Angiotensin II Stimulates Mitogen-activated Protein Kinases and Protein Synthesis by a Ras-independent Pathway in Vascular Smooth Muscle Cells*

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Angiotensin II (ANG II), a potent hypertrophic factor of vascular smooth muscle cells (VSMC), induces activation of the ras protooncogene product (Ras) and mitogen-activated protein (MAP) kinases and subsequent stimulation of protein synthesis in VSMC. In the present study, we examined whether Ras activation is required for ANG II-induced MAP kinase activation and stimulation of protein synthesis in cultured rat VSMC. Pretreatment with tyrosine kinase inhibitors, genistein and herbimycin A, or a putative phosphatidylinositol 3-kinase inhibitor, wortmannin, completely blocked ANG II-induced Ras activation, whereas neither of them had an effect on ANG II-induced MAP kinase activation. Adenovirus-mediated expression of a dominant negative mutant of Ha-Ras completely inhibited ANG II-induced Ras activation but failed to inhibit MAP kinase activation and stimulation of protein synthesis by this vasoconstrictor. These results indicate that ANG II stimulates MAP kinases and protein synthesis by a Ras-independent pathway in VSMC.

EXPERIMENTAL PROCEDURES

Materials—ANG II and MBP were obtained from Sigma. The anti-rat monoclonal antibody Y13–259 was purchased from Oncogene Science (Cambridge, MA). [1,4,5-3H]leucine (139 Ci/mmol) and [γ-32P]ATP (3000 Ci/mmol) were from Amersham Life Science (Tokyo, Japan). [32P]orthophosphate was from Du Pont. Protein G-Sepharose 4 Fast Flow and rabbit antiserum to rat IgG were from Pharmacia (Uppsala, Sweden) and Cappel (Durham, NC), respectively. Genistein and wortmannin were from Kyowa (Tokyo, Japan). Herbimycin A was from Life Technologies, Inc. Other materials and chemicals were obtained from commercial sources.

Cell Culture—VSMC were isolated from rat thoracic aorta by enzymatic dissociation as described previously (20). Cells were grown and passaged as described previously (21) and used at passage levels 7–18.

Analysis of Ras-bound GDP and GTP—Detection and quantification of Ras-bound GDP and GTP was performed as described previously (13). Briefly, the quiescent VSMC on 60-mm dishes were incubated with phosphate-free DMEM supplemented with 0.2 mCi/ml [32P]orthophosphate for 12 h. Fifty μM sodium orthovanadate was added to the cells during the last 30 min. After stimulation with ANG II, the cells were lysed. Ras was immunoprecipitated from the cell lysates with anti-Ras

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mRNA encoding the Ras proteins was inserted into adenoviral vectors containing CA promoter comprising either E1-and E3-adenoviral vectors containing CA promoter comprising a cytomegalovirus enhancer and chicken β-actin promoter were prepared as described previously (22–24). The adenoviruses expressing either a dominant negative mutant of Ha-Ras (AdRasY57), in which tyrosine replaces aspartic acid at residue 57 (25), or the bacterial β-galactosidase (AdLacZ) were constructed as described previously (24). Subconfluent VSMC grown on 60-mm dishes were incubated with DMEM containing either AdRasY57 or AdLacZ (20 plaque-forming units/cell) for 2 h at room temperature and then washed once with 2 ml of DMEM and incubated with DMEM supplemented with 10% fetal bovine serum for more 2 days. Then cells were growth-arrested for 48 h in DMEM prior to use.

Protein Synthesis Assay—Protein synthesis was measured by 3H-leucine incorporation as described previously (26). The quiescent VSMC were stimulated with ANG II in serum-free DMEM containing 0.5 μCi/ml 3H-leucine. After 24 h, the radioactivity incorporated into trichloroacetic acid-precipitable material was measured by liquid scintillation spectrometry after solubilization in 0.1 N NaOH.

Protein Determination—Cell protein was determined by the method of Bradford (27) with bovine serum albumin as a standard.

RESULTS

Effects of Tyrosine Kinase Inhibitors on ANG II-induced Ras and MAP Kinase Activation in VSMC—Since a number of investigators have shown that tyrosine kinase activity is essential for Ras and/or MAP kinase activation by various stimuli in their respective target cells (16, 19), we examined whether tyrosine kinase activity was required for ANG II-induced Ras and MAP kinase activation, using two distinct tyrosine kinase inhibitors, genistein and herbimycin A. As has been previously reported (13), the unstimulated VSMC displayed a low level of Ras activation. Treatment with either 100 μM genistein for 10 min or 3 μM herbimycin A for 30 min prior to stimulation with 100 nM ANG II for 2 min. A, positions of GDP and GTP are indicated; B, ratios of Ras-GTP to Ras-GDP plus Ras-GTP are shown as means ± S.E. of three independent trials.

FIG. 1. Effect of tyrosine kinase inhibitors on ANG II-induced Ras activation. VSMC were stimulated with or without either 100 μM genistein for 10 min or 3 μM herbimycin A for 30 min prior to stimulation with 100 nM ANG II for 2 min. A, positions of GDP and GTP are indicated; B, ratios of Ras-GTP to Ras-GDP plus Ras-GTP are shown as means ± S.E. of three independent trials.

MAP Kinase Activation—MAP kinase was activated by a MAP kinase assay in MBP-containing polyacrylamide gels as described previously (13). Briefly, the cell lysates from VSMC were electrophoresed on 10% SDS-polyacrylamide gel containing 0.5 mg/ml MBP. After washing the gel, the enzymes were denatured in 6 M guanidine HCl and then renatured in 50 mM Tris-Cl, pH 8.0, containing 5 mM 2-mercaptoethanol and 0.04% Tween 40. Kinase reaction was carried out by incubating the gel with [γ-32P]ATP. After incubation, the gel was extensively washed and dried. The radioactivity was analyzed using a Fujix bioimaging analyzer BAS2000.

FIG. 2. Effect of tyrosine kinase inhibitors on ANG II-induced MAP kinase activation. VSMC were incubated with or without either 100 μM genistein for 10 min or 3 μM herbimycin A for 30 min prior to stimulation with 100 nM ANG II for 5 min. A, positions of 44- and 42-kDa (p44 and p42, respectively) MAP kinases are indicated; B, radioactivities of phosphorylated MBP at positions of MAP kinases were quantitated and plotted as the percentage of the response to 100 nM ANG II. Values shown are means ± S.E. of three independent trials.

Cell Transfection by a Recombinant Adenovirus—Replication-defective E1-and E3-adenoviral vectors containing CA promoter comprising a cytomegalovirus enhancer and chicken β-actin promoter were prepared as described previously (22–24). The adenoviruses expressing either a dominant negative mutant of Ha-Ras (AdRasY57), in which tyrosine replaces aspartic acid at residue 57 (25), or the bacterial β-galactosidase (AdLacZ) were constructed as described previously (24). Subconfluent VSMC grown on 60-mm dishes were incubated with DMEM containing either AdRasY57 or AdLacZ (20 plaque-forming units/cell) for 2 h at room temperature and then washed once with 2 ml of DMEM and incubated with DMEM supplemented with 10% fetal bovine serum for more 2 days. Then cells were growth-arrested for 48 h in DMEM prior to use.

Protein Synthesis Assay—Protein synthesis was measured by [3H]leucine incorporation as described previously [26]. The quiescent VSMC were stimulated with ANG II in serum-free DMEM containing 0.5 μCi/ml [3H]leucine. After 24 h, the radioactivity incorporated into trichloroacetic acid-precipitable material was measured by liquid scintillation spectrometry after solubilization in 0.1 N NaOH.

Protein Determination—Cell protein was determined by the method of Bradford [27] with bovine serum albumin as a standard.

Results

Effects of Tyrosine Kinase Inhibitors on ANG II-induced Ras and MAP Kinase Activation in VSMC—Since a number of
ent pathway in VSMC. Because these pharmacological drugs may affect other reactions besides Ras activation, we used a more specific molecular tool, the adenovirus expressing a dominant negative mutant of Ha-Ras (AdRasY57) in the next set of experiments. The adenovirus expressing the bacterial β-galactosidase (AdLacZ) was used as a control. As has been described (24), AdRasY57-infected VSMC expressed markedly elevated levels of Ras protein as compared with both uninfected and AdLacZ-infected control cells (data not shown). As shown in Fig. 4, expression of RasY57 decreased the basal levels of Ras-GTP and completely prevented the stimulatory effect of ANG II on Ras activation. Expression of β-galactosidase had no effect on basal levels of Ras-GTP and ANG II-increased Ras-GTP accumulation. In consistent with the results of the pharmacological experiments described above, expression of RasY57 had no effect on ANG II-induced MAP kinase activation (Fig. 5A). Moreover, even in AdRasY57-infected VSMC, ANG II stimulated protein synthesis to extents similar to those observed in uninfected and AdLacZ-infected control cells (Fig. 5B). In all experiments so far described, we used a high concentration (100 nM) of ANG II to induce maximal responses of VSMC. It is possible that Ras activation may contribute to MAP kinase activation and stimulation of protein synthesis by lower concentrations of ANG II and that an alternative Ras-independent pathway(s) may be utilized at higher concentrations of ANG II. Therefore, in the last set of experiments, we used a lower concentration (1 nM) of ANG II and examined the effects of expression of RasY57 on Ras and MAP kinase activation and stimulation of protein synthesis. Expression of RasY57 also inhibited Ras activation by 1 nM ANG II (Fig. 6A) but failed to prevent MAP kinase activation and stimulation of protein synthesis even by this low concentration of ANG II (Fig. 6, B and C).

**DISCUSSION**

In 1992, we showed that ANG II induces tyrosine and threonine phosphorylation and activation of MAP kinases in cultured VSMC (5). ANG II also induces activation of the raf protooncogene product (Raf) and MAP kinase kinase in this cell type (35, 36). Recently, several investigators including us have clearly demonstrated that ANG II induces activation of Ras via AT1 receptors in VSMC (13–15). In our study (13), however, we found that pertussis toxin treatment, which markedly inhibited ANG II-induced Ras activation, had no inhibitory effect on ANG II-induced MAP kinase activation. This differential sensitivity to pertussis toxin of these reactions suggests that Ras activation may be dispensable for ANG II-induced MAP kinase activation in VSMC. However, since the pertussis toxin treatment did not completely inhibit ANG II-induced Ras activation, we could not rule out the possibility that ANG II was activating MAP kinases via residual Ras activity. The results reported herein extended these observations and showed that treatment with two tyrosine kinase inhibitors (genistein and herbimycin A) or wortmannin, which completely inhibited ANG II-induced Ras activation, had no effect on ANG II-induced MAP kinase activation. This differential sensitivity to pertussis toxin of these reactions suggests that Ras activation may be dispensable for ANG II-induced MAP kinase activation in VSMC. However, since the pertussis toxin treatment did not completely inhibit ANG II-induced Ras activation, we could not rule out the possibility that ANG II was activating MAP kinases via residual Ras activity. The results reported herein extended these observations and showed that treatment with two tyrosine kinase inhibitors (genistein and herbimycin A) or wortmannin, which completely inhibited ANG II-induced Ras activation, had no effect on ANG II-induced MAP kinase activation. Further, we showed that expression of a dominant negative mutant of Ha-Ras, which completely blocked ANG II-induced activation of endogenous Ras, presumably by interfering with exchange of GDP for GTP (25), had no effect on ANG II-induced MAP kinase activation. These results indicate that ANG II induces MAP kinase activation by a Ras-independ-
ent pathway in VSMC.

Previous reports showed that depletion of protein kinase C by pretreatment with a protein kinase C-activating phorbol ester, phorbol 12-myristate 13-acetate, markedly blocked ANG II-induced MAP kinase activation, suggesting that MAP kinase activation by ANG II is mediated mainly by protein kinase C activation (5, 37). Protein kinase C is known to activate Raf by direct phosphorylation (18). Indeed, protein kinase C-activating phorbol 12-myristate 13-acetate can stimulate MAP kinases without activating Ras in VSMC (5, 13). Based on the present results and these previous findings, it is likely that ANG II induces MAP kinase activation predominantly by a Ras-independent and protein kinase C-dependent pathway in VSMC. Recently, Eguchi et al. (15) also reported that ANG II induces MAP kinase activation predominantly by a Ras-independent and protein kinase C-dependent pathway in VSMC. In their system, however, ANG II-induced MAP kinase activation was only partially impaired by pretreatment with phorbol 12-myristate 13-acetate but was abolished by treatment with an intracellular Ca²⁺/calmodulin inhibitor, calmidazolium, and genistein. Furthermore, both ANG II-induced MAP kinase activation and Ras activation were insensitive to pertussis toxin treatment in their study. Based on these findings, they proposed that ANG II induces MAP kinase activation via Ras activation, which is mediated by an unidentified Ca²⁺/calmodulin-dependent tyrosine kinase in VSMC. The reason for the discrepancies between our results and theirs is unclear at present, but the discrepancies may be due to differences in phenotypes of our cells and theirs, because it is well known that various degrees of dedifferentiation occur when VSMC are placed in culture (38).

The mechanism by which ANG II activates Ras via the AT1 receptor and Gᵢ protein remains unclear. The inhibitory effect of tyrosine kinase inhibitors on Ras activation by ANG II suggests that tyrosine phosphorylation may be involved in this process. It has been shown that activation of the ANG II receptor as well as other Gᵢ-coupled-receptors such as α₂-adrenergic, lysophosphatidic acid, and endothelin receptors induces tyrosine phosphorylation of Shc, resulting in its association with a Grb2-Sos complex (39). These observations suggest that Shc-Grb2-Sos complexes may propagate signals not only from growth factor receptors but also from Gᵢ protein-coupled receptors including the ANG II receptor as well. The pathway leading to Shc phosphorylation may involve βγ subunits of Gᵢ protein because lysophosphatidic acid- and α₂-adrenergic re-
ceptor-induced Shc phosphorylation is blocked by coexpression of a 8y binding peptide derived from bARK1 (40). Recently, Schieffer et al. (14) have suggested that ANG II activates Ras by inhibiting the GAP activity through tyrosine phosphorylation of GAP by c-Src in VSMC. Therefore, it is possible that, to fully activate Ras, ANG II may utilize two mechanisms simultaneously, i.e. stimulating the exchange of GDP for GTP by Sos through tyrosine phosphorylation of Shc and inhibiting the hydrolysis of bound GTP by GAP through tyrosine phosphorylation of GAP. In the present study, we also showed that wortmannin inhibited ANG II-induced Ras activation in VSMC. Similar inhibitory effect of wortmannin on Ras activation was observed in adipocytes stimulated with insulin (32). In this cell type, inhibition of phosphatidylinositol 3-kinase with wortmannin resulted in significant activation of GAP and reduction in Ras-GTP. Therefore, it is possible that phosphatidylinositol 3-kinase may be involved in the mechanism by which ANG II induces Ras activation via inhibiting GAP activity.

Using a dominant negative strategy, Ras has been shown to be involved in stimulation of VSMC proliferation by serum, platelet-derived growth factor, acidic fibroblast growth factor, epidermal growth factor, and thrombin (24, 41). By the same strategy, we also explored the role of Ras in ANG II-induced hypertrophic response of VSMC. We found that expression of a dominant negative mutant of Ras did not inhibit ANG II-induced stimulation of protein synthesis, indicating that Ras activation is not required for ANG II-induced hypertrophic response of VSMC. It is becoming apparent that Ras may have multiple effectors besides Raf and play diverse roles in cell responses other than cell growth (16, 17). Therefore, it is possible that Ras activated by ANG II may play roles unrelated to MAP kinase activation and cell growth in VSMC. Further studies are needed to clarify the roles of Ras in ANG II actions as well as the mechanism by which ANG II activates Ras in VSMC.

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