMC processes associated with vascular damage in hypertension.\textsuperscript{30–35}

Considering that aldosterone nongenomic signaling through c-Src–dependent pathways may play a role in pathologic vascular processes in hypertension, in the present study we questioned whether upregulation of c-Src by aldosterone leads to increased MAPK phosphorylation in VSMCs from spontaneously hypertensive rats (SHR) and whether this influences collagen synthesis and oxidative stress. Our findings demonstrate that augmented c-Src activation by aldosterone in SHR VSMCs enhances MAPK phosphorylation, NAD(P)H oxidase activation, and \textsuperscript{[3H]}proline incorporation, a marker of collagen synthesis. Taken together, these data suggest that augmented nongenomic signaling by aldosterone through c-Src–dependent pathways plays an important role in profibrotic and proinflammatory actions of aldosterone in essential hypertension.

\textbf{Methods}

\textbf{Cell Culture}

The study was approved by the Animal Ethics Committee of the Clinical Research Institute of Montreal and performed according to recommendations of the Canadian Council for Animal Care. VSMCs from adult male Wistar-Kyoto rats (WKY) and SHR were studied. Rats (16 weeks old) were euthanized by decapitation. VSMCs derived from mesenteric arteries were isolated and characterized as described in detail previously.\textsuperscript{29} In brief, arteries were cleaned of adipose and connective tissue, and VSMCs were dissociated by digestion of vascular arcades with enzymatic solution (collagenase, elastase, soybean trypsin inhibitor, and bovine serum albumin type I; 60 minutes, 37°C). The tissue was filtered and the cell suspension centrifuged and resuspended in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 2 mmol/L glutamine, 20 mmol/L HEPES (pH 7.4), and antibiotics. At subconfluence, the cell culture was replaced with serum-free medium for 24 hours to render the cells quiescent. Low-passage cells (passages 4 to 7), from at least 4 different primary cell cultures, were used in the experiments. The concentration of pharmacological agents that we used was based on previous studies showing that the signaling pathways were completely abolished.\textsuperscript{25,35,36}

\textbf{Western Blotting}

The concentration of aldosterone that induced maximal c-Src phosphorylation (0.1 mmol/L) was used in this study. VSMCs from WKY and SHR were stimulated with aldosterone (1 to 60 minutes), and in some experiments, cells were preexposed (30 minutes) to 10 mmol/L eplerenone (selective aldosterone receptors antagonist) or 10 mmol/L PP2 (selective Src inhibitor). Proteins (30 µg) extracted from VSMCs were separated by electrophoresis on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane, as previously described.\textsuperscript{29} 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 phosphospecific antibodies (1:1000) overnight at 4°C. Antibodies were as follows: anti–c-Src (Tyr\textsuperscript{416}) ( Biosource International), anti–c-Src (Tyr\textsuperscript{416}), and anti–ERK1/2 (Thr\textsuperscript{202}/Tyr\textsuperscript{204}) (the latter 2 from Cell Signaling Technology, Inc.). Immunoblots for nonphosphoproteins (c-Src, p38 MAPK, and ERK1/2) were carried out in the same membranes used to evaluate their phosphorylated forms. After incubation with secondary antibodies, signals were revealed with chemiluminescence, visualized by autoradiography, and quantified densitometrically. Results were normalized by the total protein and expressed as percentage of vehicle used in the experimental protocols.

\textbf{Determination of Proline Incorporation}

\textsuperscript{[3H]}proline incorporation was used as a marker of collagen synthesis and was measured according to the protocol of Dubey et al.\textsuperscript{97} Quiescent cells were stimulated for 24 hours with aldosterone (0.01 mmol/L to 0.1 µmol/L). In some experiments, cells were preexposed to eplerenone, PP2, or SB212190 (a selective p38 MAPK inhibitor; 10 µmol/L) and stimulated with 0.1 µmol/L aldosterone.

\textbf{Measurement of NAD(P)H Oxidase Activity}

NAD(P)H oxidase activity was measured as previously described.\textsuperscript{29} VSMCs were stimulated with 0.1 mmol/L aldosterone (1 to 60 minutes). In some experiments, cells were preexposed for 30 minutes to eplerenone or PP2 (10 µmol/L) and then stimulated with aldosterone for 60 minutes, when activity of the oxidase was maximal. The lucigenin-derived chemiluminescence assay was used to determine NAD(P)H oxidase activity in cell homogenates. Activity was expressed as arbitrary units per milligram protein.

\textbf{Data Analysis}

Aldosterone-stimulated effects were expressed as the percent increase over control, with the control normalized to 100%. Results are presented as mean±SEM and were compared by ANOVA or by Student t test when appropriate. Probability values <0.05 were considered significant.

\textbf{Results}

\textbf{Aldosterone Effects on c-Src, p38 MAPK, and ERK1/2 Phosphorylation in VSMCs From WKY and SHR}

As shown in Figure 1, aldosterone time-dependently increased c-Src phosphorylation in VSMCs from WKY and SHR. The time-course curve of c-Src activation by aldosterone was shifted to the left in VSMCs from SHR. Aldosterone receptor antagonism with eplerenone, as well as inhibition of c-Src with PP2, reduced aldosterone-induced c-Src activation in both WKY and SHR cells. Aldosterone induced a biphasic increase in p38 MAPK activation, with a peak response obtained within 1 minute, followed by a second response within 30 minutes (Figure 2). Significantly greater effects at 1 minute were observed in SHR versus WKY cells (P<0.05). The phosphorylation of ERK1/2 induced by aldosterone at 15 minutes was also increased in SHR cells compared with WKY (Figure 3). PP2 pretreatment inhibited both aldosterone-induced p38 MAPK (Figure 2) and ERK1/2 activation (Figure 3). Total protein expression of c-Src, ERK1/2, and p38 MAPK was unaltered by aldosterone stimulation.

\textbf{Aldosterone Effects on NAD(P)H Oxidase Activity}

Aldosterone induced a time-dependent increase in NAD(P)H oxidase activity with maximal effect at 60 minutes in VSMCs from both WKY and SHR (Figure 4A). Significantly greater responses were observed in VSMCs from SHR versus WKY (P<0.05). Figure 4B shows that pretreatment with eplerenone or PP2 significantly reduced aldosterone-mediated activation of NAD(P)H oxidase in VSMCs from WKY and SHR. Neither PP2 nor eplerenone influenced the basal state of NAD(P)H oxidase activity.

\textbf{Aldosterone Effects on \textsuperscript{[3H]}Proline Incorporation}

Aldosterone induced a concentration-dependent increase in \textsuperscript{[3H]}proline incorporation, a marker of collagen synthesis, in VSMCs from WKY and SHR (Figure 5A). This effect was
significantly increased in cells from SHR. Eplerenone did not change the basal levels of [3H]proline incorporation and inhibited aldosterone effects in VSMCs from both groups (Figure 5B). PP2 and SB212190 decreased basal levels of [3H]proline incorporation. In the presence of these inhibitors, aldosterone-induced effects were blocked.

Discussion

c-Src is an important signaling molecule in aldosterone-induced acute effects in VSMCs. However, the functional significance of this nonreceptor tyrosine kinase in aldosterone signaling in hypertension remains unclear. Major findings from the present study demonstrate that (1) aldosterone-induced activation of c-Src, ERK1/2, and p38 MAPK is increased in SHR cells; (2) c-Src inhibition abolishes aldosterone effects on p38 MAPK and ERK1/2 activation; (3) augmented aldosterone-stimulated [3H]proline incorporation in SHR VSMCs is mediated through p38 MAPK in a c-Src–dependent manner; and (4) NAD(P)H-driven generation of superoxide anion by aldosterone involves c-Src and is enhanced in SHR. These data suggest that upregulated c-Src signaling by aldosterone plays an important role in vascular cellular changes in SHR.

Increasing evidence suggests that aldosterone influences structural and functional changes in the vasculature.9,11,18,29 Besides its well-known genomic actions, aldosterone induces rapid cellular responses by activating signaling pathways independently of genomic effects.17–29 The protein tyrosine kinase c-Src is abundant in the vasculature and appears to be an important signaling molecule in VSMCs. c-Src induces activation of MAPKs (p38MAPK, c-Jun NH2-terminal kinase, and ERK1/2), which are associated with cell growth, apoptosis, and collagen deposition.30 In addition, c-Src is a critical proximal regulator of NAD(P)H oxidase–driven superoxide anion generation.30 We previously reported that aldosterone increases c-Src phosphorylation in VSMCs.29 In the present study, we questioned whether c-Src regulation by aldosterone is altered in VSMCs from SHR and whether this is associated with changes in VSMC signaling. Our findings demonstrate that aldosterone stimulation rapidly increases phosphorylation of c-Src with enhanced effects in cells from SHR. Aldosterone receptor antagonism with eplerenone, as well as inhibition of c-Src with PP2, reduced

Figure 1. Effects of PP2 and eplerenone on aldosterone-induced c-Src phosphorylation in VSMCs from WKY and SHR. Top, Representative immunoblots. Bottom, Corresponding line graphs demonstrate time course of aldosterone (0.1 μmol/L) on c-Src phosphorylation in the absence and presence of PP2 (A, 10 μmol/L) or eplerenone (B, 10 μmol/L). Results are mean±SEM of 5 or 6 experiments. *P<0.05, SHR vs WKY; **P<0.05, PP2 or eplerenone vs aldosterone alone. Abbreviations are as defined in text.
aldosterone-induced c-Src activation. The time course of c-Src phosphorylation by aldosterone was shifted to the left in VSMCs from SHR, suggesting increased Src-dependent signaling in genetically hypertensive rats.

Because MAPKs are downstream targets of c-Src, we assessed whether aldosterone-mediated c-Src–dependent MAPK activation, specifically ERK1/2 and p38 MAPK, are also augmented in SHR VSMCs. Aldosterone induced a biphasic increase in ERK1/2 phosphorylation, with augmented activation in SHR VSMCs. PP2 inhibited the aldosterone-induced ERK1/2 effects, indicating that c-Src is an important upstream regulator of this MAPK. Because ERK1/2 activation in SHR is associated with exaggerated growth of VSMCs, we suggest that aldosterone contributes to vascular hypertrophy in SHR through c-Src–dependent, ERK1/2-mediated pathways.

Activation of p38 MAPK by aldosterone was also increased in SHR VSMCs and inhibited by PP2, indicating that c-Src is involved in p38 MAPK signaling by aldosterone in hypertension. Previous studies demonstrated that in SHR, p38 MAPK phosphorylation is important in collagen production stimulated by agonists of G protein–coupled receptors.38,39 Here we found that aldosterone increases [3H]proline incorporation, particularly in cells from SHR, indicating a stimulatory effect of aldosterone on collagen synthesis. This effect of aldosterone occurs through a c-Src–dependent p38 MAPK pathway in VSMCs, as evidenced by the inhibitory actions of PP2 and SB212190.

In addition to its profibrotic effects, aldosterone is proinflammatory. We assessed whether aldosterone-mediated, NAD(P)H-driven generation of superoxide anion, an index of oxidative stress and inflammation, is influenced by c-Src in SHR. Our data demonstrate that aldosterone stimulates c-Src–dependent NAD(P)H oxidase activation in rat VSMCs and that this is increased in cells from SHR. Because c-Src appears to be both upstream and downstream of NAD(P)H oxidase, activation of c-Src by aldosterone could result in amplification of NAD(P)H oxidase–mediated generation of reactive oxygen species and, consequently oxidative stress–induced vascular damage by aldosterone. Moreover, reactive oxidative species cooperate with aldosterone-induced c-Src activation to amplify NAD(P)H oxidase activity.

Figure 2. Effect of PP2 on aldosterone-induced p38 MAPK phosphorylation in VSMCs from WKY and SHR. Top, Representative immunoblots. Bottom, Corresponding line graphs demonstrate time course of aldosterone (0.1 μmol/L) on p38 MAPK phosphorylation in the absence and presence of PP2 (10 μmol/L). Results are mean ± SEM of 4 or 5 experiments. *P < 0.05, SHR vs WKY; **P < 0.05, PP2 vs aldosterone alone. Abbreviations are as defined in text.

Figure 3. Effect of PP2 on aldosterone-induced ERK1/2 phosphorylation in VSMCs from WKY and SHR. Top, Representative immunoblots. Bottom, Corresponding line graphs demonstrate time course of aldosterone (0.1 μmol/L) on ERK1/2 phosphorylation in the absence and presence of PP2 (10 μmol/L). Results are mean ± SEM of 4 or 5 experiments. *P < 0.05, SHR vs WKY; **P < 0.05, PP2 vs aldosterone alone. Abbreviations are as defined in text.
Oxygen species have been recognized as important mediators that regulate signal transduction pathways, including MAPKs.\textsuperscript{40–42} It could be suggested that aldosterone-induced activation of c-Src associated with increased ERK1/2 and p38 MAPK phosphorylation in SHR VSMCs may be because of redox-sensitive pathways. Furthermore, it is possible that alterations in regulatory mechanisms of c-Src may be involved. c-Src is activated by autophosphorylation of Tyr416 and is inactivated by carboxy-terminal Src kinase (Csk), which induces the phosphorylation of Tyr527. We previously demonstrated that upregulation of c-Src by angiotensin II in SHR VSMCs is associated with altered activation of Csk, which negatively regulates Src.\textsuperscript{34} We speculate that increased activation of c-Src by exogenous aldosterone may be due, in part, to decreased activation of Csk.

Interestingly, elevated blood pressure and abnormal physiological parameters in the SHR microcirculation are normalized by adrenalectomy,\textsuperscript{43} implicating a role for mineralocorticoids and glucocorticoids in blood pressure regulation in SHR. There is a paucity of information on the status of mineralocorticoid receptors in SHR. However, DeLano and Schmid-Schonbein\textsuperscript{44} demonstrated an increased density of mineralocorticoid receptors in the SHR microcirculation. In view of these observations, the enhanced sensitivity of SHR VSMCs to aldosterone-induced c-Src phosphorylation may be in part associated with enhanced mineralocorticoid receptor density. This awaits further clarification. Eplerenone, a selective mineralocorticoid receptor antagonist, inhibited phosphorylation of c-Src and p38 MAPK (data not shown), as well as activation of NAD(P)H oxidase activity and [\textsuperscript{3}H]proline incorporation induced by aldosterone in both WKY and SHR. Taken together, these observations indicate that rapid non-genomic effects of aldosterone are mediated by activation of the classic mineralocorticoid receptors.
Results from our study identify a signaling cascade through which aldosterone could influence vascular cellular changes in hypertension. Aldosterone stimulation increased MAPK activation and NAD(P)H-mediated generation of superoxide anion through c-Src–dependent mechanisms, and these effects are increased in SHR. Augmented c-Src–dependent signaling by aldosterone in SHR is associated with processes that stimulate collagen synthesis, cell growth, and inflammation. Taken together, our findings suggest that nongenomic signaling by aldosterone through c-Src–dependent pathways plays an important role in profibrotic and proinflammatory actions of aldosterone in essential hypertension.

Perspectives

The nongenomic signaling pathway for aldosterone, involving c-Src–modulated activation of p38 MAPK, ERK1/2, and NAD(P)H oxidase, is increased in essential hypertension. Activation of this pathway may be important in the profibrotic, proinflammatory, and mitogenic actions of aldosterone. The advent of selective aldosterone receptor antagonists, such as eplerenone, could have important therapeutic value in the prevention of end-organ damage induced by aldosterone in essential hypertension. Moreover, findings from our study highlight the functional importance of c-Src/MAPK/NAD(P)H oxidase and suggest that the inhibition of c-Src in antagonizing nonclassic actions of mineralocorticoids may also have beneficial effects.

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