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Involvement of a Tyrosine Kinase Pathway in the Growth-Promoting Effects of Angiotensin II on Aortic Smooth Muscle Cells

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SUMMARY

Angiotensin II (AII) is a growth factor that stimulates protein synthesis and induces cellular hypertrophy in aortic smooth muscle cells (SMC). This trophic effect is mediated by the AT_1 subtype of AII receptors. However, very little is known about the cellular signaling pathways involved in this response. In the present study, we examined the role of protein tyrosine phosphorylation in the growth-promoting effects of AII on rat aortic SMC. The addition of AII to quiescent aortic SMC induced tyrosine phosphorylation of multiple substrates, as revealed by antiphosphotyrosine immunoblotting. This response was blocked by preincubation with the AT_1-selective antagonist losartan. To explore the functional role of this signaling pathway, we performed experiments with two mechanistically distinct tyrosine kinase inhibitors. Treatment of quiescent aortic SMC with genistein and herbimycin A abolished the stimulatory effect of AII on overall protein tyrosine phosphorylation. Similarly, the two inhibitors prevented AII-induced tyrosine phosphorylation of the cytoskeletal protein paxillin. Under the same conditions, incubation with genistein or herbimycin A did not interfere with AII binding to the AT_1 receptor and did not significantly affect AII-stimulated inositol-1,4,5-trisphosphate production and Ca^{2+} mobilization. In parallel to their selective action on tyrosine phosphorylation, both genistein and herbimycin A completely inhibited AII-stimulated protein synthesis in a dose-dependent manner. In contrast, the two inhibitors were much less potent in preventing the trophic effect of phorbol-12-myristate 13-acetate in these cells. We further demonstrate that genistein and herbimycin A did not prevent mitogen-activated protein kinase activation and c-*fos* gene induction, which is consistent with the notion that these downstream effectors do not link AII-induced tyrosine phosphorylation to protein synthesis. These results provide evidence that tyrosine phosphorylation has a critical role in cellular hypertrophy and is involved in AII action in vascular SMC.

The role of the vasoconstrictive hormone AII as a growth factor has received considerable attention in recent years. This interest was triggered by the exciting possibility that AII may play an important role in the development of vascular diseases such as hypertension and atherosclerosis, which are characterized by abnormal growth of vascular SMC (1, 2). AII has been reported to stimulate protein synthesis and to induce cellular hypertrophy in vascular SMC derived from normal rats (3–6). Other studies have shown that the hormone can induce a delayed mitogenic effect in cultured SMC (7, 8). The growth-promoting effects of AII were also demonstrated *in vivo* after the observation that treatment with angiotensin-converting enzyme inhibitors or AII receptor antagonists significantly reduced neointimal proliferation after acute arterial injury (9–12). Consistent with this notion, prolonged infusion of AII was shown to stimulate vascular SMC DNA synthesis in the normal and injured rat arterial wall (13).

The molecular basis of the hypertrophic effect of AII remains largely unknown. AII binds to two major subtypes of receptors, designated AT_1 and AT_2, to activate multiple signal transduction pathways in target cells. Molecular cloning has revealed that both receptor subtypes belong to the superfamily of receptors with seven transmembrane helices (14–17). AT_1 receptor activation stimulates the activity of phospholipases C, D, and A_2 and inhibits the activity of adenylyl cyclase (reviewed in Refs. 18 and 19). Little is known regarding the biochemical events triggered by the AT_2

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ABBREVIATIONS: AII, angiotensin II; SMC, smooth muscle cells; MAP, mitogen-activated protein; sarile, [Sar^1,Ile^8]angiotensin II; PMA, phorbol-12-myristate-13-acetate; IP_3, inositol-1,4,5-trisphosphate; mAb, monoclonal antibody; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; MBP, myelin basic protein; EGF, epidermal growth factor; PKC, protein kinase C; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $[Ca^{2+}]_i$, intracellular calcium concentration; SDS, sodium dodecyl sulfate; kb, kilobase(s).

receptor. In common with other growth factors, AII induces the expression of mRNAs for early growth response genes, such as c-*fos*, c-*jun*, and c-*myc* (20–24) and activates the Na^+/H^+ antiporter (25–27).

Accumulating evidence suggests that tyrosine phosphorylation may have a significant role in the growth response to G protein-coupled receptor agonists. A number of studies have shown that growth factors such as thrombin, bombesin, vasopressin, endothelin, bradykinin, and lysophosphatidic acid can stimulate tyrosine phosphorylation of multiple substrates in their target cells (28–31). AII was also reported to induce rapid tyrosine phosphorylation in liver epithelial cells (32), glomerular mesangial cells (28), vascular SMC (Refs. 33 and 34 and the present study), and cardiac fibroblasts (35). However, it is not known whether tyrosine phosphorylation has a role in the hypertrophic action of AII.

In the present study, we used two distinct tyrosine kinase inhibitors to explore the role of tyrosine phosphorylation in the hypertrophic effect of AII on vascular SMC. We show that treatment of aortic SMC with herbimycin A and genistein prevents AII-induced tyrosine phosphorylation and potently inhibits the increased rate of protein synthesis. The two inhibitors do not reduce AII binding to its receptor or interfere significantly with phospholipase C activation, MAP kinase activation, or c-*fos* mRNA expression. We further show that tyrosine kinase inhibitors are much less potent in inhibiting protein synthesis induced by the phorbol ester PMA. Together, our data suggest that a tyrosine kinase pathway has an important role in the hypertrophic response to AII.

Experimental Procedures

Materials. AII and sarile were purchased from Hukabel Scientific. [^{125}I]Sarile was prepared by radioiodination of sarile using a solid-phase method with Iodo-Beads as previously described (36). The receptor antagonist losartan was generously provided by DuPont-Merck. Insulin was a gift of Dr. Jean-Louis Chiasson (Hôtel-Dieu de Montréal, Quebec, Canada). Genistein, daidzein, PMA, and IP_3 were obtained from LC Services. Herbimycin A was obtained from GIBCO or BIOMOL. Genistein and daidzein were dissolved in dimethylsulfoxide to give stock solutions of 50 mM, and herbimycin A was dissolved in dimethylsulfoxide to give 1 mM solutions. Fura-2 was obtained from Molecular Probes. Protease inhibitors and ionomycin were obtained from Sigma Chemical Co. Iodo-Beads were obtained from Pierce. Protein A-Sepharose was purchased from Pharmacia LKB. [^{125}I]Na, [^{3}H]leucine, and [^{3}H]IP_3 were purchased from Amersham. Agarose-linked PY-20 antiphosphotyrosine mAb and antipaxillin mAb 165 were obtained from ICN, whereas 4G10 antiphosphotyrosine mAb was obtained from Upstate Biotechnology. Antiserum SM1 was raised by immunization of rabbits with purified glutathione *S*-transferase-ERK1 fusion protein and is specific to $p44^{mapk}$ protein (37).

Cell culture. Vascular SMC were isolated from the thoracic aortas of 12-week-old male Brown-Norway rats by an explant procedure as described (38). The cells were grown in low-glucose DMEM supplemented with 10% calf serum, 2 mM L-glutamine, and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin). Cultures were maintained at 37° in a humidified atmosphere of 95% air/5% CO_2. All experiments were conducted on cells at passages 9–14. Quiescent aortic SMC were obtained by incubation of 95%-confluent cell cultures in serum-free DMEM/F12 (1:1) containing 15 mM HEPES, pH 7.4, 0.1% bovine serum albumin, and 5 μg/ml transferrin for 48 hr. Where indicated, quiescent cells were treated with vehicle alone or with the indicated concentrations of genistein for the last 30 min or herbimycin A for the last 18 hr before the addition of AII.

Immunoprecipitations. Quiescent cultures of aortic SMC in 60-mm petri dishes were washed once and stimulated with AII as indicated at 37°. The cells were then washed twice in ice-cold PBS and lysed in 0.4 ml of Triton X-100 lysis buffer (50 mM Tris·HCl, pH 7.4, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10% glycerol, 10^{-4} M phenylmethylsulfonyl fluoride, 10^{-6} M leupeptin, 10^{-6} M pepstatin A, 1% Triton X-100) for 25 min at 4°. Cell lysates were scraped from the plates and clarified by centrifugation at 13,000 × *g* for 10 min, and normalized amounts of lysate proteins (150–200 μg) were subjected to immunoprecipitation. Phosphotyrosil proteins were immunoprecipitated by incubation with 20 μl of agarose-coupled PY-20 mAb for 2 hr at 4°. For immunoprecipitation of paxillin, lysate proteins were incubated for 2 hr at 4° with 20 μl of mAb 165 preadsorbed to rabbit anti-mouse IgG-coated protein A-Sepharose beads. Immune complexes were washed three times with lysis buffer before electrophoresis on 7.5% acrylamide gels and immunoblot analysis.

Immunoblot analysis. After electrophoresis, proteins were electrophoretically transferred to Hybond-C nitrocellulose membranes (Amersham) in 25 mM Tris and 192 mM glycine and fixed for 15 min in methanol/acetic acid/glycerol (40:7:3). For immunoblotting of phosphotyrosil proteins, membranes were blocked in TBS containing 1 mg/ml bovine serum albumin and 1 mg/ml ovalbumin for 1 hr at 37° before incubation for 2 hr at 25° with mAb 4G10 (1:5000) in blocking solution. For immunoblotting of paxillin, the blocking solution consisted of TBS containing 5% nonfat dry milk, and the primary incubation was carried out for 2 hr at 25° with a 1:500 dilution of mAb 165. The membranes were washed five times with TBS and 0.1% Tween 20 and once with TBS before incubation with horseradish peroxydase-conjugated goat anti-mouse IgG (1:10,000) in TBS and 0.1% Tween 20 for 1 hr. For evaluation of the tyrosine phosphorylation status of MAP kinases, the membrane was blocked for 1 hr at 37° in TBS, 1% bovine serum albumin, and 0.1% Tween 20 and then incubated for 1 hr at 25° with mAb 4G10 (1:5000) in blocking solution. The membrane was washed as described before incubation with horseradish peroxydase-conjugated goat anti-mouse IgG (1:10,000) in TBS, 5% milk, and 0.1% Tween 20 for 1 hr. Immunoreactive bands were visualized by enhanced chemilluminescence (Amersham).

Receptor binding assay. Confluent aortic SMC were treated or were not treated with 30 μM genistein for 30 min or 1 μM herbimycin A for 18 hr before binding assay. Cell membranes were isolated essentially as described (39). Competition binding studies were carried out by incubating 40 μg of aortic SMC membranes with 0.2 nM [^{125}I]sarile and varying concentrations of the agonist AII. Bound [^{125}I]sarile was separated from free ligand by rapid filtration through GF/B filters presoaked with 0.2% bovine serum albumin. The filters were counted for radioactivity. Average values of duplicate determinations of bound [^{125}I]sarile were used for data analysis. Binding data were analyzed by nonlinear least-squares curve fitting with the SCAFIT computer program (40).

Preparation of adrenocortical membranes. Bovine adrenal glands were obtained soon after slaughter and placed in cold PBS. All subsequent procedures were carried out at 4°. The glands were cleansed of fat, and the medulla was removed by scraping with a scalpel. The entire cortex was placed in 10 volumes of homogenization buffer (25 mM Tris·HCl, pH 8.5, 110 mM KCl, 10 mM NaCl, 5 mM KH_2PO_4, 1 mM dithiothreitol, 2 mM EDTA, 10^{-4} M phenylmethylsulfonyl fluoride, 10^{-6} M leupeptin, 10^{-6} M pepstatin A) and briefly homogenized (2 × 10 sec, setting medium) with an ULTRA-TURRAX disperser (IKA) followed by five strokes of a Potter-Elvehjem tissue grinder. The homogenate was centrifuged at 500 × *g* for 15 min, and the resulting supernatant was then centrifuged at 35,000 × *g* for 20 min. The pellet was resuspended by gentle homogenization in homogenization buffer at a concentration of 20–40 mg protein/ml. Membranes were frozen in liquid nitrogen and stored at −70° until use.

Measurement of IP_3. The intracellular mass of IP_3 was measured with a specific radioreceptor assay (41). Quiescent aortic SMC

in 35-mm petri dishes were stimulated with the indicated concentrations of AII for 15 sec at 37°. The incubation was terminated by the addition of 100 μl of cold 40% trichloroacetic acid (final concentration of 10%). After extraction for 10 min on ice, the cells were scraped and centrifuged at 1000 × *g* for 15 min at 4°. The resulting supernatant was extracted five times with 5 volumes of water-saturated diethyl ether and neutralized with 10 μl of 1 M $NaHCO_3$. An aliquot of 50–100 μl of cell extract was then assayed for IP_3 mass. For IP_3 binding assays, adrenocortical membranes (750 μg protein) were incubated with 1 nM [^{3}H]IP_3 and an aliquot of cell extract or varying concentrations of unlabeled IP_3 for 30 min at 4° in a total volume of 500 μl of IP_3 binding buffer (25 mM Tris·HCl, pH 8.5, 100 mM KCl, 20 mM NaCl, 5 mM KH_2PO_4, 1 mM EDTA, 0.1% bovine serum albumin). Bound [^{3}H]IP_3 was separated from free ligand by centrifugation at 13,000 × *g* for 5 min. The supernatant was removed by aspiration, and the radioactivity in the pellet was determined by liquid scintillation counting. Average values of duplicate determinations of bound [^{3}H]IP_3 were used for data analysis. The mass of IP_3 are expressed as pmol of IP_3 produced/mg of protein.

Measurement of intracellular Ca^{2+}. Aortic SMC were grown to 95% confluence on 22-mm round glass coverslips (Fisher Scientific) and synchronized in the quiescent state as described. The cells were loaded with the fluorophore Fura-2 by incubation at room temperature for 60 min in a HEPES-buffered solution (138 mM NaCl, 3.8 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM HEPES, pH 7.4) containing 2.5 μM Fura-2. The coverslips were then mounted in a specially designed incubation chamber (100 μl trough) set on the stage of an Olympus IMT-2 inverted microscope. Cells were perifused with bicarbonate-buffered Krebs-Henseleit (120 mM NaCl, 25 mM $NaHCO_3$, 3.8 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4) equilibrated with 95% O_2/5% CO_2 at 37°. Control buffer or buffer containing the indicated concentrations of drugs was delivered by an eight-way solenoid valve manifold (Flow Control Systems) through a heating tube ensuring rapid solution exchange without mixing or temperature changes. Fluorescence was monitored with a Deltascan model 4800 spectrofluorometer (Photon Technology) coupled to the microscope by fiber optics. Cells were alternatively excited at 350 and 380 nm, and the emitted light was collected through a band-pass filter at 505 nm. Fura-2 signals were calibrated *in situ* at the end of each experiment by perfusing solutions containing 5 μM ionomycin and 4 mM $CaCl_2$ (R_{max}) or 4 mM EGTA (R_{min}). Autofluorescence was then obtained by quenching with a solution containing 2 mM $MnCl_2$. $[Ca^{2+}]_i$ was calculated from the 350/380-nm fluorescence ratio after autofluorescence subtraction with the software provided by Photon Technology that is based on the method described by Grynkiewicz *et al.* (42).

Assay of $p44^{mapk}$ activity. The activity of $p44^{mapk}$ was measured by an immune-complex kinase assay as described (37). Quiescent aortic SMC in 60-mm petri dishes were stimulated with the indicated concentrations of AII for 5 min at 37°. The cells were then washed twice with ice-cold PBS and lysed in 0.4 ml of Triton X-100 lysis buffer for 25 min at 4°. Cell lysates were clarified by centrifugation at 13,000 × *g* for 10 min at 4°, and 100 μg of lysate proteins were incubated for 4 hr at 4° with 5 μl of antiserum SM1 preadsorbed to protein A-Sepharose beads. The immune complexes were washed three times with lysis buffer and once with MAP kinase assay buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM *p*-nitrophenylphosphate). MBP kinase activity was determined by resuspending the beads in a total volume of 40 μl of MAP kinase assay buffer containing 0.25 mg/ml MBP, 50 μM ATP, and 5 μCi [γ-^{32}P]ATP. Reactions were initiated with ATP, incubated at 30° for 10 min, and stopped by the addition of 2× Laemmli's sample buffer (1 × = 62.5 mM Tris · HC1, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, bromphenol blue, pH 6.8). The samples were analyzed by SDS-polyacrylamide gel electrophoresis on 12% acrylamide gels, and the band corresponding to MBP was excised and counted with a liquid scintillation counter. Protein kinase activities are expressed as pmol of phosphate incorporated into MBP/min/mg of lysate protein.

Northern blot analysis. Quiescent aortic SMC in 150-mm dishes were stimulated with 100 nM AII for 30 min at 37°. Total RNA was extracted with guanidinium thyocyanate as described previously (43). Equal amounts of total RNA (10 μg) were denatured by heating for 15 min at 65° in 2.2 M formaldehyde and 50% formamide and resolved by electrophoresis in a 1% agarose gel containing 1.8% formaldehyde. The RNA was transferred to Hybond-N (Amersham) nylon membranes by vacuum blotting, fixed, and hybridized with ^{32}P-labeled c-*fos* cDNA. Hybridization was carried out in hybridization medium [5× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate), 0.1% SDS, 5× Denhardt's solution (1× Denhardt's solution = 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 50% formamide, and 100 μg/ml herring sperm DNA] containing the labeled probe (1 × 10^6 cpm/ml) for 16 hr at 42°. The membranes were washed twice at 25° for 15 min in 2× SSC and 0.1% SDS and once at 60° for 30 min in 2× SSC and 0.1% SDS. The extent of hybridization was analyzed with a phosphorimager apparatus (Molecular Dynamics). The results were normalized to glyceraldehyde-3-phosphate-dehydrogenase mRNA.

The probes used were a 0.9-kb *Pst*I fragment of mouse c-*fos* cDNA (provided by Dr. Mona Nemer, Clincal Research Institute of Montreal) and a 1.2-kb *Xba*I/*Pst*I fragment of rat glyceraldehyde-3-phosphate-dehydrogenase cDNA (provided by Dr. Trang Hoang, Clinical Research Institute of Montreal) labeled by random priming.

Protein synthesis measurements. Quiescent aortic SMC in triplicate wells of 24-well plates were stimulated with indicated concentrations of growth factors in serum-free quiescence medium containing 0.5 μCi/ml [^{3}H]leucine. After 24 hr of stimulation, the medium was aspirated, and the cells were incubated for a minimum of 30 min in cold 5% trichloroacetic acid. The wells were then washed once with trichloroacetic acid and three times with tap water. The radioactivity incorporated into trichloroacetic acid-precipitable material was measured by liquid scintillation counting after solubilization in 0.1 M NaOH. For experiments with tyrosine kinase inhibitors, quiescent cells were treated with the indicated concentrations of either genistein for 30 min or herbimycin A for 18 hr before stimulation for 24 hr with growth factors in the continuous presence of the inhibitor.

Other methods. Protein concentrations were measured using the BCA protein assay kit (Pierce) with bovine serum albumin as standard. Dose-response curves were analyzed according to a four-parameter logistic equation using the ALLFIT computer program (44).

Results

AII stimulates protein tyrosine phosphorylation in aortic SMC. AII potently stimulates protein synthesis in rat aortic SMC, with a half-maximal effect observed at 0.5 nM (6). No effect on DNA synthesis was observed in these cells. To gain insight into the cellular mechanisms involved in this hypertrophic response, we examined the effect of AII on protein tyrosine phosphorylation. Quiescent cultures of aortic SMC were stimulated with 100 nM AII for different times and lysed. The cell lysates were subjected to immunoprecipitation with agarose-linked PY-20 antiphosphotyrosine mAb, and the immunoprecipitated proteins were analyzed by immunoblotting with the 4G10 antiphosphotyrosine mAb. As shown in Fig. 1, the addition of AII to quiescent aortic SMC caused a rapid increase in tyrosine phosphorylation of multiple cellular proteins. The most prominent substrates migrated with apparent molecular mass of 120–125 and 65–75 kDa. We (45) and others (46) have recently identified the M_r 65,000–75,000 band as the focal adhesion-associated protein paxillin. Other less prominent bands of 180, 80–85, and

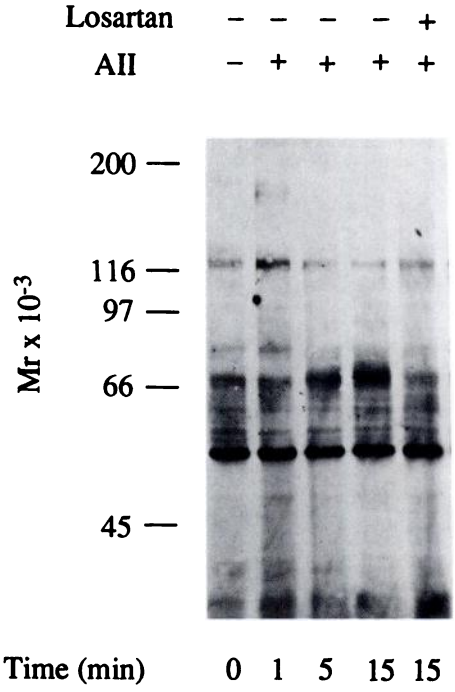

Fig. 1. AII stimulates protein tyrosine phosphorylation in rat aortic SMC. Quiescent rat aortic SMC were preincubated or not preincubated for 10 min with the AT_1-selective antagonist losartan (10^{-5} M) and then stimulated for the indicated times with 100 nM AII. Cells were lysed, and the lysates were subjected to immunoprecipitation with agarose-coupled anti-phosphotyrosine mAb PY-20. Proteins were resolved by SDS-polyacrylamide gel electrophoresis on 7.5% acrylamide gels and transferred to nitrocellulose membranes before analysis by immunoblotting with anti-phosphotyrosine mAb 4G10. Data are representative of four independent experiments with comparable results.

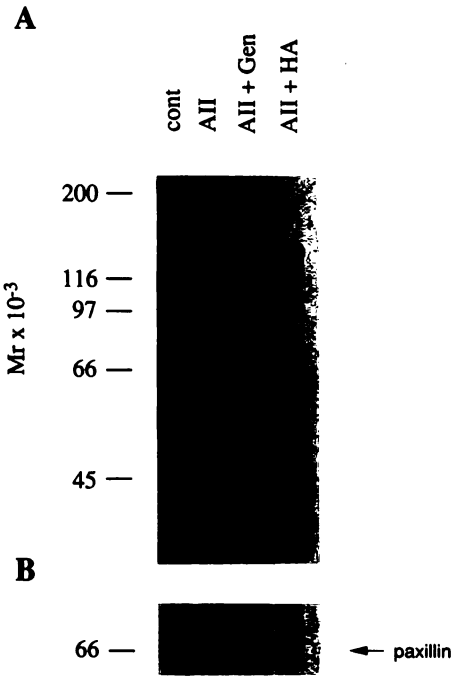

Fig. 2. Genistein (*Gen*) and herbimycin A (*HA*) inhibit the stimulatory effect of AII on tyrosine phosphorylation. Quiescent rat aortic SMC were pretreated in the absence or presence of 30 μM genistein for 30 min or 1 μM herbimycin A for 18 hr. Cells were then stimulated or not stimulated (*cont*) with 100 nM AII for 5 min. Tyrosine-phosphorylated proteins were immunoprecipitated with agarose-coupled PY-20 anti-phosphotyrosine mAb, resolved on 7.5% acrylamide gels, and transferred to nitrocellulose membranes. A, Membranes were analyzed by immunoblotting with anti-phosphotyrosine mAb 4G10. B, Membranes were analyzed by immunoblotting with anti-paxillin mAB 165. *Arrow*, position of paxillin. Similar results were obtained in three independent experiments.

50–55 kDa were also observed in some experiments. It is interesting to note that the kinetics of tyrosine phosphorylation stimulated by AII varied for the different substrates. The phosphorylation of the M_r 120,000–125,000 band was the most rapid, reaching a maximum at 1–5 min and declining to baseline level after 5–15 min. Incubation with losartan completely suppressed AII-induced tyrosine phosphorylation of all substrates, demonstrating that the AT_1 receptor is also linked to the stimulation of tyrosine phosphorylation (Fig. 1).

Genistein and herbimycin A inhibit AII-stimulated tyrosine phosphorylation without interfering with AII binding to the AT_1 receptor or AII-dependent phospholipase C activation. To investigate the role of tyrosine phosphorylation in the growth-promoting effects of AII, we used two chemically and mechanistically distinct tyrosine kinase inhibitors, genistein and herbimycin A, to block cellular tyrosine kinase activity. Genistein is a competitive inhibitor of ATP binding (47), whereas herbimycin A is believed to inactivate cellular tyrosine kinases by interacting with reactive sulfhydryl groups (48). We first determined whether the two tyrosine kinase inhibitors could prevent the increase in tyrosine phosphorylation induced by AII in aortic SMC. Quiescent cells were pretreated with 30 μM genistein for 30 min or 1 μM herbimycin A for 18 hr and then stimulated with 100 nM AII. Cell lysates were prepared, and tyrosine phosphorylated proteins were immunoprecipitated and analyzed by Western blotting. Fig. 2A shows that both genistein and herbimycin A abolished AII-stimulated tyrosine phosphorylation of the M_r 180,000, 120,000–125,000, and 65,000–75,000 bands. In the same experiment, the membrane was stripped and probed again with an mAb to paxillin. The addition of AII significantly increases the tyrosine phosphorylation of paxillin in quiescent aortic SMC, and this response was completely prevented by preincubation with either tyrosine kinase inhibitor (Fig. 2B). Otherwise, herbimycin A also reduced background tyrosine phosphorylation at high concentrations.

We then tested the specificity of genistein and herbimycin A action by examining their effects on the binding of AII to its receptor and the subsequent activation of phospholipase C. To evaluate the effect of the compounds on the binding properties of the AT_1 receptor, we performed competition binding studies with the agonist AII in membranes derived from either control aortic SMC or cells treated with 30 μM genistein or 1 μM herbimycin A. Computer analysis of AII binding data revealed that the two inhibitors neither changed the total number of AT_1 receptor sites nor significantly affected the slope factor of the curves or the proportion of high affinity sites (Fig. 3). The mean ED_{50} value was 3.9 ± 0.6 nM in untreated cells (slope factor = 0.85 ± 0.01), 3.4 ± 0.8 nM in cells treated with genistein (slope factor = 0.78 ± 0.01), and 3.6 ± 0.9 nM in herbimycin A-treated cells (slope factor = 0.85 ± 0.04). Next, we examined the effect of the tyrosine kinase inhibitors on AII-stimulated IP_3 production and Ca^{2+} mobilization. AII rapidly stimulated the formation of IP_3 in quiescent aortic SMC, with a maximal accumulation

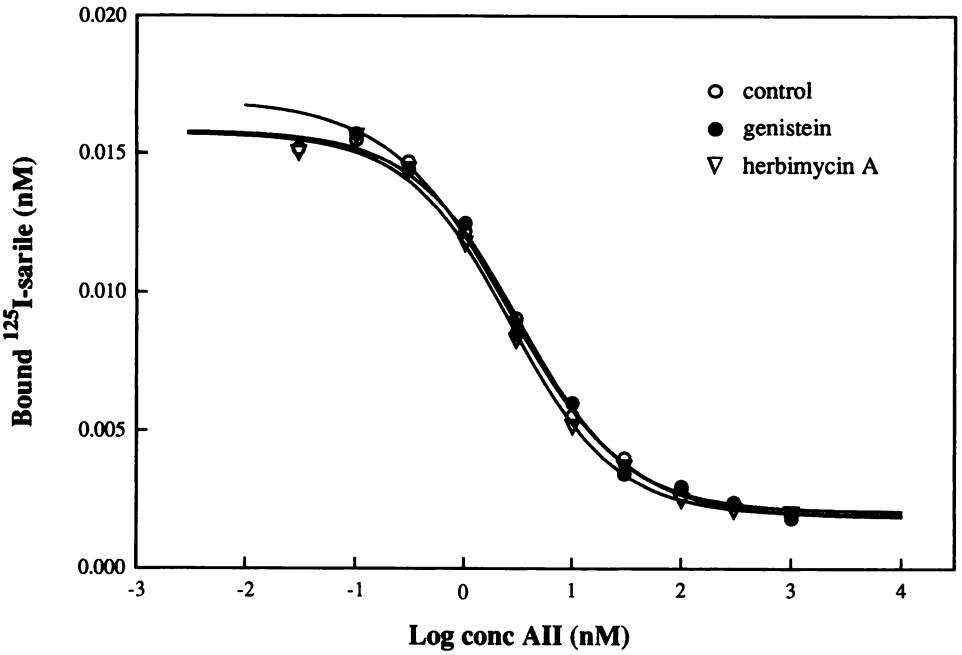

Fig. 3. Effects of genistein and herbimycin A on the binding properties of the AT_1 receptor. Rat aortic SMC were pretreated in the absence (*control*) or presence of 30 μM genistein for 30 min or 1 μM herbimycin A for 18 hr. Cell membranes were incubated for 1 hr at 25° with 0.2 nM [^{125}I]sarile and varying concentrations of AII. Bound [^{125}I]sarile was determined with rapid filtration on GF/B filters. Data for each curve were obtained from a single experiment with duplicate determinations of each point. *Solid lines*, computerized least-squares fit of the data. Data are representative of three independent experiments with similar results.

detected at ~15 sec.[1] As shown in Fig. 4A, pretreatment of the cells with genistein or herbimycin A did not prevent the rapid increase in the level of IP_3 induced by 100 nM AII. Detailed pharmacological studies further indicated that the two compounds had no effect on the dose dependence of AII to stimulate the production of IP_3 (Fig. 4B). As expected from these findings, the tyrosine kinase inhibitors also had little effect on AII-induced Ca^{2+} mobilization in these cells (Fig. 4C). The mean area under the Ca^{2+} curves, calculated over a 3-min period, was 9.92 ± 1.58 μM in untreated cells (13 experiments), 6.42 ± 2.42 μM in genistein-treated cells (6 experiments), and 9.61 ± 1.97 μM in cells treated with herbimycin A (7 experiments). The slight reduction in the calcium response observed in cells treated with genistein was not significant. These results are consistent with other reports showing that genistein does not inhibit the release of intracellular Ca^{2+} induced by AII in rat hepatocytes and vascular SMC (49, 50). Together, these results indicate that genistein and herbimycin A at concentrations that markedly inhibit AII-stimulated tyrosine phosphorylation do not interfere with AT_1 receptor function or phospholipase C activation.

Genistein and herbimycin A inhibit AII-stimulated protein synthesis in aortic SMC. To investigate the biological significance of AII-stimulated tyrosine phosphorylation in rat aortic SMC, we examined the effect of the tyrosine kinase inhibitors on the hypertrophic response to the hormone. For these experiments, quiescent aortic SMC were pretreated with various concentrations of genistein or herbimycin A before stimulation with 100 nM AII for 24 hr in the continuous presence of the drug. Fig. 5 shows that the two inhibitors completely blocked the increased rate of protein synthesis induced by AII in a dose-dependent manner. Half-maximal inhibition was observed at a concentration of 9 μM of genistein and 220 nM of herbimycin A. On the other hand, the biologically inactive structural analogue of genistein, daidzein, had little effect at the highest concentration tested (Fig. 5A). Genistein did not significantly affect the basal rate of protein synthesis at concentrations of <30 μM, whereas herbimycin A also reduced basal leucine incorporation in unstimulated cells at high concentrations. No cytotoxicity was observed at concentrations of genistein up to 100 μM and at concentrations of herbimycin A up to 1 μM (data not shown).

To exclude the possibility that genistein and herbimycin A exert nonselective inhibitory effects on the growth response, we tested their effects in aortic SMC exposed to the phorbol ester PMA, a potent activator of PKC isozymes. In control cells, PMA alone caused a 2.0-fold increase in [^{3}H]leucine incorporation at a concentration of 10 nM (Fig. 6). No significant effect of genistein was observed on the rate of protein synthesis stimulated by PMA at concentrations up to 10 μM (Fig. 6A). In cells pretreated with herbimycin A, the hypertrophic response to the phorbol ester was not affected at concentrations of the drug up to 0.3 μM (Fig. 6B). Analysis of dose-response curves confirmed that the effect of lower doses of PMA was not blocked by these concentrations of genistein or herbimycin A (Fig. 6C). These results strongly suggest that tyrosine phosphorylation has a critical role in the hypertrophic effect of AII on vascular SMC.

Genistein and herbimycin A do not prevent AII-stimulated MAP kinase activation and c-*fos* gene induction. AII potently stimulates the enzymatic activity of MAP kinases in vascular SMC (6, 51, 52). In view of the findings that genistein and herbimycin A inhibit AII-stimulated protein synthesis, we sought to determine whether the inhibitors could interfere with the signaling pathways leading to MAP kinases activation. Fig. 7A shows that pretreatment of aortic SMC with 30 μM genistein or 0.3 μM herbimycin A, concentrations that completely abolish the hypertrophic effect of AII, had no effect on the ability of the hormone to maximally activate MAP kinase. The two inhibitors also did not influence the dose dependence of AII to stimulate MAP kinase activity (Fig. 7B). As predicted from the mechanism of

[1] M. Servant and S. Meloche, unpublished observations.

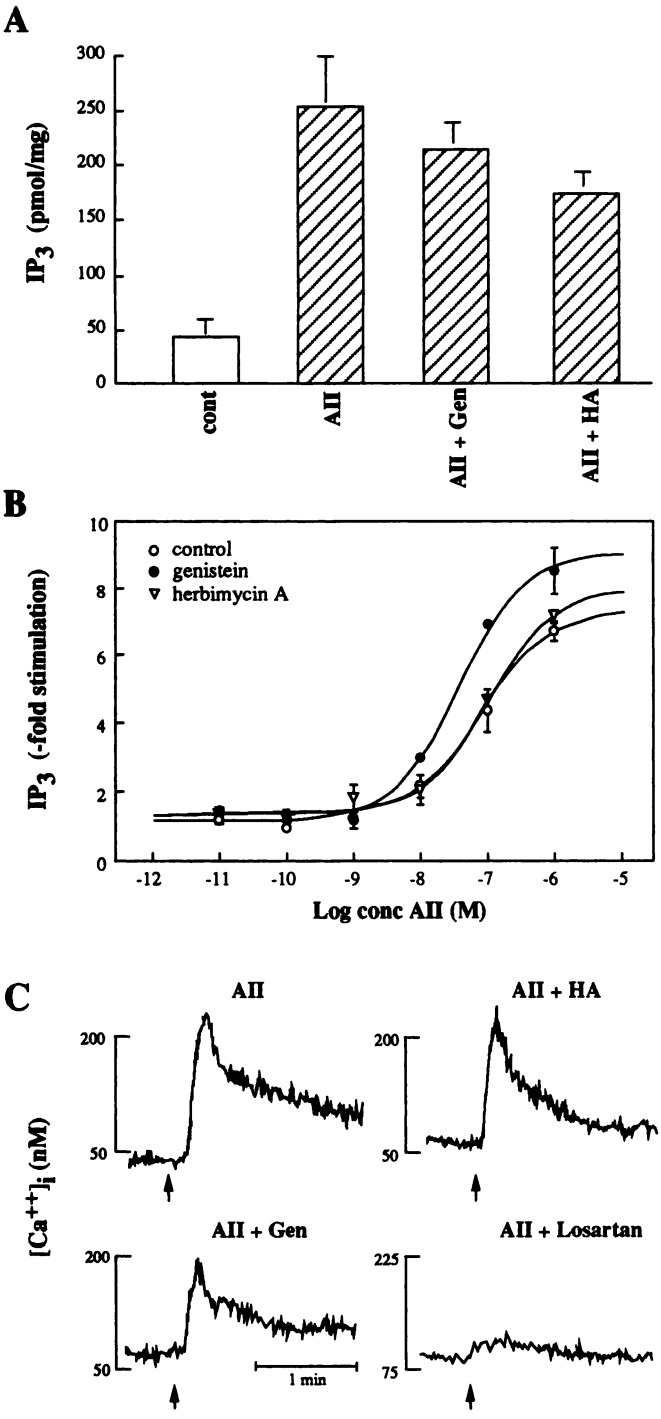

Fig. 4. Effects of genistein (*Gen*) and herbimycin A (*HA*) on the activation of phospholipase C. Quiescent rat aortic SMC were pretreated in the absence or presence of 30 μM genistein for 30 min or 1 μM herbimycin A for 18 hr before stimulation with AII as described. A, Bar graph of intracellular IP_3 mass. Cells were stimulated or not stimulated (*cont*) with 100 nM AII for 15 sec. Intracellular content of IP_3 was measured as described in Experimental Procedures. Results represent the mean ± standard error of triplicate determinations. Similar results were obtained in five separate experiments. B, Dose-response curves for the effect of AII on phospholipase C activity. Cells were stimulated or not stimulated (*control*) with the indicated concentrations of AII for 15 sec before IP_3 assays. Results are presented as -fold stimulation relative to the activity in quiescent cells. Similar results were obtained in two separate experiments. C, Traces of mobilization of Ca^{2+}. Cells were loaded with Fura-2 and then stimulated with 100 nM AII in the absence or presence of 10^{-5} M losartan. $[Ca^{2+}]_i$ was measured as described in Experimental Procedures.

activation of MAP kinases, treatment of cells with these concentrations of genistein and herbimycin A had no influence on the tyrosine phosphorylation of MAP kinase isoforms (Fig. 7C). We noted, however, that incubation with 1 μM herbimycin A reduced by 50% the activation of the enzyme by AII. The reason for this partial inhibition at high concentrations of herbimycin A is not known.

We also evaluated the effect of the compounds on induction of the early growth response gene c-*fos*. AII stimulation leads to increased c-*fos* mRNA expression in vascular SMC, and this response has been associated with the growth-promoting effects of the hormone (20–22). As shown in Fig. 8, neither genistein nor herbimycin A affected the induction of the c-*fos* gene in response to 100 nM AII or to lower concentrations of the peptide (not shown). Moreover, incubation with the tyrosine kinase inhibitors did not alter the kinetics of c-*fos* mRNA expression in AII-stimulated aortic SMC (data not shown). Thus, these data suggest that tyrosine phosphorylation has only a minor role, if any, in the regulation of the c-*fos* gene by AII.

Discussion

Protein tyrosine phosphorylation has recently been implicated in the action of G protein-coupled receptor agonists after the observation that these agonists increase tyrosine phosphorylation of multiple proteins in their target cells (28–34). However, in contrast to its well-defined and fundamental role in the action of receptor tyrosine kinases, the biological significance of tyrosine phosphorylation in G protein-coupled receptors signaling remains largely unknown. The observation that tyrosine kinase inhibitors can block the stimulation of DNA synthesis induced by thrombin (53, 54), endothelin (55), and bombesin (56) suggests that tyrosine phosphorylation might also have an important role in the mitogenic response to these agents. We examined the role of tyrosine phosphorylation in the hypertrophic effect of AII on vascular SMC and show that treatment of intact aortic SMC with tyrosine kinase inhibitors completely inhibits AII-induced tyrosine phosphorylation without significantly interfering with receptor binding, phospholipase C activation, MAP kinase activation, or c-*fos* gene induction. Most important, we demonstrate that the reduction of tyrosine phosphorylation is associated with complete inhibition of AII-stimulated protein synthesis in these cells.

In the present study, we used two distinct tyrosine kinase inhibitors, genistein and herbimycin A, to investigate the contribution of tyrosine phosphorylation in the hypertrophic response. The two inhibitors were selected on the basis of their different chemical nature, their high selectivity for protein tyrosine kinases, and their different mechanism of action. Genistein is an isoflavone derivative that acts as a competitive inhibitor of ATP binding to the kinase (47). This compound has been demonstrated to inhibit *in vitro* the tyrosine kinase activity of the EGF receptor and of nonreceptor tyrosine kinases such as $p60^{v\text{-}src}$ and $p110^{gag\text{-}fes}$ (47). In contrast, genistein scarcely inhibited the serine/threonine kinase activity of cAMP-dependent protein kinase, PKC, and phosphorylase kinase at concentrations up to 370 μM (47). In intact cells, genistein was found to block the stimulation of tyrosine phosphorylation by EGF (47), phytohemagglutinin (57), and lysophosphatidic acid (58). Herbimycin A is a ben-

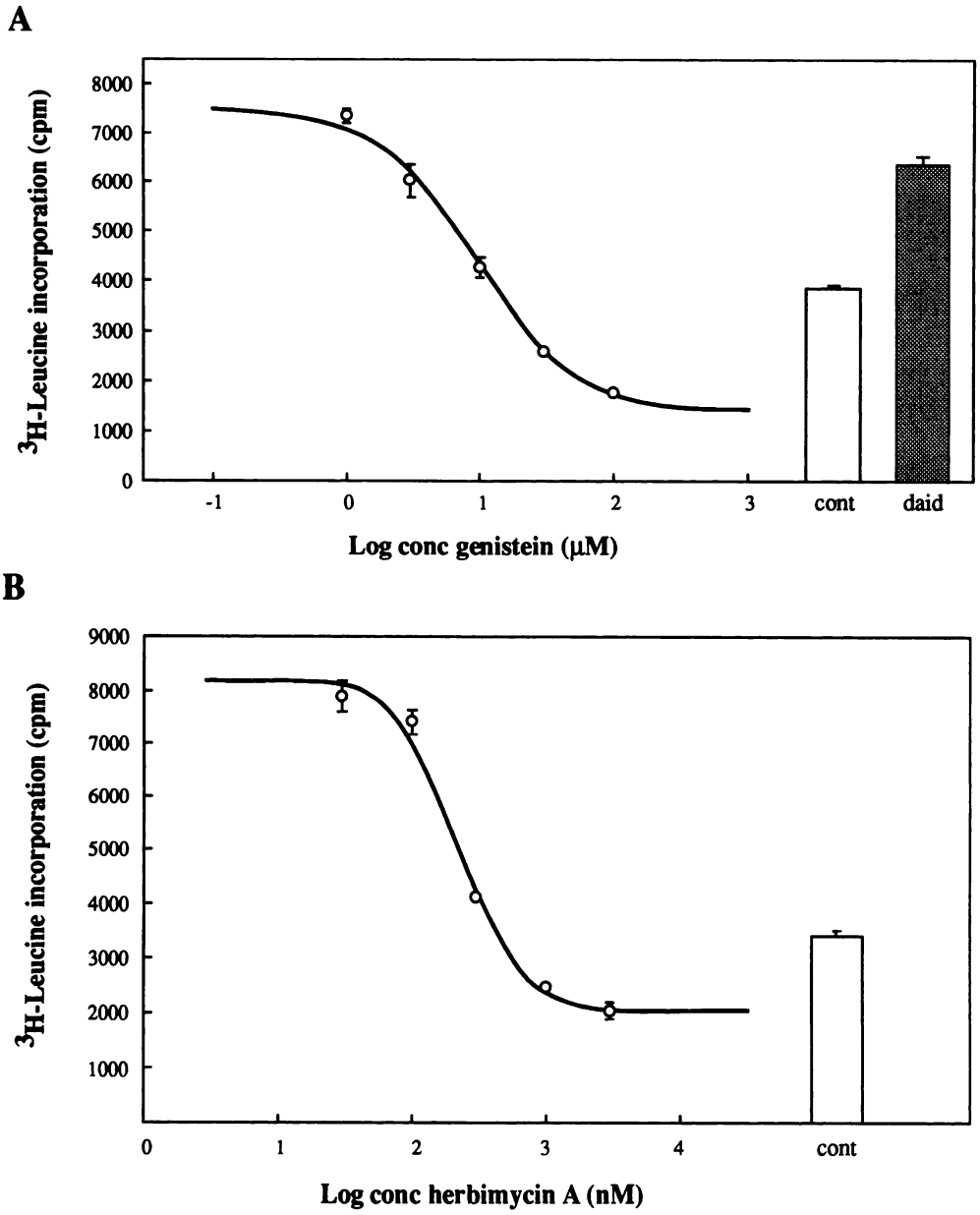

Fig. 5. Dose-dependent inhibition of AII-induced protein synthesis by genistein and herbimycin A. A, Quiescent rat aortic SMC were pretreated with the indicated concentrations of genistein for 30 min. B, Quiescent rat aortic SMC were pretreated with the indicated concentrations of herbimycin A for 18 hr. Cells were then stimulated for 24 hr with 100 nM AII in the continuous presence of the inhibitors. Protein synthesis was measured by [^{3}H]leucine incorporation. Each value represents the mean ± standard error of triplicate determinations. Basal rate of protein synthesis (*cont*) is shown. Biologically inactive analogue daidzein (*daid*) at 30 μM had little effect on the induction of protein synthesis by AII. Similar results were obtained in three independent experiments.

zoquinonoid antibiotic that binds to reactive sulfhydryl groups of tyrosine kinases (48, 59). This compound has been reported to block the tyrosine kinase activity of $p60^{v\text{-}src}$, $p210^{bcr\text{-}abl}$, and $p130^{v\text{-}fps}$ *in vitro* (48, 60) and to inhibit the transforming activity of tyrosine kinase oncogenes such as *src*, *yes*, *fps*, and *abl* (59, 61). Herbimycin A has also been shown to inhibit *in vivo* the induction of tyrosine phosphorylation by various growth factors, including anti-IgM (62), bacterial lipopolysaccharide (63), interleukin-3 (64), thrombin (54), and endothelin (55). Herbimycin A had no inhibitory effect on cAMP-dependent protein kinase or PKC (60) and did not reverse the transformed phenotype induced by the oncogenes *raf*, *ras*, and *myc* (61). We report that both genistein and herbimycin A potently inhibited AII-stimulated protein synthesis in aortic SMC. The inhibition was dose dependent and was complete at concentrations known to be selective for tyrosine kinases. Identical concentrations of daidzein, a biologically inactive analogue of genistein, did not significantly affect the stimulatory effect of AII. These results strongly support the premise that tyrosine phosphorylation has a role in cell hypertrophy and is involved in the action of AII. However, it is still theoretically possible that genistein or herbimycin A reduces protein synthesis by other mechanisms. For example, a recent report demonstrated that genistein inhibits certain metabolic effects of insulin in rat adipocytes without inhibiting the insulin receptor tyrosine kinase (65). Obviously, the observation that two mechanistically distinct tyrosine kinase inhibitors produce similar inhibitory effects favors a major involvement of tyrosine phosphorylation in this response.

The site of action and the specificity of the tyrosine kinase inhibitors were investigated by considering the binding properties and early signaling events of AII. We first demonstrated that genistein and herbimycin A did not modify the density of AT_1 receptors or its affinity for AII in competition binding assays. The best documented signaling pathway coupled to the AT_1 receptor is the activation of phospholipase C, which generates the intracellular second messengers IP_3 and

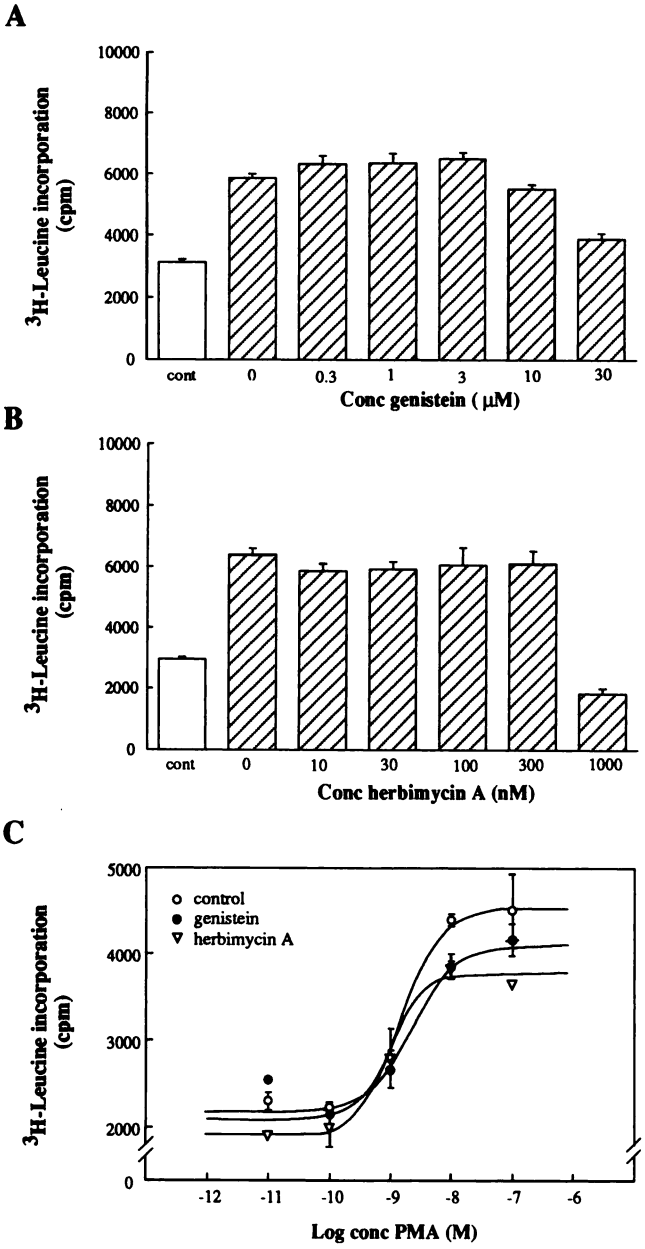

Fig. 6. Effects of genistein and herbimycin A on the hypertrophic effect of PMA in rat aortic SMC. A, Quiescent rat aortic SMC were pretreated with the indicated concentrations of genistein for 30 min. B, Quiescent rat aortic SMC were pretreated with the indicated concentrations of herbimycin A for 18 hr. Cells were then stimulated for 24 hr with 10 nM PMA in the presence of the inhibitor drugs. Protein synthesis was measured by [^{3}H]leucine incorporation. Each value represents the mean ± standard error of triplicate determinations. Basal rate of protein synthesis (*cont*) is shown. Similar results were obtained in three independent experiments. C, Dose-response curves for the stimulatory effect of PMA on protein synthesis. Quiescent rat aortic SMC were pretreated in the absence (*control*) or presence of 10 μM genistein for 30 min or 0.3 μM herbimycin A for 18 hr. Cells were then stimulated for 24 hr with the indicated concentrations of PMA. Protein synthesis was measured as described. Similar results were obtained in two separate experiments.

diacylglycerol (18, 19). We show that the two inhibitors did not significantly interfere with the formation of IP_3 and the subsequent mobilization of calcium. These results confirm and extend previous observations showing that genistein does not inhibit the mobilization of intracellular Ca^{2+} and

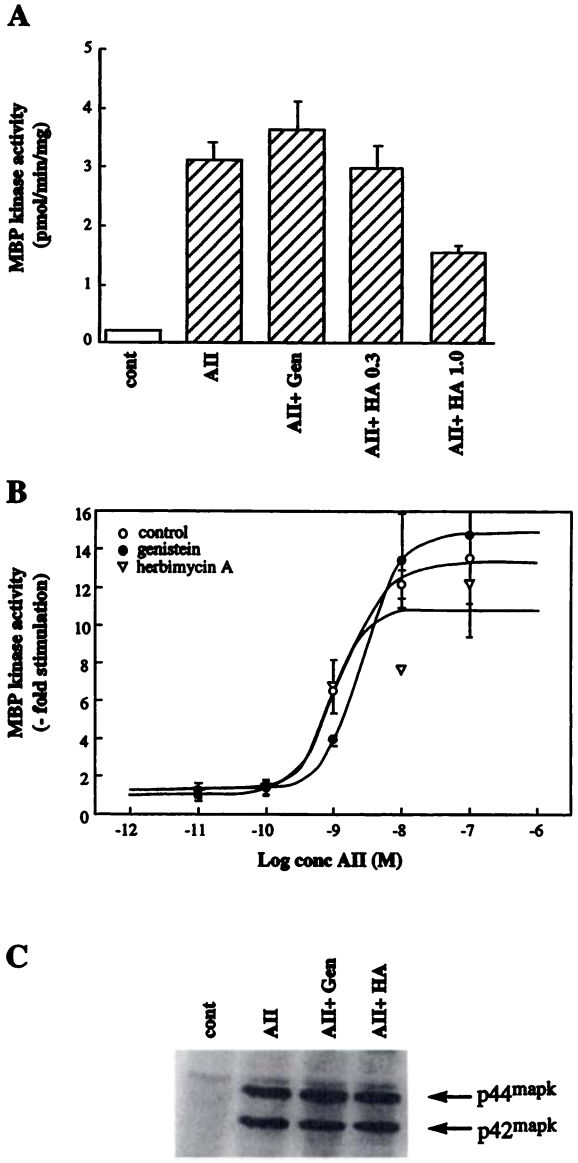

Fig. 7. Effects of genistein (*Gen*) and herbimycin A (*HA*) on AII-stimulated MAP kinase activation in rat aortic SMC. A, Quiescent rat aortic SMC were pretreated in the absence or presence of 30 μM genistein for 30 min or the indicated concentrations of herbimycin A (HA) for 18 hr. Cells were then stimulated or not stimulated (*cont*) with 100 nM AII for 5 min. Cell lysates were prepared, and $p44^{mapk}$ was immunoprecipitated with antiserum SM1 preadsorbed to protein A-Sepharose beads. Immune complexes were washed, and phosphotransferase activity was assayed with MBP as a substrate (see Experimental Procedures). Enzymatic activities are expressed as pmol of PO_4 incorporated into the substrate/min/mg of lysate protein and represent the mean ± standard error of duplicate determinations. Data are representative of five independent experiments with comparable results. B, Dose-response curves for the stimulatory effect of AII on $p44^{mapk}$ activity. Quiescent rat aortic SMC were pretreated in the absence (*control*) or presence of 30 μM genistein for 30 min or 0.3 μM herbimycin A for 18 hr. Cells were then stimulated with the indicated concentrations of AII for 5 min. Enzymatic activity of $p44^{mapk}$ was measured as described. Results are presented as -fold stimulation relative to the activity in quiescent cells. Similar results were obtained in two separate experiments. C, Immunoblot analysis of MAP kinases tyrosine phosphorylation. Quiescent rat aortic SMC were treated as in B before stimulation or without stimulation (*cont*) with 100 nM AII for 5 min. Equal amounts of lysate proteins (90 μg) were resolved by SDS-polyacrylamide gel electrophoresis on 7.5% acrylamide gel and transferred to nitrocellulose membrane. Membrane was probed with anti-phosphotyrosine mAb 4G10, and the proteins were visualized with chemilluminescence detection. *$p44^{mapk}$* and *$p42^{mapk}$*, Positions of isoforms.

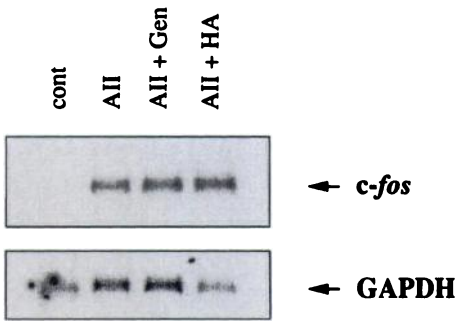

Fig. 8. Effect of genistein (*Gen*) and herbimycin A (*HA*) on AII-induced c-*fos* gene expression. Quiescent rat aortic SMC were pretreated in the absence or presence of 30 μM genistein for 30 min or 1 μM herbimycin A for 18 hr. Cells were then stimulated or not stimulated (*cont*) with 100 nM AII for 30 min. Total RNA was extracted from the cells and analyzed by Northern hybridization with a ^{32}P-labeled c-*fos* cDNA fragment as described in Experimental Procedures. Results were normalized by rehybridization of the membrane with a glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) probe. Extent of hybridization was visualized with phosphorimaging.

the accumulation of diacylglycerol induced by AII in rat liver cells (49) and vascular SMC (49). In contrast, genistein and herbimycin A were shown to markedly inhibit the stimulation of inositol-phosphate formation and Ca^{2+} signaling by platelet-derived growth factor (49, 50), T cell receptor/CD3 activation (57, 62), IgM cross-linking (62), and platelet-activating factor (66). Together, these findings indicate that (a) the inhibitory effects of genistein and herbimycin A are not due to cell injury because early signaling events of AII are left intact, and (b) the inhibitory action of the two compounds does not result from interference with phospholipase C activation. The observation that much higher concentrations of genistein and herbimycin A are required to inhibit the increased rate of protein synthesis stimulated by PMA further indicate that PKC is not the primary target of these compounds.

The biochemical events leading to AII-stimulated tyrosine phosphorylation and the identity of the tyrosine kinase(s) remain to be determined. Previous studies by Huckle *et al.* (32, 67) have shown that the stimulatory effect of AII on tyrosine phosphorylation in liver epithelial cells was dependent on Ca^{2+} mobilization but not PKC activation. Other studies in renal mesangial cells (28) and vascular SMC (34) have suggested a partial role for PKC in AII-induced tyrosine phosphorylation. More recently, we identified the focal adhesion-associated protein paxillin as the prominent tyrosine-phosphorylated substrate of AII in aortic SMC (45). We found that AII-induced paxillin phosphorylation was completely dependent on the activation of phospholipase C. Chelation of $[Ca^{2+}]_i$ completely abolished the ability of AII to stimulate tyrosine phosphorylation of paxillin, whereas selective inhibition of PKC partially attenuated the response. In contrast, neither mobilization of $[Ca^{2+}]_i$ nor activation of PKC appears to contribute to the stimulation of tyrosine phosphorylation by the G protein-coupled receptor agonists bombesin and lysophosphatidic acid (68–71). The identification of the protein tyrosine kinases involved in the action of these different agonists will help determine whether their mode of activation is specific to the agonist or to particular cell types.

Very little is known regarding the downstream targets coupling the tyrosine phosphorylation pathway to the trophic effect of AII. As mentioned, it was recently shown that AII potently stimulates tyrosine phosphorylation of the cytoskeletal protein paxillin in aortic SMC (45, 46). Interestingly, paxillin was also found to be a prominent tyrosine phosphorylated substrate of bombesin (70) and lysophosphatidic acid (71) in Swiss 3T3 cells, but its relationship to growth control remains to be established. Given their central role in the integration of growth factor signaling pathways, MAP kinases represent potential downstream effectors of AII-activated protein tyrosine kinase(s). Moolenaar *et al.* (58) suggest that a putative pertussis toxin- and genistein-sensitive protein tyrosine kinase mediates the activation of $p21^{ras}$ and MAP kinase by the G protein agonist lysophosphatidic acid in Rat-1 fibroblasts. Consistent with this possibility, AII has been shown to stimulate the phosphorylation and enzymatic activation of MAP kinase isoforms in vascular SMC (6, 51, 52). However, our results clearly show that both genistein and herbimycin A inhibit AII-induced protein synthesis at concentrations that do not interfere with MAP kinase activation. We also examined the expression of the c-*fos* gene in cells treated with the tyrosine kinase inhibitors. AII stimulation is known to increase c-*fos* mRNA expression in vascular SMC (20–22), although there is no evidence that induction of c-*fos* gene is required for the hypertrophic response. Interestingly, treatment with herbimycin A and tyrphostin was found to strongly attenuate the induction of c-*fos* gene by endothelin (55) and bombesin (56), respectively. In contrast to these studies, we did not observe any inhibitory effect of genistein and herbimycin A on AII-induced c-*fos* mRNA expression, indicating that tyrosine phosphorylation is unlikely to be involved in the regulation of the c-*fos* gene by AII. Taken together, these findings support the notion that G protein-regulated tyrosine kinases may use specific downstream effectors in response to different agonists. An interesting possibility is that distinct targets may specifically be involved in the response to hypertrophic agents such as AII or mitogenic agents such as bombesin and lysophosphatidic acid.

In conclusion, we propose that tyrosine phosphorylation is part of the signal transduction pathways that mediate the growth-promoting effects of AII on vascular SMC. Further experiments are required to address the regulation of this pathway and to identify the putative tyrosine kinase(s) involved in AII action.

Acknowledgments

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