Angiotensin II Controls p21\textsuperscript{ras} Activity via pp60\textsuperscript{src*}

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Angiotensin II is the major effector molecule of the renin-angiotensin system, and it exerts its physiologic functions via a G protein-coupled cell surface receptor called AT\textsubscript{1}. We found that in rat aortic smooth muscle cells, angiotensin II stimulated the formation of Ras-GTP, Ras-Raf-1 complex formation, and the tyrosine phosphorylation of two important Ras GTPase-activating proteins (GAPs), p120 Ras-GAP and p190 Rho-GAP. Electroporation of anti-pp60\textsuperscript{src} antibody into cultured, adherent smooth muscle cells blocked the angiotensin II stimulation of Ras-GAP and Rho-GAP tyrosine phosphorylation. In contrast electroporation of antibodies against c-Yes or c-Fyn had no effect. Anti-pp60\textsuperscript{src} antibody also blocked angiotensin II-stimulated Ras activation and Ras-Raf-1 complex formation. These data strongly suggest that a G protein-coupled receptor such as the AT\textsubscript{1} receptor can activate the Ras protein cascade via the tyrosine kinase pp60\textsuperscript{src}.

Angiotensin II is the major effector molecule of the renin-angiotensin system. This octapeptide stimulates vascular smooth muscle contraction, elevates vascular resistance, and increases intravascular volume. These effects combine to raise systemic blood pressure (1, 2). There is also substantial experimental evidence that angiotensin II acts as a growth factor (3). For instance, cultured vascular smooth muscle cells respond to angiotensin II by expressing early growth response genes such as c-fos, c-jun, and c-myc by and increasing thymidine incorporation (4, 5). This correlates with in vivo data that infusion of angiotensin II into animals injured with a vascular balloon catheter markedly exacerbates the resulting myointimal proliferation (6). A role for angiotensin II in cell growth and tissue remodeling has also been shown in animal models of hypertension, heart failure, and atherosclerosis (7–9).

Angiotensin II exerts its physiologic functions via a high affinity cell surface receptor now called the AT\textsubscript{1} receptor. This receptor was first cloned in 1991, and it contains the structural features of a seven transmembrane, heterotrimeric G protein-associated receptor (10). In vascular smooth muscle cells, ligand activation of the AT\textsubscript{1} receptor leads to the rapid activation of phospholipase C and the production of 1,4,5-inositol triphosphate (11).

Recently it has become clear that many of the intracellular signals mediated by the AT\textsubscript{1} receptor are similar to the signaling pathways activated by receptor tyrosine kinases. For instance, ligand activation of the AT\textsubscript{1} receptor leads to the rapid tyrosine phosphorylation and activation of phospholipase C-\gamma1 in vascular smooth muscle cells (12). This is a critical event for downstream signaling, because inhibition of tyrosine phosphorylation markedly reduces angiotensin II stimulation of 1,4,5-inositol triphosphate production. Separate studies have also shown that angiotensin II, acting through the AT\textsubscript{1} receptor, stimulates JAK2 tyrosine phosphorylation and activation (13, 14). Finally, several studies have indicated that angiotensin II leads to the phosphorylation and activation of mitogen-activated protein kinases (15, 16). The known link between p21\textsuperscript{ras} activity and mitogen-activated protein kinase stimulation, as well as the central role of p21\textsuperscript{ras} in cell growth, prompted us to ask if this molecule is activated by angiotensin II.

p21\textsuperscript{ras} and other small G proteins are membrane bound guanine nucleotide binding proteins that are active when complexed with GTP and inactive when bound to GDP (17, 18). The ratio of bound GTP to GDP is regulated by the rate of nucleotide exchange and by the rate of GDP hydrolysis. Proteins such as mammalian son-of-sevenless (mSOS) stimulate the exchange of GTP for bound GDP on p21\textsuperscript{ras}. The deactivation of p21\textsuperscript{ras} is regulated by GTPase-activating proteins, called GAPs, which markedly accelerate intrinsic Ras GTPase activity and lead to Ras-GDP. The best defined GAP is Ras-GAP, a 120-kDa protein that binds p21\textsuperscript{ras} via a Src homology II domain (19, 20). A second protein implicated in Ras deactivation is Rho-GAP, a 190-kDa protein (21). Although the exact role of Rho-GAP is not known, it is thought to have a regulatory role in stress fiber induction and focal adhesion (22). In cells stimulated with growth factors the GAPs are phosphorylated on tyrosine and form a complex that enhances the GTPase activity of p21\textsuperscript{ras} (23).

The biochemical pathway by which a G protein-coupled receptor such as the AT\textsubscript{1} receptor regulates p21\textsuperscript{ras} activity is not known. These cell surface receptors lack intrinsic kinase activity and must activate Ras in a fashion different from that of tyrosine kinase receptors. In this study we show that angiotensin II stimulates p21\textsuperscript{ras}, leading to p21\textsuperscript{ras}-GTP and Ras-Raf-1 complex formation. Angiotensin II also stimulates the tyrosine phosphorylation of Ras-GAP and Rho-GAP. The tyrosine kinase pp60\textsuperscript{src} appears to play a critical early role in angiotensin II signaling; neutralization of pp60\textsuperscript{src} activity by the electroporation of anti-pp60\textsuperscript{src} antibodies into smooth muscle cells blocked both Ras-GTP accumulation and Ras-Raf-1 complex formation.
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**Experimental Procedures**

- **Materials:** Human recombinant epidermal growth factor and all cell culture media were purchased from Life Technologies, Inc. Monoclonal antibodies against Rho-GAP (p190), Ras-GAP (p120), p21ras, and phosphotyrosine (clone FY20) were obtained from Transduction Laboratories (Kensington, KY). Polyclonal anti-p21ras-rcp, pp60c-src peptide, anti-c-fos, c-Fyn, and monoclonal rat anti-p21ras (Y13-259) antibody-agarose conjugate were obtained from Santa Cruz Biotech. (Santa Cruz, CA). [32P]Orthophosphate was obtained from DuPont NEN. The enhanced chemiluminescence kit was obtained from Amersham Corp.

Cell Culture—Rat vascular smooth muscle (RASM) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 10 μg/ml streptomycin, and 100 units/ml penicillin. Cells to be used for experiments were grown to 75–85% confluence, washed once with serum-free Dulbecco’s modified Eagle’s medium, and grown at 37°C in a humidified atmosphere of 10% CO2 in air.

Growth-arrested RASM cells were cultured in serum-free Dulbecco’s modified Eagle’s medium supplemented with 1 mM sodium pyruvate, 10 ng/ml aprotinin, 10 ng/ml penicillin, and 100 μg/ml streptomycin. After 12 h, [32P]orthophosphate was added (0.2 mCi/plate), and the cells were cultured for 6 h. Cells were lysed and immunoprecipitated with anti-p21ras (2 μg/ml), anti-Rho-GAP (2 μg/ml), or anti-phosphotyrosine (FY20 clone, 5 μg/ml). The immunoprecipitates were then recovered, separated on SDS-polyacrylamide gel electrophoresis, and visualized as described previously (12). The tyrosine phosphorylation of Ras-GAP and Rho-GAP was scanned by using a Silverscanner (La Cie, Inc.) interfaced with a personal computer. Each band was background present in the lane. The tyrosine phosphorylation of Ras-GAP and Rho-GAP was scanned by using a Silverscanner (La Cie, Inc.) interfaced with a personal computer. Each band was background present in the lane.

Electroporation of RASM Cells—Growth-arrested RASM cells were electroporated using a petri dish electrode as described previously (25). In brief, electroporation was performed in Ca2+- and Mg2+-free Hank’s balanced salt solution (5 mM KCl, 0.3 mM KH2PO4, 138 mM NaCl, 4 mM NaHCO3, 0.3 mM Na2HPO4, 0.3 mM NaH2PO4) at pH 7.4 containing a final concentration of 10 μg/ml antibodies (29). Cells were exposed to 1 pulse at 100 V for 40 ms (square wave) using an Electrosquare Porator, model T820 (BTX Inc., San Diego CA). Following electroporation, cells were incubated for an additional 30 min at 37°C (5% CO2).

Antibody Preabsorption—The anti-p21ras-rcp antibody used for electroporation was raised against residues 509–533 of the N-terminal site of human Src. It was preabsorbed with a 10-fold excess of peptide for 2 h at 37°C in Hank’s balanced salt solution. The preabsorbed antiserum was immediately used for electroporation experiments. An aliquot of antibody was also sham absorbed without peptide.

Ras-GTP/Ras-GDP Analysis—Rat vascular smooth muscle cells were quiesced in serum and phosphate-free Dulbecco’s modified Eagle’s medium supplemented with 1 mM sodium pyruvate, 100 units/ml penicillin and 100 μg/ml streptomycin. After 12 h, [32P]orthophosphate was added (0.2 μCi/plate), and the cells were cultured for 6 h. Cells were then lysed and immunoprecipitated with 100 ng/ml recombinant human epidermal growth factor (EGF) or 10–7 M angiotensin II. The results are plotted as the ratio of GDP to the total of GDP plus GTP. After 1 min, angiotensin II was added (0.2 mCi/plate), and the cells were cultured for 6 h. Cells were then lysed and immunoprecipitated with anti-p21ras-rcp antibody. The ratio of GTP to GDP was calculated.

**RESULTS**

Angiotensin II Activation of p21ras—Activation of p21ras is dependent upon the formation of a Ras-GTP complex. To measure the effects of angiotensin II on the formation of this complex, cultured RASM cells were quiesced for 24 h and then loaded with [32P]orthophosphate. After exposure to angiotensin II for 0, 1, 5, or 10 min, cells were lysed and immunoprecipitated with anti-p21ras-rcp antibody. The ratio of GTP to GDP bound to p21ras was then determined by thin-layer chromatography. Without angiotensin II, p21ras is found associated with GDP (Fig. 1A, lane C0). However, 1 min after the addition of 10–7 M angiotensin II, there is a significant increase in the amount of Ras-GTP complex formation (Fig. 1A, lane A0).

Levels of Ras-GTP then slowly decline. An identical experiment was performed using 100 ng/ml EGF in place of angiotensin II (Fig. 1, lanes E1, E5, and E10). As previously demonstrated, this growth factor stimulated Ras-GTP complex formation (27). In order to quantitate the shift of Ras from the GDP to the GTP binding forms, the ratio of GTP to the total of GTP plus GDP was determined by densitometry. The averages of five separate experiments are shown in Fig. 1B. These data show that although the angiotensin II-mediated stimulation of Ras-GTP (3-fold) was less than that observed with EGF (4.5-fold), both

**Fig. 1.** Ras activation. RASM cells were labeled with [32P]orthophosphate, and p21ras was collected by immunoprecipitation. Associated GTP and GDP were analyzed by thin-layer chromatography. A, the binding of [32P]-labeled guanine nucleotides to p21ras under unstimulated conditions (C0) and after 1, 5, and 10 min of exposure to 10–7 M angiotensin II (A0, A5, and A10) or 100 ng/ml EGF (E1, E5, and E10). B, data from five separate experiments were quantitated by densitometry. The results are plotted as the ratio of GTP to the total of GTP plus GDP. After 1 min, angiotensin II (●) induced a 3-fold increase of p21ras-GTP, whereas EGF (■) induced an 4.5-fold increase.
agents induced maximal Ras-GTP complex formation by 1 min. At this time point, the angiotensin II-stimulated ratio of Ras-GTP to the total of Ras-GTP plus Ras-GDP ranged from 58 to 74% of that stimulated by EGF (mean response 66% ± 11).

Activated p21ras is known to recruit Raf-1 into a protein complex (28). If angiotensin II stimulated Ras-GTP formation, we then hypothesized that angiotensin II would also induce Ras-Raf-1 complex formation. To measure this, RASM cells were exposed to angiotensin II, lysed, and immunoprecipitated with anti-p21ras antisera. The immunoprecipitated proteins were probed by Western blot analysis using anti-Raf-1 antibody. This showed that after angiotensin II addition, there is a marked increase in Ras-Raf-1 complex formation (Fig. 2). As with Ras-GTP levels, the greatest Ras-Raf-1 association was present at 1 min, after which levels declined. We have also measured Ras-Raf-1 complex formation by stimulating cells with angiotensin II, immunoprecipitating with anti-Raf-1 antibody, and probing by Western blot analysis using anti-Ras antibody. This protocol gave identical results to those shown in Fig. 2.

GAP proteins play an intimate role in the Ras activation-deactivation cycle. Analysis of growth factor signaling has indicated that these proteins serve to limit the time Ras remains in the active Ras-GTP form. To investigate if angiotensin II stimulates the tyrosine phosphorylation of Rho-GAP and Ras-GAP, RASM cells were exposed to angiotensin II, lysed, and immunoprecipitated with an anti-phosphotyrosine antibody. A Western blot of the precipitated proteins was then probed with monoclonal antibodies against either Rho-GAP or Ras-GAP. In unstimulated cells, very little Ras-GAP was phosphorylated on tyrosine (Fig. 3A). However, 1 min after the addition of angiotensin II, Ras-GAP tyrosine phosphorylation increased to about 16-fold greater than at time 0. Even at 10 min, levels of Ras-GAP tyrosine phosphorylation remained elevated. By comparison, Rho-GAP showed less change in tyrosine phosphorylation levels after angiotensin II exposure (Fig. 3B). Densitometry of five experiments showed that on average the tyrosine phosphorylation of Rho-GAP increased 4-fold 1 min after angiotensin II addition. The tyrosine phosphorylation of Rho-GAP was also studied by immunoprecipitation with monoclonal anti-Rho-GAP following by Western blot analysis using anti-phosphotyrosine. This experiment gave data virtually identical to that of Fig. 3B.

The Role of Src in Angiotensin II p21ras Regulation—A central question in understanding the interaction of the AT1 receptor with the p21ras pathway is how a seven transmembrane receptor initiates phosphorylation of downstream signaling molecules. We have focused on the role of the soluble tyrosine kinase pp60src, because previous work has demonstrated that this enzyme plays an important role in angiotensin II-induced signaling (25). To inactivate Src activity, we inserted polyclonal anti-Src antibody into cultured, adherent RASM cells using an electroporation technique previously shown effective in introducing immunoglobulins into these cells (25). We first asked if the neutralization of intracellular Src activity affected angiotensin II-stimulated phosphorylation of Ras-GAP and Rho-GAP. As control experiments, cells were electroporated in the presence of vehicle (Hanks’ balanced salt solution), bovine serum albumin (BSA), or pooled rabbit IgG (Fig. 4A: E, E1, and EB). None of these reagents affected the tyrosine phosphorylation of Ras-GAP 1 min after the addition of angiotensin II. Electroporation itself does appear to augment the angiotensin II-mediated tyrosine phosphorylation of Rho-GAP (Fig. 4A, E).
In contrast to the results with BSA or pooled rabbit IgG, electroporation of rabbit anti-pp60src antibody inhibited the angiotensin II-induced tyrosine phosphorylation of both Ras-GAP and Rho-GAP (Fig. 4A, ES). The specificity of the pp60src antibody was controlled using two separate protocols. First, electroporation experiments were performed using antiserum antibody. We have also used electroporation to test the effects of rabbit monoclonal antibodies directed against the Src family proteins c-Yes (pp62) and c-Fyn (pp59). As shown in Fig. 4C, neither anti-c-Yes (EY) nor anti-c-Fyn (EF) antibody blocked the angiotensin II-stimulated tyrosine phosphorylation of Ras-GAP or Rho-GAP. Western analysis had previously demonstrated that RASM cells lack c-Yes but contain c-Fyn (data not shown). These experiments suggest a major role for pp60src in angiotensin II-mediated phosphorylation of Rho-GAP and Ras-GAP.

We also studied the role of pp60src in Ras-GTP formation. When cells were electroporated in the presence of vehicle, they responded to angiotensin II or to EGF with the rapid conversion of Ras-GDP to Ras-GTP, very similar to nonelectroporated cells (Fig. 5A, E). When cells were electroporated with either rabbit IgG or BSA, these cells responded to angiotensin II or EGF in a fashion indistinguishable from cells electroporated with vehicle alone (Fig. 5A, E and EB). In contrast, when cells were electroporated with rabbit anti-pp60src antisera, Ras activation in response to angiotensin II was abolished (Fig. 5A, ES). This was not a toxic effect because these cells remained fully capable of activating Ras in response to EGF.

We also used the electroporation technique to investigate the role of pp60src on angiotensin II-mediated formation of a Ras-Raf-1 complex. Consistent with the above data, anti-pp60src antibody completely blocks Ras-Raf-1 complex formation (Fig. 5B, ES). No effect on angiotensin II-mediated complex formation was observed with either rabbit IgG or BSA (Fig. 5B, E and EB). These data strongly suggest that pp60src or a highly related enzyme plays a critical role in angiotensin II-mediated activation of the Ras signaling pathway.

**DISCUSSION**

The AT1 receptor is a seven transmembrane receptor responsible for virtually all of the physiologic actions of angiotensin II (1, 2). Whereas the process of angiotensin II-mediated smooth muscle contraction has been intensely studied, the intracellular signals associated with angiotensin II-mediated cell growth are less understood. Previous investigators have established that angiotensin II activates the mitogen-activated protein kinase cascade (15, 16). Here we show that angiotensin II stimulates the activation of p21ras (Ras). This was first established by directly verifying the conversion of Ras-GDP to Ras-GTP and by demonstrating Ras-Raf-1 association. Thus, angiotensin II appears to use the Ras pathway to activate mitogen-activated protein kinase in a fashion analogous to growth factors...
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Critical to angiotensin II-mediated activation of the Ras pathway is the intracellular kinase pp60src. This enzyme plays a role upstream of p21ras activation. It also leads to the tyrosine phosphorylation of GAPs perhaps through direct pp60src kinase activity.

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such as EGF. That said, there must be differences in the signaling initiated by angiotensin II and by classic growth factors because angiotensin II is a less potent growth factor than EGF or similar molecules. In analyzing the Ras activation in response to angiotensin II, we noted two differences from the Ras activation in response to EGF. The magnitude of angiotensin II-induced Ras-GTP formation was lower, and Ras was more rapidly inactivated. A major intracellular mechanism used to regulate Ras activity is the action of Ras-GTPase proteins or GAPs. In response to angiotensin II, there is a rapid and marked tyrosine phosphorylation of Ras-GAP. In association with Rho-GAP, this molecule converts the active Ras molecule back to the Ras-GDP form (17). Thus, we hypothesize that the rapid activation of the Ras-GAP system by tyrosine phosphorylation acts to modulate the stimulatory potential of angiotensin II.

Another obvious difference between angiotensin II signaling and that of classic growth factors is that the AT1 receptor lacks intrinsic kinase domains (10). Thus, the AT1 receptor must recruit an intracellular kinase to initiate any type of kinase cascade. Recent experimental evidence indicates that pp60src is activated by angiotensin II (29). In vascular smooth muscle cells, it appears to play a major role in angiotensin II-mediated tyrosine phosphorylation acts to modulate the stimulatory potential of angiotensin II.

Critical to angiotensin II-mediated activation of the Ras pathway is the intracellular kinase pp60src. This enzyme plays a role upstream of p21ras activation. It also leads to the tyrosine phosphorylation of GAPs perhaps through direct pp60src kinase activity.

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