0026-895X/94/040653-07$03.00/0

MOLECULAR PHARMACOLOGY, 46:653-659, 1994.

Down-regulation by Growth Factors of Vascular Smooth Muscle Angiotensin Receptor Gene Expression

GEORG NICKENIG and T. J. MURPHY

Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322

Received April 8, 1994; Accepted July 12, 1994

SUMMARY

The effects of epidermal growth factor, basic fibroblast growth factor, and platelet-derived growth factor-BB on angiotensin type 1 (AT1) receptor gene expression were examined in rat thoracic aorta vascular smooth muscle cells (VSMC) in culture. Incubation of serum-deprived VSMC with 20 ng/ml epidermal growth factor, 20 ng/ml basic fibroblast growth factor, or 50 ng/ml platelet-derived growth factor-BB reduces AT1 receptor mRNA levels, as assessed by Northern hybridization analysis, to approximately 30% of control levels. This effect is maximal 4 hr after addition of each growth factor to the culture medium and is sustained for up to 24 hr of incubation after a single dose. There is a correlative loss of membrane-associated AT1 receptors and angiotensin II-stimulated inositol phosphate production after 24 hr of growth factor treatment. The half-life of AT1 receptor mRNA is reduced significantly by growth factors, compared with that for cells treated with actinomycin D alone to block transcription. This suggests that growth factors activate a mechanism that involves post-transcriptional destabilization of AT1 receptor mRNA. This effect can be blocked by prior treatment of VSMC with actinomycin D or cycloheximide, suggesting that the effect of the growth factors on AT1 receptor gene expression is mediated through induction of an unknown gene or genes that function to destabilize AT1 receptor mRNA and that mRNA translation is essential for the destabilizing effect. Nuclear run-on assays reveal that the growth factors also significantly reduce the rate of *de novo* AT1 receptor gene transcription. Thus, down-regulation of AT1 receptor gene expression by growth factors also appears to involve mechanisms that decrease the rate of AT1 receptor gene transcription. These data reveal marked down-regulation of AT1 receptor gene expression in VSMC by growth factor receptor activation, through mechanisms that involve both attenuation of transcription and post-transcriptional mRNA destabilization.

Chronic diseases such as hypertension, diabetes, and atherosclerosis are associated with abnormalities in vascular function. These can be manifested as changes in VSMC ultrastructure, gene expression patterns, and growth properties. A pathophysiological role for numerous mitogenic factors acting upon smooth muscle cells is becoming increasingly clear. Mitogenic factors elaborated within the blood vessel wall by invading leukocytes, macrophages, and platelets, in addition to sources from vascular cells, are known to profoundly influence the properties of VSMC. Timely and extensive reviews of this field by Jackson and Schwartz (1), by Bobik and Campbell (2), and by Ross (3) have been published recently.

The mitogenic factors EGF, bFGF, and PDGF-BB have, among other properties, the ability to induce de-differentiation, proliferation, and chemotaxis of VSMC *in vitro* and *in vivo* (4–8). Their effects are transduced by cognate cell surface receptors, which initiate signaling cascades that include tyrosine-directed phosphorylation and the activation of *ras* and phospholipase C pathways (see Ref. 2). The AT1 receptor is a G protein-coupled receptor expressed in several tissues and cellular phenotypes in vertebrates (9). However, VSMC are principal physiological effector targets for circulating angiotensin II. Indeed, the acute pressor responses of vertebrates to increments of angiotensin II result from activation of smooth muscle cell AT1 receptors (10).

In vascular injury models *in vivo*, angiotensin II has been shown to enhance smooth muscle cell proliferation, whereas angiotensin-converting enzyme inhibitors and Dup753, an AT1 receptor-selective antagonist, have been shown to attenuate blood vessel smooth muscle cell proliferation, migration, and intimal thickening (11–13). Irrespective of the possible involvement of AT1 receptors in these responses, the growth factors bFGF and PDGF have been implicated as significant mediators

This work was supported by National Institutes of Health Grant HL48252 and by the American Heart Association, Georgia Affiliate. G.N. is supported by Deutsche Forschungsgemeinschaft Grant DFG Ni398/1-1.

ABBREVIATIONS: VSMC, vascular smooth muscle cell(s); EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; PDGF-BB, platelet-derived growth factor BB; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; PDGF, platelet-derived growth factor; AT1 receptor, angiotensin type 1 receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

of VSMC proliferation in these models (5, 14). Because of this, as well as continuing controversy (1) regarding whether angiotensin II is a direct mitogenic factor or has an indirect mode of action, it is of interest to explore to what extent growth factors may alter the responsiveness of smooth muscle cell AT1 receptors. Studies were conducted to investigate the effects of the growth factors EGF, bFGF, and PDGF-BB on AT1 receptor gene expression in cultured VSMC. These results demonstrate that VSMC AT1 receptor gene expression is markedly downregulated after growth factor receptor activation. This appears to result from two complementary mechanisms. The first involves the induction by the growth factors of an unknown gene or genes that function to destabilize AT1 receptor mRNA. Superimposed upon this response is growth factor-mediated attenuation of *de novo* AT1 receptor gene transcription. Ultimately, this growth factor response leads to a significant decrease in the expression and function of AT1 receptors in the cell membrane.

Experimental Procedures

Materials. Growth factors, cycloheximide, salts, and buffers were purchased from Sigma Chemical Co. (St. Louis, MO) or Life Technologies (Gaithersburg, MD). Antibiotics, actinomycin D, serum, and cell culture media were from Life Technologies. Hybond N membranes, [^{32}P]UTP, [^{32}P]dCTP, and Na^{125}I were purchased from Amersham (Arlington Heights, IL). TRI-reagent was from Molecular Research Center (Cincinnati, OH), and angiotensin peptides were from Bachem (Torrance, CA) or from Sigma. *myo*-[^{3}H]Inositol was purchased from American Radiolabeled Chemicals (St. Louis, MO). Dup753 was a gift from DuPont-Merck. Oligonucleotides were synthesized using Millipore chemicals, with a Millipore Cyclone Plus automated DNA synthesizer.

Cell culture. VSMC, generously provided by the laboratory of R. Wayne Alexander (Emory University, Atlanta), are a continuous cell line obtained from a primary culture of rat thoracic aorta (15). These were grown in a 5% CO_2 atmosphere at 37° in DMEM supplemented with 10% heat-inactivated calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. VSMC were passaged by harvesting with 0.25% trypsin-versine and seeding at a 1:6 ratio. Experimental cells (passages 10–20) were seeded onto 60- or 100-mm dishes, grown to confluence, and deprived of serum for 24 hr by incubation in serum-free medium (DMEM with 100 units/ml penicillin and 100 μg/ml streptomycin). Growth factors were dissolved in sterile phosphate-buffered saline; actinomycin D and cycloheximide were dissolved in sterile 100% ethanol before addition to culture dishes at a 100× concentration.

mRNA isolation and Northern analysis. After experimental treatments, culture medium was aspirated and the cells were lysed *in situ* with 1 ml of TRI-reagent, scraped, and processed according to the manufacture's protocol, to obtain total cellular RNA. This was quantified spectrophotometrically by measuring absorbance values at 260 and 280 nm. Ten-microgram aliquots were electrophoresed through 1.2% agarose-0.67% formaldehyde gels and stained with ethidium bromide to verify the quantity and quality of the RNA. After capillary transfer to Hybond N membranes in 20× SSC (1× = 150 mM sodium chloride, 15 mM sodium citrate), the RNA was cross-linked to the membranes using a Stratalinker 2400 (Stratagene, La Jolla, CA). The ethidium staining patterns on these blots were inspected after cross-linking, to ensure uniform nucleic acid transfer. Those blots for which this was not evident were excluded from further study. Northern blots were prehybridized for 2 hr at 42° in a buffer containing 50% deionized formamide, 0.5% SDS, 6× SSC, 10 μg/ml denatured salmon sperm DNA, and 5× Denhardt's solution and were then hybridized for 16 hr at 42° with a random-primed, [^{32}P]dCTP-labeled, rat AT1 receptor cDNA probe, in the same buffer except without Denhardt's solution. The rat AT1 receptor cDNA probe was a 824-base pair fragment generated from the pCa18b template (16) by the polymerase chain reaction, using the primer pair 5′-GTCATGATCCCTACCCTCTACAGC-3′ and 5′-CCGTAGAACAGAGGGTTCAGGCAG-3′ and Vent polymerase (New England Biolabs, Beverly, MA). The hybridized filters were washed for 15 min at room temperature with 2× SSC and for 15 min at 50° with 2× SSC/0.1% SDS and were exposed for 4–12 hr to Hyperfilm (Amersham) at −80°.

Laser densitometry. A volume integration protocol was used to quantify the autoradiographic hybridization signals with a personal densitometer and the Imagequant program from Molecular Dynamics (Santa Clara, CA). Hybridization signal borders of scanned autoradiograms were marked manually by rectangulation, total pixel values within a signal area were summed, and pixel values within an identically sized, rectangular background area on the same film were subtracted. For comparisons of treatments and for presentation, these integrated volumes were normalized as a percentage of the hybridization values from control, vehicle-treated cells.

Nuclear run-on assays. These assays are a slight modification of protocols developed by Groudine and colleagues (17). After a 4-hr incubation of VSMC with growth factors, cells grown on five 100-mm dishes were dispersed with trypsin, collected, and washed with 150 mM KCl, 4 mM magnesium acetate, 10 mM Tris·HCl, pH 7.4. After centrifugation in a Beckman GS-GR tabletop centrifuge, using a GH 3.8 rotor (1200 rpm, for 5 min, at 4°), the cell pellet was resuspended in 2 ml of the same buffer containing 0.5% Nonidet P-40 (Sigma). After lysis for 10 min on ice, the nuclei were isolated by centrifugation (2000 rpm, for 10 min, at 4°) through 4 ml of 0.6 M sucrose, with the same equipment. The supernatants were carefully removed and the nuclear pellet was resuspended in a buffer containing 40% glycerol, 50 mM Tris, 5 mM $MgCl_2$, and 0.1 mM EDTA. These were stored at −80° until used for assays. Nuclei (~1–3 × 10^7 nuclei/reaction) were used to carry out the transcription, in a reaction mixture containing 40% glycerol, 50 mM Tris, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM levels of CTP, GTP, and ATP, and 0.2–0.3 μM [^{32}P]UTP (>3000 μCi/mmol), at 30° for 30 min. Reactions were terminated by addition of 800 μl of TRI-reagent, and the radioactive RNA was isolated and purified by collection of the eluate from a Bio-Rad P-30 spin column. Approximately 5 × 10^6 to 2 × 10^7 cpm of the [^{32}P]UTP-labeled RNA were dissolved in hybridization solution (100 mM TES, pH 7.4, 0.3 M NaCl, 100 μg/ml *Escherichia coli* tRNA). Plasmids (5 μg) containing cDNAs for the AT1 receptor (a *Hind*III-*Not*I cDNA insert from pCa18b subcloned in pKS$^+$-Bluescript) (16) or GAPDH (rat GAPDH in pIBI30; International Biotechnology, New Haven, CT) and a plasmid (KS$^+$-Bluescript) without insert were linearized, denatured, and applied to nylon membranes using a slot-blot apparatus. These membranes were prehybridized for 2 hr at 42° in 100 mM TES, 0.3 M NaCl, 100 μg/ml *E. coli* tRNA, 5× Denhardt's solution, and were hybridized at 42° for 16 hr. Membranes were washed for 10 min at room temperature in 2× SSC and for 15 min at 50° in 2× SSC/0.1% SDS. The filters were exposed to film for 12–48 hr, and autoradiographic signals were quantified by laser densitometry.

Membrane preparation and binding assays. VSMC grown and treated on 100-mm diameter dishes were rinsed three times with phosphate-buffered saline, pH 7.4, and collected by scraping with a rubber policeman. After a brief centrifugation at 4° in a microfuge, the pellets were resuspended in 1 ml of ice-cold 50 mM Tris·HCl, pH 8.0, and homogenized by repeated trituration through a 22-gauge needle. The membranes were pelleted by centrifugation at 12,000 rpm for 10 min at 4° in a microcentrifuge. The resulting pellet was washed and recentrifuged once. ^{125}I-[Sar^1,Ile^8]-Angiotensin II was synthesized from Na^{125}I (2175 Ci/mmol) and [Sar^1,Ile^8]-angiotensin II using chloramine T. After an initial purification on a Sep-Pak cartridge (Millipore) (18), monoiodinated peptide was separated from other reaction products by high performance liquid chromatography on a Vydak 0.46- × 25-cm, 5-μm, C18 column, using a linear gradient of 6–60% acetonitrile in water, with 0.1% trifluoroacetic acid in both phases, over 45 min, at a flow rate of 1 ml/min. The monoiodinated peptide peak eluted routinely at

45% acetonitrile. Membrane proteins were determined by the Bradford method, using a Bio-Rad kit and bovine γ-globulin as a standard. For binding assays 2 μg of membrane protein were resuspended in a buffer containing 0.1% bovine serum albumin, 25 mM Tris, pH 7.4, and 5 mM $MgCl_2$, in a final assay volume of 250 μl. Total and nonspecific binding points were measured in triplicate or duplicate, respectively. Nonspecific binding was determined in the presence of 10 μM Dup753. The samples were incubated for 60 min at 20° after addition of radioligand, followed by aspiration over 0.25% polyethyleneimine-treated GF/B filter strips and three washes with ice-cold 50 mM Tris·HCl, pH 8.0, using a Brandel cell harvester. The samples were counted for 1 min in a Beckman 4000 γ counter at an efficiency of 80%.

Inositol phosphate assays. Confluent VSMC seeded on 24-well cell culture plates were deprived of serum for 24 hr in the presence of 1 μCi/ml *myo*-[^{3}H]inositol. Vehicle or bFGF, to a final concentration of 20 ng/ml, was added for an additional 24-hr incubation. After this, the cells were rinsed three times with DMEM containing 20 mM HEPES, pH 7.4, and 10 mM LiCl. The cells were then incubated with angiotensin II in the same medium for 60 min at 37°. The reactions were stopped by aspiration of the medium and addition of 0.5 ml of ice-cold 20 mM formic acid. The plates were frozen and thawed three times before 50 μl of each sample were removed and extracted with chloroform. The organic phase from each sample was dried, redissolved in scintillation fluid, and counted, to determine label incorporation into the lipid pool. The remainders of the samples were overlaid onto 0.5-ml bed volumes of Dowex AG 1X8 ion exchange resin (200–400 mesh, formate form). After 10-ml washes each with deionized water and 5 mM sodium borate/60 mM sodium formate mixture, total inositol phosphates were eluted into scintillation vials with 3 ml of 0.1 M formic acid/2 M ammonium formate. The data are presented as a percentage of inositol lipid hydrolysis (cpm in total inositol phosphate pool divided by cpm × 10 in organic pool).

Results

The mitogenic and functional responsiveness of rat thoracic aortic smooth muscle cells to the concentrations of EGF, bFGF, and PDGF-BB used in this study has been characterized extensively by others (4, 19, 20). For the present studies, these VSMC were cultured under conditions similar to those reported previously. Under these conditions AT1 receptor protein levels and mRNA levels can be detected readily by standard radioligand binding and Northern hybridization methodology. Serum was removed from the culture medium 24 hr before initiation of experimental treatments, to obviate its effects.

Fig. 1 illustrates representative autoradiographic results from Northern hybridizations of a rat vascular AT1 receptor cDNA probe to 10 μg of electrophoretically separated, total cellular RNA extracted from VSMC at various time points after addition of the growth factors to the culture medium. The probe hybridizes to an abundant 2.2-kilobase transcript and a minor 3.2-kilobase transcript, as observed previously (16), which represent alternatively processed transcripts from a single gene (21). For the purposes of quantification, only the autoradiographic signals generated from hybridization to the 2.2-kilobase transcript were measured and compared in this study. However, the expression of the two transcripts is reduced similarly by growth factor treatment. These autoradiograms reveal a significant time-dependent attenuation of the transcript levels. This is evident by inspection of the hybridization pattern in response to each of the growth factors, wherein the AT1 receptor mRNA signal appears nearly maximally reduced 4 hr after exposure to the growth factor and this reduction is sustained for up to 24 hr. RNA was also obtained from control, vehicle-treated cells 4 hr, 12 hr, and 24 hr after growth factor addition in all experiments. The hybridization signals for these samples are not significantly different from that for the 0-hr control. Thus, the loss in AT1 receptor mRNA hybridization signal in the growth factor-treated cells does not result from a nonspecific degradation of the system over the time course of the experimental protocol. Also shown in Fig. 1 is hybridization of a GAPDH cDNA probe to the same Northern blot of RNA from PDGF-BB-treated cells. Compared with the AT1 receptor mRNA signal, GAPDH mRNA appears relatively stable over the time course of the assay. Using GAPDH mRNA as a reference, these observations indicate that growth factors selectively down-regulate the expression of the vascular AT1 receptor mRNA in this system.

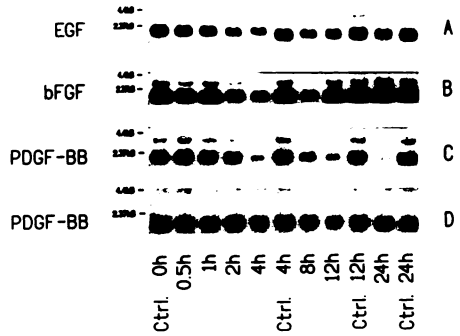

Fig. 1. Representative autoradiograms of Northern hybridizations after VSMC treatment with growth factors. A, B, and C, Hybridization of an AT1 receptor cDNA probe to Northern blots of 10 μg of total RNA extracted from cells treated for the indicated times with 20 ng/ml EGF, 20 ng/ml bFGF, or 50 ng/ml PDGF-BB, respectively. D, Hybridization of a GAPDH cDNA probe to the same blot as shown in C, after the AT1 receptor cDNA probe was stripped off. In every experiment, RNA was extracted from cells treated with vehicle (*Ctrl.*) in parallel with the growth factors at the 0-, 4-, 12-, and 24-hr time points. The 4.4-kilobase and 2.37-kilobase markers indicate the relative positions of the 28 S and 18 S rRNA bands, respectively. Each *panel* is representative of three separate experiments for each growth factor and probe.

Autoradiographic data, generated from at least three separate experiments for each growth factor, were analyzed by laser densitometry. Fig. 2 graphically shows the growth factor-induced reduction in the AT1 receptor mRNA hybridization signal, relative to vehicle-treated control levels. This method of analysis reveals that each growth factor induces the same relative effect, with maximum attenuation being achieved 4 hr after addition of the growth factor. The levels of AT1 receptor mRNA expression after 4 hr of treatment, relative to the 0-hr control signals (100%), are as follows: EGF, 38 ± 4%; bFGF, 26 ± 3%; and PDGF-BB, 23 ± 8%. Fig. 2 also illustrates the stability of AT1 receptor mRNA in vehicle-treated VSMC over the 24-hr experimental period. GAPDH RNA levels are also stable over this time course, both in the absence and in the presence of growth factors. However, bFGF appears to consistently induce GAPDH mRNA levels to approximately 150% of control.

Experiments were performed to gain insight into general mechanisms involved in this response to the growth factors. In one set of experiments, VSMC gene transcription was inhibited by preincubation of the cells for 30 min with 20 μg/ml actinomycin D before exposure to growth factors. Northern hybridizations were then performed on RNA extracted from VSMC at various time points after the addition of vehicle or growth factors (Fig. 3). In vehicle-treated cells, AT1 receptor mRNA levels are reduced to ~50% of control levels 6 hr after the

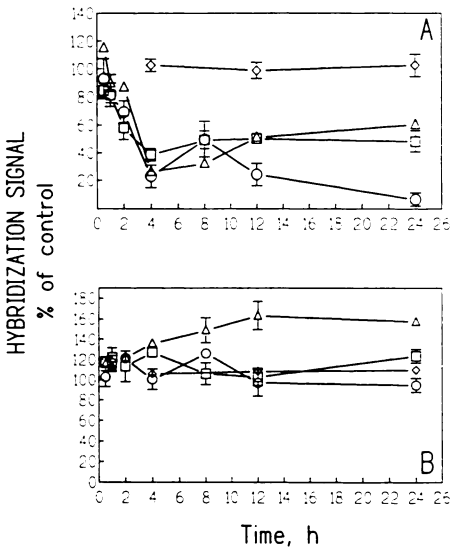

Fig. 2. Time course of growth factor-mediated mRNA responses, determined by densitometric analysis. A, Response of AT1 receptor mRNA to growth factors. B, Response of GAPDH mRNA to growth factors. Confluent VSMC on 6-cm dishes were exposed for the indicated times to vehicle (◇), 20 ng/ml EGF (□), 20 ng/ml bFGF (△), or 50 ng/ml PDGF-BB (○). Northern blots were hybridized with the rat AT1 receptor cDNA probe, washed, and exposed to film for 4–12 hr. After autoradiography, each blot was stripped and rehybridized with a GAPDH cDNA probe. Hybridization signals were obtained by determination of the integrated pixel values in a rectangulated autoradiographic transcript signal, using a laser densitometer, and pixel values from a background area of the film were subtracted. The data points were normalized to the hybridization signal at the 0-hr time point for control cells that were not exposed to growth factors. AT1 receptor mRNA and GAPDH receptor mRNA signals were normalized separately. Each *point* represents the mean ± standard error of hybridization signals from three separate experiments for each growth factor or vehicle.

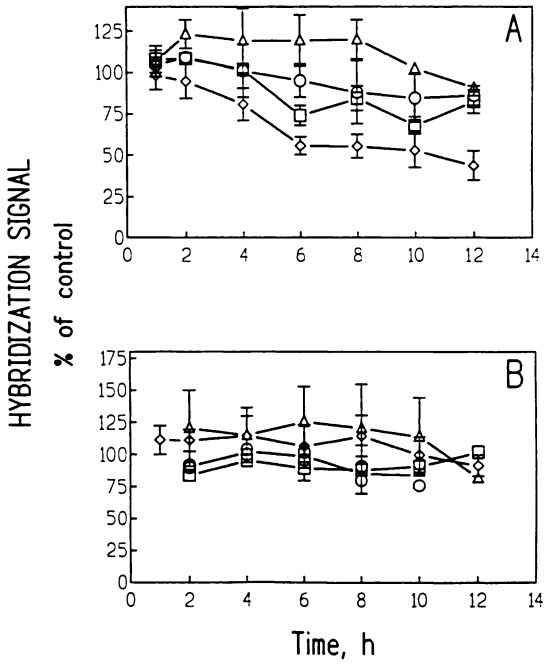

Fig. 3. Time course of growth factor-mediated mRNA response and effect of actinomycin D. A, Densitometric analysis of the response of AT1 receptor mRNA to growth factors after 30-min pretreatment with actinomycin D. B, Response of GAPDH mRNA to growth factors after actinomycin D pretreatment. Confluent VSMC on 6-cm dishes were pretreated for 30 min with 20 μg/ml actinomycin D and then exposed for the indicated times to vehicle (◇), 20 ng/ml EGF (□), 20 ng/ml bFGF (△), or 50 ng/ml PDGF-BB (○). After Northern hybridization with the AT1 receptor cDNA probe, the blots were stripped and rehybridized with a GAPDH cDNA probe. The data points were normalized to the mRNA hybridization signal obtained from cells treated for 30 min with actinomycin D alone. This signal was not significantly different from that obtained from cells not treated with actinomycin D. Each *point* represents the mean ± standard error of three experiments for each growth factor or vehicle.

addition of actinomycin D. The rate of AT1 receptor mRNA decay over this period is therefore taken to represent a measure of the stability of the mRNA under otherwise normal conditions. This decay rate and amplitude contrast markedly with those for AT1 receptor mRNA in vehicle-treated cells in the absence of actinomycin D (see Fig. 1), suggesting that the half-life of AT1 receptor mRNA is approximately 6 hr. Pretreatment of VSMC with actinomycin D blocks the growth factor-induced down-regulation of AT1 receptor mRNA (Fig. 3; compare with results shown in Fig. 1). Indeed, in the absence of cellular transcription the stability of AT1 receptor mRNA appears to be enhanced slightly by the addition of growth factors. Together, these observations suggest that growth factors attenuate AT1 receptor mRNA expression by decreasing the stability of the transcripts. Furthermore, because this destabilizing effect of the growth factors is blocked by actinomycin D, the simplest interpretation of these data is that growth factors induce a gene or set of genes that function to destabilize AT1 receptor mRNA.

Because the effect of growth factors appears to require transcription, it is of interest to determine whether the effect is dependent upon new protein synthesis (Fig. 4). Cycloheximide (15 μg/ml) treatment alone for 4 hr causes a slight increase in VSMC AT1 mRNA levels, compared with vehicle-treated controls. However, the inhibition of AT1 receptor mRNA by a 4-hr treatment with EGF, bFGF, and PDGF-BB is effectively attenuated by coincubation with cycloheximide. These results indicate that mRNA translation is necessary for growth factor-mediated destabilization of AT1 receptor mRNA.

Nuclear run-on assays were performed to determine whether VSMC growth factor receptor activation also affects AT1 receptor gene transcription. Nuclei were isolated from cells exposed for 4 hr to vehicle, 20 ng/ml EGF, 20 ng/ml bFGF, or 50 ng/ml PDGF-BB. As shown in Fig. 5, the intensity of hybridization by nascently transcribed, ^{32}P-labeled RNA from nuclei isolated from cells treated with growth factors is markedly reduced, compared with vehicle-treated cells. From three run-on experiments, these reductions in signal represent 28 ± 3% (EGF), 29 ± 5% (bFGF), and 17 ± 5% (PDGF-BB) of the AT1 receptor gene transcription rate in nuclei isolated from vehicle-treated cells. No significant change in response to growth factors is observed for the GAPDH mRNA transcription rate in the same nuclei. For quantitative purposes, the level of hybridization signal for the AT1 receptor cDNA-containing slots was normalized to that for the vector signal, because a measurable amount of nonspecific background hybridization occurred in the pKS$^+$-Bluescript slots. These observations indicate that growth factor receptor activation also attenuates the rate of transcription of the AT1 receptor gene in VSMC.

Radioligand binding assays were performed to assess whether the decreased level of AT1 receptor mRNA is coincident with a loss of AT1 receptor protein. To test this, total membrane receptors were measured after a 24-hr treatment of VSMC with

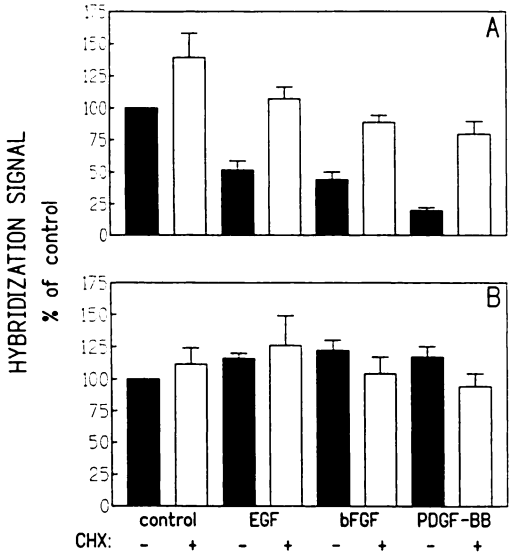

Fig. 4. Effect of cycloheximide (*CHX*) on growth factor-mediated changes in VSMC mRNA. A, Response of AT1 receptor mRNA. B, Response of GAPDH mRNA. VSMC were incubated for 4 hr in the absence (■) or presence (□) of 15 μg/ml cycloheximide, without (vehicle) or with 20 ng/ml EGF, 20 ng/ml bFGF, or 50 ng/ml PDGF-BB. After hybridization with the AT1 receptor cDNA probe, the blots were stripped and rehybridized with a GAPDH cDNA probe. The data points were normalized to the hybridization signal of vehicle-treated cells in the absence of cycloheximide. Each *bar* represents the mean ± standard deviation of two separate experiments for each growth factor.

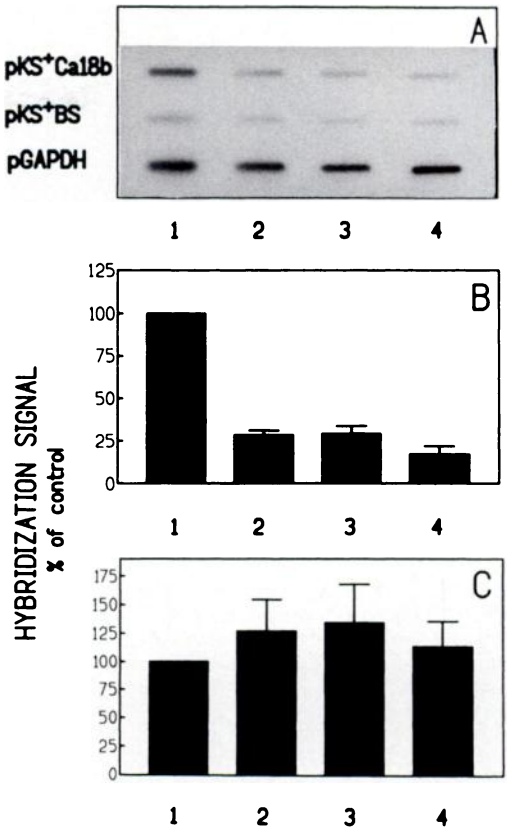

Fig. 5. Nuclear run-on analysis of transcription after growth factor treatment of VSMC. A, Representive autoradiographic result. B and C, Quantitative analysis of autoradiographic signals for nascent AT1 receptor transcripts and PDH transcripts, respectively, for vehicle (*1*), 20 ng/ml EGF (*2*), 20 ng/ml bFGF (*3*), and 50 ng/ml PDGF-BB (*4*). After incubation of VSMC with the growth factors for 4 hr, run-on transcription reactions were performed on isolated nuclei. [^{32}P]UTP-labeled RNA transcripts were hybridized to denatured cDNA plasmids encoding the rat AT1 receptor (*pKS⁺Ca18b*) or GAPDH (*pGAPDH*) or the KS⁺-Bluescript (*pKS⁺BS*) plasmid without an insert. After washing, filters were exposed to film for 12–48 hr. Hybridization signals from the pKS⁺-Bluescript slots were subtracted from those for the AT1 receptor. The pGAPDH hybridization signals were not corrected similarly. The data presented in B and C represent corrected or uncorrected, respectively, mRNA hybridization signals normalized to that from vehicle-treated cells. Each *bar* represents the mean ± standard error of three separate experiments for each growth factor.

the growth factors. Fig. 6 shows representative Rosenthal transformations of ^{125}I-[Sar^1,Ile^8]-angiotensin II saturation binding to membranes prepared from experimentally treated cells. Binding to membranes prepared from vehicle-treated cells reveals a K_d value of 0.29 ± 0.01 nM and a B_{max} value of 1.90 ± 0.01 pmol/mg of protein (three experiments). Binding to membranes from the growth factor-treated cells shows a decrease in the B_{max} value, without changes in the affinity for the radioligand. The values for EGF (B_{max} = 0.74 ± 0.17 pmol/mg of protein, K_d = 0.33 ± 0.22 nM), bFGF (B_{max} = 0.88 ± 0.25 pmol/mg of protein, K_d = 0.34 ± 0.27 nM), and PDGF-BB (B_{max} = 0.24 ± 0.02 pmol/mg of protein, K_d = 0.21 ± 0.05 nM) are consistent in demonstrating this effect. These binding data reveal that growth factor receptor activation markedly attenuates AT1 receptor protein expression in VSMC. This finding is consistent with the observed inhibitory effects of the growth factors on AT1 receptor mRNA expression and *de novo* gene transcription.

To examine this further, the VSMC inositol phosphate production response to angiotensin II was measured after a 24-hr treatment with 20 ng/ml bFGF. Fig. 7 reveals that growth factor treatment markedly attenuates AT1 receptor function, as evident by inspection of the maximal inositol phosphate production response to 1.0 μM angiotensin II. In growth factor-treated cells this is <30% of that observed in vehicle-treated cells. Coupled with the effects of growth factors on AT1 receptor gene transcription, mRNA stability, and protein expression, these data are consistent with the general conclusion that growth factor receptor activation decreases AT1 receptor gene expression and function in VSMC.

Discussion

The appearance of smooth muscle cell surface AT1 receptors can be potentially regulated at several mechanistic levels. Among the regulatory paradigms one can postulate are general control mechanisms modulating the rate of transcript formation, the degree of transcript stability, and the efficiency of mRNA translation to protein and molecular interactions with the cell surface-associated receptor protein that modulate its abundance and sensitivity to hormone in that domain. The principal observations of this study are that growth factor receptor activation markedly attenuates AT1 receptor gene expression in cultured VSMC, as measured by the disappearance of mRNA transcript and cell membrane-associated AT1 receptors and by measurements of the rate of gene transcription using a nuclear run-on approach. These data suggest that inhibition by growth factors of AT1 receptor gene expression in VSMC is secondary to two complementary mechanisms of action. The first of these involves attenuation of the rate of AT1 receptor gene transcription. The second appears to involve growth factor-induced destablization of the AT1 receptor-coding mRNA transcript.

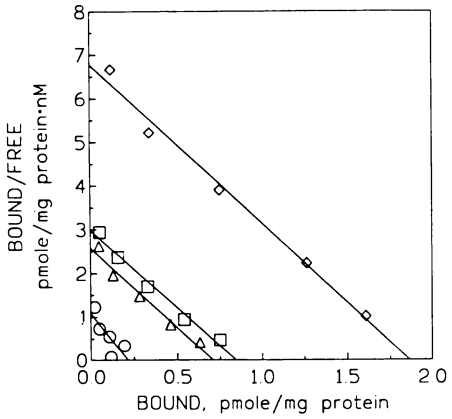

Fig. 6. Rosenthal analysis of the effect of growth factors on membrane saturation binding of ^{125}I-[Sar^1,Ile^8]-angiotensin II. Confluent VSMC on 100-mm dishes were exposed for 24 hr to either vehicle (◇), 20 ng/ml EGF (□), 20 ng/ml bFGF (△), or 50 ng/ml PDGF-BB (○). Saturation binding assays were performed with membranes prepared from these cells, using ^{125}I-[Sar^1,Ile^8]-angiotensin II and 10 μM Dup753, to define nonspecific binding. Nonlinear curves of specific binding (total bound minus nonspecifically bound) versus free radioligand (total added minus total bound) were transformed by the method of Rosenthal (26), to obtain linear plots. The data are representative of two separate experiments performed identically.

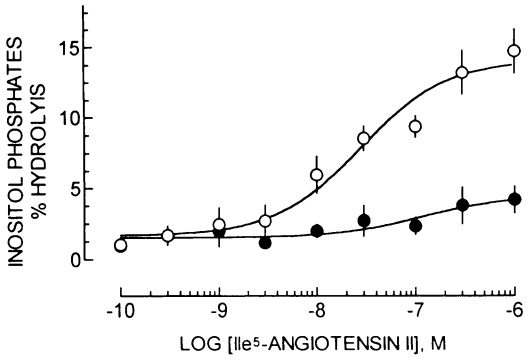

Fig. 7. Effect of bFGF treatment on the inositol phosphate response to angiotensin II in VSMC. Confluent VSMC grown on 24-well culture plates were exposed to vehicle (○) or 20 ng/ml bFGF (●) for 24 hr, in the presence of 1 μCi/ml *myo*-[^{3}H]inositol. After this, angiotensin II-mediated inositol phosphate production was determined by incubating the cells for 60 min at 37° with the indicated concentrations of angiotensin II in the presence of 10 mM LiCl. Each *point* represents the percentage hydrolysis of ^{3}H-labeled inositol phosphates from the total labeled pool from each well. The data shown are the mean ± standard error of three experiments, with duplicate determinations in each experiment.

Comparison of the kinetics of AT1 receptor mRNA expression in cells treated with growth factors and those treated with actinomycin D reveals that a growth factor-induced mRNA destabilization process appears to be the principal mechanism whereby AT1 receptor mRNA expression is reduced by growth factors. This is concluded from the observation that the normal AT1 receptor mRNA decay rate is accelerated in cells treated with growth factors. Fig. 3 reveals a reduction of AT1 receptor mRNA to 50% of control levels 6 hr after addition of actinomycin D. Because nascent AT1 receptor gene transcription is inhibited by actinomycin D treatment, the decrease in transcript abundance over time in actinomycin D-treated cells reflects the normal post-transcriptional processes leading to AT1 receptor mRNA decomposition. In contrast, growth factor treatment for 3 hr reduces AT1 receptor mRNA to a level equivalent to that observed after 6 hr of transcriptional inhibition using actinomycin D. Furthermore, the maximal level of AT1 receptor mRNA reduction by growth factors exceeds that observed in cells treated only with actinomycin D at any time point. These observations indicate that growth factors initiate active cellular processes leading to accelerated AT1 receptor mRNA degradation.

It is of interest that the destabilizing effect of growth factors can be effectively blocked by pretreatment of cells with either actinomycin D or cycloheximide before exposure to growth factors. One interpretation of these observations, taken together, is that the growth factors induce transcription of a protein-coding gene or set of genes whose products function to degrade AT1 receptor mRNA. Putative induced products such as these may act by themselves on the target mRNA or function by activating latent cellular factors. A general hypothesis is that such growth factor-induced destabilizing factors bind to cognate recognition domains in AT1 receptor mRNA and either degrade the RNA specifically by this interaction or enhance the degradation of the RNA through interaction with latent, constitutively expressed, RNase-like activities. This hypothesis is being tested at this time utilizing mutational approaches and analysis of AT1 receptor RNA-protein binding interactions.

Alternatively, mutational approaches in other systems have shown generally that translation of destabilized mRNAs is an essential step for initiation of subsequent degradation pathways (for review, see Ref. 22). The effect of cycloheximide in blocking the effect of the growth factors can be interpreted in this light. The inability of growth factors to induce AT1 receptor mRNA degradation after cycloheximide treatment may simply reflect a requirement for AT1 receptor mRNA translation to precede its degradation. Consistent with this is the observation (shown in Fig. 4) that, 6 hr after cycloheximide treatment, AT1 receptor mRNA levels are higher than those in vehicle-treated control cells. Thus, translation blockade in the absence of growth factors appears to increase the stability of AT1 receptor mRNA.

The data shown in Fig. 3 demonstrate a tendency for growth factor-mediated stabilization of AT1 receptor mRNA when transcription is blocked using actinomycin D. This observation may indicate that latent factors, capable of regulation by the growth factors, are expressed constitutively in VSMC to control AT1 receptor mRNA stability. Adenosine- and uridine-rich sequences in the 3′ untranslated region of certain cytokine and early response genes are known to mediate accelerated RNA degradation (23, 24). In contrast to multiple AUUUA repeats in a uridine-rich environment in these generally unstable RNAs, the AT1a receptor mRNA contains only two AUUUA elements in its 3′ untranslated region and, after transcriptional block with actinomycin D alone, is down-regulated in a less immediate pattern than is typically observed for mRNAs containing functional AUUUA elements. Thus, it is currently doubtful that these represent functional elements in AT1 receptor mRNA. However, additional experimentation will be necessary to demonstrate which among these, and other, possibilities are involved in the growth factor response of AT1 receptor mRNA.

Interestingly, a similar mRNA destabilization process appears to be activated by PDGF in VSMC to down-regulate the expression of smooth muscle α-actin gene expression (25). PDGF-BB-induced smooth muscle actin destabilization can also be blocked by actinomycin D treatment. It is attractive to

speculate that destabilization of AT1 receptor mRNA and that of smooth muscle actin mRNA share similar mechanisms of action. Corjay *et al.* (25) have proposed that growth factor-induced destabilization of smooth muscle-specific mRNAs may reflect a more general process involved in de-differentiation of smooth muscle cells to a mitotic phenotype. If so, continued investigation of the molecular pathways involved in regulating AT1 receptor gene expression in VSMC may lead to a better understanding of the cellular processes involved in initiation of smooth muscle cell proliferation in vascular diseases.

References

1. Jackson, C. L., and S. M. Schwartz. Pharmacology of smooth muscle cell replication. *Hypertension (Dallas)* **20**:713–736 (1992).
2. Bobik, A., and J. H. Campbell. Vascular derived growth factors: cell biology, pathophysiology and pharmacology. *Pharmacol. Rev.* **45**:1–42 (1993).
3. Ross, R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature (Lond.)* **362**:801–809 (1993).
4. Berk, B. C., T. A. Brock, R. C. Webb, M. B. Taubman, W. J. Atkinson, M. A. Gimbrone, Jr., and R. W. Alexander. Epidermal growth factor, a vascular smooth muscle mitogen, induces rat aortic contraction. *J. Clin. Invest.* **75**:1083–1086 (1985).
5. Lindner, V., and M. A. Reidy. Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. *Proc. Natl. Acad. Sci. USA* **88**:3739–3743 (1991).
6. Sachinidis, A., R. Locher, W. Vetter, D. Tatje, and J. Hoppe. Different effects of platelet-derived growth factor isoforms on rat vascular smooth muscle cells. *J. Biol. Chem.* **265**:10238–10243 (1990).
7. Jawien, A., D. F. Bowen-Pope, V. Lindner, S. M. Schwartz, and A. W. Clowes. Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. *J. Clin. Invest.* **89**:507–511 (1992).
8. Blank, R. S., and G. K. Owens. Platelet-derived growth factor regulates actin isoform expression and growth state in cultured rat aortic smooth muscle cells. *J. Cell. Physiol.* **142**:635–642 (1990).
9. Caponi, A. M., G. Aguilera, J. L. Fakunding, and K. J. Catt. Angiotensin II: receptors and mechanisms of action, in *Biochemical Regulation of Blood Pressure* (R. L. Soffer, ed.). John Wiley & Sons, New York, 205–262 (1981).
10. Peach, M. J. Renin-angiotensin system: biochemistry and mechanisms of action. *Physiol. Rev.* **57**:313–370 (1977).
11. Powell, J. S., J.-P. Clozel, R. K. M. Muller, H. Kuhn, F. Hefti, M. Hosang, and H. R. Baumgartner. Inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. *Science (Washington D. C.)* **24**:186–188 (1989).
12. Azuma, H., Y. Niimi, and H. Hamasaki. Prevention of intimal thickening after endothelial removal by a non-peptide angiotensin II receptor antagonist, losartan. *Br. J. Pharmacol.* **10**:665–671 (1992).
13. Osterrieder, W., R. K. M. Muller, J. S. Powell, J.-P. Clozel, F. Hefti, and H. R. Baumgartner. Role of angiotensin II in injury-induced neointima formation in rats. *Hypertension (Dallas)* **18** (Suppl. II):II60–II64 (1991).
14. Ferns, G. A. A., E. W. Raines, K. H. Sprugel, A. S. Motani, M. A. Reidy, and R. Ross. Anti-PDGF antibody significantly inhibits neointimal smooth muscle accumulation after angioplasty. *Science (Washington D. C.)* **25**:1129–1132 (1991).
15. Gunther, S., R. W. Alexander, W. S. Atkinson, and M. A. Gimbrone. Functional angiotensin II receptors in cultured vascular smooth muscle cells. *J. Cell Biol.* **92**:289–298 (1982).
16. Murphy, T. J., R. W. Alexander, K. K. Griendling, M. S. Runge, and K. E. Bernstein. Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor. *Nature (Lond.)* **351**:233–236 (1991).
17. Linial, M., N. Gunderson, and M. Groudine. Enhanced transcription of c-*myc* in bursal lymphoma cells requires continuous protein synthesis. *Science (Washington D. C.)* **230**:1126–1132 (1985).
18. Miller, J. J., G. S. Schultz, and R. S. Levy. Rapid purification of radioiodinated peptides with Sep-Pak reversed phase cartridges and HPLC. *Int. J. Peptide Protein Res.* **24**:112–122 (1984).
19. Berk, B. C., E. Elder, and M. Mitsuka. Hypertrophy and hyperplasia cause differing effects on vascular smooth muscle cell Na^+/H^+ exchange and intracellular pH. *J. Biol. Chem.* **265**:19632–19637 (1990).
20. Ververis, J. J., and P. Delafontaine. Regulation of insulin-like growth factor 1 receptors on vascular smooth muscle cells by growth factors and phorbol esters. *Circ. Res.* **72**:1285–1292 (1993).
21. Takeuchi, K., R. W. Alexander, Y. Nakamura, T. Tsujino, and T. J. Murphy. Molecular structure and transcriptional function of the rat vascular AT_{1a} angiotensin receptor gene. *Circ. Res.* **73**:612–621 (1993).
22. Sachs, A. B. Messenger RNA degradation in eukaryotes. *Cell* **74**:413–421 (1993).
23. Brewer, G. An A+U-rich element RNA-binding factor regulates c-*myc* mRNA stability *in vitro*. *Mol. Cell. Biol.* **11**:2460–2466 (1991).
24. You, Y., C. Y. A. Chen, and A. B. Shyu. U-rich sequence-binding proteins (URBPs) interacting with a 20-nucleotide U-rich sequence in the 3′ untranslated region of c-*fos* mRNA may be involved in the first step of c-*fos* mRNA degradation. *Mol. Cell. Biol.* **12**:2931–2940 (1992).
25. Corjay, M. H., R. S. Blank, and G. K. Owens. Platelet-derived growth factor-induced destabilization of smooth muscle α-actin mRNA. *J. Cell. Physiol.* **145**:391–397 (1990).
26. Rosenthal, H. E. Graphical method for the determination and presentation of binding parameters in a complex system. *Anal. Biochem.* **20**:525–532 (1967).

Send reprint requests to: T. J. Murphy, Department of Pharmacology, Room 5031, O. W. Rollins Research Building, Emory University School of Medicine, Atlanta, GA 30322.