Angiotensin II Induces Delayed Mitogenesis and Cellular Proliferation in Rat Aortic Smooth Muscle Cells

Correlation with the Expression of Specific Endogenous Growth Factors and Reversal by Suramin

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Abstract

By means of a rat aortic smooth muscle (RASM) cell culture model, the effects of angiotensin II (AII) on early proto-oncogene gene expression, DNA synthesis, and cell proliferation were measured and compared to known mitogens. In 24-h [^{3}H]-thymidine incorporation assays, AII was found to be a weak mitogen when compared to potent mitogens such as fetal bovine serum and platelet-derived growth factor (PDGF). In contrast, when assays were carried out for 48 h, AII induced a significant dose-dependent stimulation of DNA synthesis, which more than doubled at 3 nM AII, and was maximal (five- to eightfold above control) at 100 nM AII. Treatment of cells with the AII type 1 receptor antagonist losartan inhibited the mitogenic effects of AII. AII also stimulated smooth muscle cell proliferation, as indicated by an absolute increase in cell number after AII stimulation of RASM cells for 5 d. AII stimulation of RASM cell growth correlated with the increased expression of specific endogenous growth factors, including transforming growth factor β_1 (TGF-β_1) and PDGF A-chain. However, addition of either PDGF- or TGF-β_1-neutralizing antibodies failed to significantly reduce the delayed mitogenic effects induced by AII. In contrast, we found that AII-stimulated mitogenesis could be inhibited in a dose-dependent manner by the growth factor inhibitor drug suramin. Taken together, our results indicate that enhanced endogenous growth factor expression may represent the direct mechanism by which AII promotes smooth muscle cell growth in some vascular hyperproliferative diseases. (*J. Clin. Invest.* 1994. 93:788–798.) Key words: atherosclerosis • platelet-derived growth factor • restenosis • smooth muscle • transforming growth factor-β

Introduction

Excessive proliferation of vascular smooth muscle cells is implicated as a critical event in atherosclerosis and restenosis after balloon angioplasty. However, the exact cellular mechanisms responsible for smooth muscle cell proliferation are not yet defined. Several lines of evidence suggest a role for the renin-angiotensin system in the marked smooth muscle cell growth observed as a consequence of hypertension, vascular injury, and atherosclerosis (reviewed in references 1–4). For example, in the spontaneously hypertensive rat, inhibition of angiotensin II (AII)[1] production by angiotensin-converting enzyme (ACE) inhibitors causes a decrease in overall smooth muscle cell mass (5). Furthermore, recent studies demonstrate that treatment of rats with ACE inhibitors or an angiotensin receptor antagonist significantly decreases myointimal proliferation after acute arterial injury with a balloon catheter (6, 7). In related studies, infusion of AII in rats before balloon injury greatly exacerbates the myoproliferative lesions in treated vessels (8). AII is also reported to stimulate vascular smooth muscle cell growth directly or enhance the growth response of cells to serum (9–13). However, these findings conflict with other studies using rat aortic smooth muscle (RASM) cell lines which indicate that AII stimulates cellular hypertrophy but not proliferation (14, 15).

AII is known to bind to specific high-affinity receptors on the surface of responsive cells and distinct AII receptor genes have been identified (16–18). In vascular smooth muscle cells, activation of the AII type 1 (AT_1) receptor stimulates phosphatidylinositol-specific phospholipase C activity through the activation of at least one closely coupled G protein (1, 19). This results in the generation of the second messengers inositol trisphosphate and diacylglycerol, leading to intracellular Ca^{2+} mobilization and protein kinase C activation, respectively. AII also stimulates rapid protein tyrosine phosphorylation in cultured RASM cells, and the activation of other serine, threonine protein kinases, including Raf-1 and mitogen-activated protein kinases that are implicated in mitogenic signal transduction (20). AII stimulation of RASM cells increases the production of presumed autocrine factors such as PDGF A-chain and TGF-β, suggesting that it may be affecting cell growth in an indirect mechanism (13, 21). Like other growth factors, AII induces a rapid increase of the growth-associated nuclear proto-oncogenes c-*myc*, c-*fos*, and c-*jun* (21–23). These studies all indicate that AII can induce rapid changes in gene expression and function that may ultimately lead to increased cell growth.

In the present study, we evaluate the effects of AII, in comparison to other known smooth muscle cell mitogens, on DNA synthesis and cell proliferation in RASM cells. Like other mitogens, AII stimulates a rapid increase in c-*fos* proto-oncogene expression, but an accompanying increase in DNA synthesis is delayed compared to responses induced by FBS or PDGF. We also demonstrate that AII stimulates RASM cell proliferation and that the effects of AII are inhibited by AII receptor antago-

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1. *Abbreviations used in this paper:* AII, angiotensin II; ACE, angiotensin-converting enzyme; anti-P-TYR, anti-phosphotyrosine antibodies; AT_1, and AT_2, angiotensin type 1 and 2 receptors; FGF, fibroblast growth factor; SF-DME, serum-free DME.

nists. AII stimulated RASM cell growth correlates with increased expression of endogenous growth factors, including PDGF A-chain and TGF-β_1. However, our studies indicate that autocrine production of these factors alone may not be sufficient for AII-induced smooth muscle cell growth. Our results support the concept that AII stimulates smooth muscle cell growth by induction of a productive autocrine loop(s), and suggest novel target sites for therapeutic intervention in AII-stimulated vascular smooth muscle cell proliferation.

Methods

Cell culture. RASM cells, prepared from adult Sprague-Dawley rats, were generously provided by Dr. Marschall Runge (Emory University, Atlanta, GA). Cells were propagated in DME (D-glucose [4,500 mg/liter], L-glutamine [584 mg/liter], and Hepes (25 mM]) supplemented with FBS (10% vol/vol, Hyclone Laboratories Inc., Logan, UT), penicillin (100 U/ml), and streptomycin (100 mg/ml). Cells were passaged at 80–90% confluence with 0.25% (wt/vol) trypsin and utilized between passages 10 and 18. NIH/3T3 mouse fibroblasts were generously provided by Dr. Stuart Aaronson (National Cancer Institute, Bethesda, MD). Fibroblasts were maintained in DME plus 10% calf serum and antibiotics. All culture reagents were obtained from Gibco-BRL (Grand Island, NY) unless otherwise specified.

Growth factors, antibodies, and other reagents. Recombinant human PDGF-AA and BB homodimers were purchased from Amgen Biologicals (Thousand Oaks, CA). Polyclonal goat anti-PDGF (A and B chain) and turkey anti-TGF-β_1 neutralizing antisera were from Collaborative Research (Bedford, MA). Recombinant human basic FGF was from Genzyme Corp. (Boston, MA). Murine monoclonal antibodies directed against basic fibroblast growth factor (FGF) (bovine) and phosphotyrosine (anti-P-TYR) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Recombinant human and mouse EGF, and polyclonal goat anti-human EGF and rabbit anti-mouse EGF neutralizing antisera were supplied by the same vendor. Porcine TGF-β_1 and the corresponding polyclonal rabbit anti-TGF-β_1 neutralizing antisera were from R&D Systems Inc. (Minneapolis, MN). Recombinant human TGF-β_1 was generously provided by Dr. Tony Purchio, Bristol-Myers Squibb Pharmaceutical Research Institute (Seattle, WA). Purified monoclonal anti-TGF-β antibody, 1D.11 (24), was provided by Kris Safford, Biological Process Research, Bristol-Myers Squibb (Seattle, WA). Suramin (the hexasodium salt of 8,8′-{carbonyl-bis[imino-3,1-phenylenecarbonylimino (4-methyl-3,1-phenylene) carbonylimo]} bis-1,3,5-naphthalenetrisulfonic acid) was kindly provided by the Pharmaceutical Resources Branch of the National Cancer Institute, Bethesda, MD.

Conditioned media. To obtain samples of conditioned media, confluent cultures of RASM cells were first growth arrested by incubation for 48 h in a chemically defined serum-free medium (SF-DME), DME supplemented with 10 nM selenium (Gibco/BRL) and 10 μg of transferrin per ml (Collaborative Research). The cells were then either left untreated or stimulated with AII (1 μM) for the indicated times (up to 48 h). Samples of conditioned media from the RASM cells were collected, and aliquots were either used immediately or were frozen on dry ice and stored at −80°C.

Mitogenic assays. RASM cells were plated at a density of 1×10^5 cells/ml in serum-containing medium on fibronectin-coated multi- (24- or 48-) well tissue culture plates. After attachment, cells were rinsed twice with PBS and incubated for 72 h in SF-DME. Quiescent cells were labeled with [^{3}H]thymidine (1 μCi/ml, 70–90 Ci/mmol) (DuPont/NEN Research Products, Boston, MA) in the absence or presence of FBS (10% vol/vol) or test mitogens for 24–48 h. In some experiments, AII receptor antagonists or suramin were included 30 min before the addition of test mitogens, and co-incubated throughout the experiment. Relative [^{3}H]thymidine incorporation was determined by liquid scintillation spectrometry after precipitation with ice-cold TCA (5% wt/vol) and solubilization (37°C) in NaOH (0.25 M).

For assays with AII-conditioned media as agonist, NIH/3T3 cells were plated as described and then quiesced for 18 h in SF-DME supplemented with 0.1% BSA. Control growth factors and conditioned media were incubated (37°C, 30 min) without or with either PDGF neutralizing antibody (50 μg/ml) or suramin (100 μM) before addition to the NIH/3T3 cells. Cells were incubated for 18 h and then labeled with [^{3}H]thymidine for 6 h. Relative [^{3}H]thymidine incorporation was determined as described.

Cell proliferation assays. Cells (5×10^4 cells/ml) were plated on 100-mm dishes in serum-containing media. After attachment, cells were growth arrested for 48 h in serum-free medium (SF-DME). Cells were then divided into three treatment groups: 1% FBS, 10% FBS, and AII (1 μM) plus 1% FBS. Cells were incubated for 4 d in the presence or absence of the angiotensin receptor antagonist losartan (1 μM). AII and antagonist were added every 24 h. After 4 d, cells were recovered using trypsin (0.25%)/EDTA (1 mM) and cell counts were performed using a Coulter Counter (Model ZM, Coulter Corp., Hialeah, FL). Data analysis was performed using the StatView computer program (Abacus Concepts, Inc., Berkeley, CA). Means were separated by analysis of variance using Dunnett's t test. Data were deemed significantly different at $P < 0.05$.

RNA extraction and Northern analyses. Monolayer cultures were incubated overnight in SF-DME. Cells were then left untreated or were stimulated with AII (1 μM) for various times. Plates were immediately washed twice with cold PBS and thoroughly drained. Total cellular RNA was extracted using guanidinium isothiocyanate essentially as described (25). In some experiments poly (A)$^+$ RNA was purified from cell lysates using the Quick-Prep mRNA Purification Kit (Pharmacia/LKB, Piscataway, NJ) according to the manufacturer's instructions. Samples of total RNA (10 μg/lane) or poly (A)$^+$ selected RNA (2.5 μg/lane) were electrophoretically resolved in formaldehyde-denaturing agarose gels, electro- or capillary-transferred and UV-crosslinked (Stratalinker, Stratagene, Inc., La Jolla, CA) to Nytran nylon membranes (Schleicher & Schuell, Inc., Keene, NH). Specific mRNAs were detected by hybridization with the appropriate random primed ^{32}P-labeled cDNA probes. These included a mouse c-*fos* cDNA probe (American Type Culture Collection, Rockville, MD), a rat TGF-β_1 (generously provided by Dr. Su Wen Qian, National Cancer Institute), and a human PDGF A-chain cDNA probe (designated LA28, kindly provided by Dr. W. La Rochelle, National Cancer Institute). Blots were washed thoroughly at room temperature in 2×-SSPE buffer (300 mM NaCl, 20 mM sodium phosphate, 2 mM EDTA) containing 0.1% SDS and finally for 1 h at 65°C in (0.2×) SSPE/0.1% SDS. Hybridization of ^{32}P-labeled probes was quantitated using a Model 400E Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Relative levels of mRNA were normalized by comparison to control bands detected after hybridization of the blots with a ^{32}P-labeled probe for the murine ribosomal protein gene L32 (rPL32/4a (26); provided by Dr. Robert Perry, Fox Chase Cancer Center, Philadelphia, PA).

Analysis of NIH/3T3 cellular protein tyrosine phosphorylation induced by RASM-conditioned media. Conditioned media samples were added to confluent cultures of NIH/3T3 fibroblasts that had been serum-starved for 24 h. In some samples, media was incubated with an anti-PDGF neutralizing antiserum (50 μg/ml) for 30 min before addition to the NIH/3T3 cells. As controls, NIH/3T3 cells were stimulated with recombinant growth factors (PDGF-AA [10 ng/ml], PDGF-BB [10 ng/ml], or basic FGF [10 ng/ml]), or AII (1 μM). After 10 min, cultures were rinsed twice in ice-cold PBS containing 1 mM Na_3VO_4, and then lysed on ice in 0.5 ml of P-TYR lysis buffer (50 mM Hepes, pH 7.5, 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, plus 10 μg/ml of aprotinin and leupeptin). Lysates were sonicated for 10 s and then centrifuged at 14,000 g for 10 min at 4°C. Equal protein aliquots (typically, 1–2 mg protein) were used for anti-phosphotyrosine immunoprecipitations in which 2 μg of anti-P-TYR was used per mg of cell lysate. Immunoprecipitations were carried out for 2 h at 4°C, and immune complexes were recovered using protein G-agarose (Gamma-Bind G, Genex Corp., Gaithersburg, MD). Immunoprecipitates were

washed five times in P-TYR lysis buffer, resolved by SDS-PAGE, and immunoblotted as previously described (27, 28). Aliquots of the whole-cell lysates (100 μg protein), removed before immunoprecipitation, were routinely analyzed by immunoblotting. Immunoreactive bands were visualized using ^{125}I-protein A (Amersham Inc., Arlington Heights, IL), followed by autoradiography. In control experiments, anti-P-TYR immunoreactive bands were specifically competed with phosphotyrosine (1 mM) but not phosphoserine or phosphothreonine.

Radioligand binding. Whole-cell binding of AII was performed with modifications of the procedure described by Crozat et al. (29). Cells (5×10^4 cells/cm^2) in complete media were plated and allowed to attach overnight. Cells were washed with PBS then incubated (2 h, 37°C, 5% CO_2) in DME + 0.5% BSA with 0.6 nM [^{125}I]Sar^1, Ile^8-AII (2,200 Ci/mmol, DuPont/NEN) and various concentrations (10^{-10} to 10^{-5} M) of unlabeled Sar^1, Ile^8-AII. The media was removed and cells washed with cold PBS + 1% BSA. Cells were dissolved in 0.25 M NaOH with 0.4% Triton X-100 and bound radioactivity was measured in a Cobra Gamma Counter (Packard Instrument Co., Downers Grove, IL) at 80% efficiency.

Results

AII induces the rapid expression of the growth-associated nuclear proto-oncogene c-fos. Rapid induction of the nuclear proto-oncogenes such as c-*fos*, c-*myc*, and c-*jun*, corresponding to specific proteins involved in gene transcription, is recognized as a useful marker for cell growth stimulation (30). To examine the effects of AII on induction of c-*fos*, we analyzed expression of mRNA in RASM cells treated with AII in comparison to the known mitogens FBS and PDGF-BB. Northern blot analysis of total RNA was performed using samples isolated from quiescent cells and cells incubated for 30 min with FBS (10% vol/vol), PDGF-BB homodimer (50 ng/ml), or AII (1 μM). All treatments stimulated pronounced induction of mRNA for c-*fos*. As shown in Fig. 1, AII induced a 19-fold stimulation of c-*fos* mRNA, compared to PDGF-BB (~ 25-fold), or FBS (~ 34-fold). These results demonstrate that AII is able to produce efficient stimulation of c-*fos* expression in our RASM cell lines, and are in general consistent with previous studies (22).

AII is a weak mitogen after 24 h, but stimulates a pronounced increase in DNA synthesis after 48 h. To evaluate the effects of AII on vascular cell growth, mitogenic assays were performed using RASM cells (Fig. 2). Cells were incubated without (serum free) or with FBS (10% vol/vol) or AII (1 μM). In these experiments, AII stimulated only a minor (2-fold) increase in DNA synthesis when measured after 24 h. In contrast, FBS induced a 10–15-fold increase over the serum free control by 24 h. PDGF-BB (50 ng/ml) was the most potent mitogen in these experiments and stimulated a 30–35-fold increase DNA synthesis (data not shown). These data imply that the marked stimulation of c-*fos* by AII is not sufficient for a prompt mitogenic response in these cells.

To determine if AII effects were delayed in comparison to FBS or PDGF, in parallel experiments, mitogenic assays were extended to 48 h. Relative levels of DNA synthesis stimulated by FBS and PDGF-BB were similar to those observed after 24 h (10–15- and 20–30-fold, respectively). However, by 48 h, AII (1 μM) now induced a consistent 5- to 8-fold stimulation of DNA synthesis in comparison to quiescent controls (Fig. 2, *shaded bars*). Furthermore, AII-stimulated DNA synthesis was inhibited by pretreatment (30 min) and co-incubation of the cells with the AT_1 receptor antagonist losartan (5 μM).

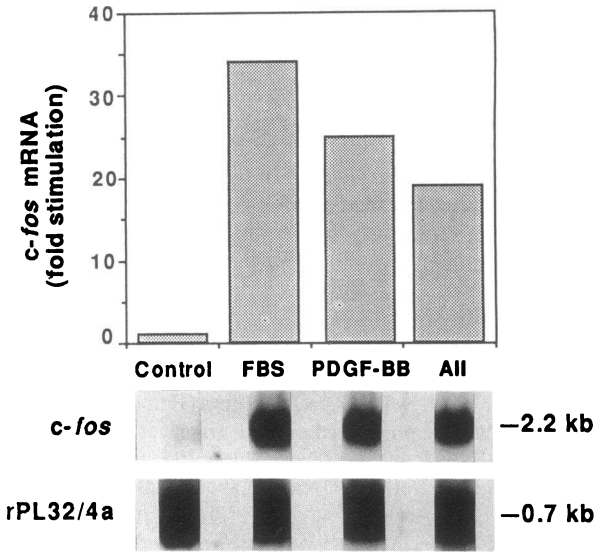

Figure 1. Rapid induction of c-*fos* mRNA by AII. Quiescent RASM cells were stimulated with FBS (10% vol/vol), PDGF-BB (50 ng/ml) or AII (1 μM) for 30 min. Total RNA was extracted and analyzed by Northern blotting using a ^{32}P-labeled probe for mouse c-*fos* cDNA (*bottom*). Relative levels of c-*fos* mRNA in each sample (*top*), determined by phosphorimaging analysis, were adjusted by comparison to positively hybridized internal control bands for the murine ribosomal protein L32 (rPL32/4a) mRNA.

The specificity of AII-stimulated DNA synthesis in RASM cells was evaluated using AII receptor antagonists. For these experiments, the AT_1 receptor-specific antagonist losartan was compared to the AT_2 receptor-specific antagonist PD 123177 (31). The effects of the compounds on control and serum-stimulated DNA synthesis were also determined to evaluate nonspecific or toxic effects. We found that AII (1 μM)-stimulated DNA synthesis was inhibited by losartan at a concentration of

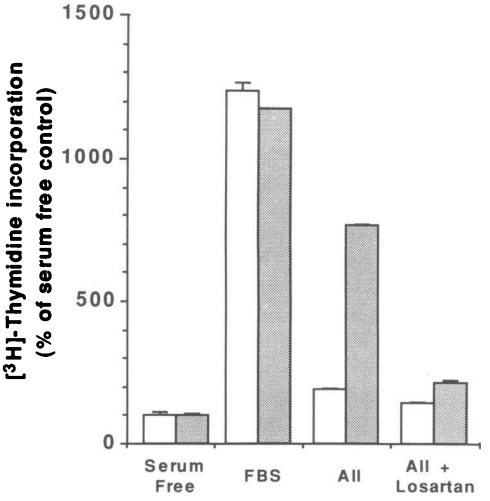

Figure 2. AII stimulates delayed mitogenesis in cultured RASM cells. Cells were incubated for 24 h (*open bars*) or 48 h (*shaded bars*) with [^{3}H]thymidine in SF-DME, FBS (10% vol/vol), AII (1 μM) or AII plus losartan (5 μM). Results are expressed as percentage of the unstimulated serum-free control. Baseline [^{3}H]thymidine incorporation was 21,200±3,200 cpm at 24 h; 35,200±3,900 at 48 h. Bars are the mean±SD of three treatment wells from a representative experiment.

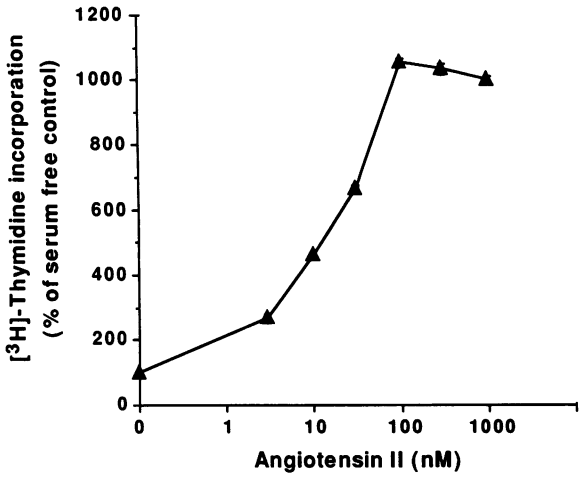

Figure 3. AII induction of RASM cell DNA synthesis is dose dependent. Cells were incubated with [^{3}H]thymidine and AII for 48 h. Results are expressed as percentage of the unstimulated serum-free control. Baseline [^{3}H]thymidine incorporation was 22,400±900 cpm. Points are the mean±SD of three treatment wells from a representative experiment.

5 μM. In contrast, the AT_2 receptor antagonist PD 123177 (5 μM) had no effect on the AII-stimulated increase in DNA synthesis (data not shown). Neither compound had a significant effect on incorporation of [^{3}H]thymidine in the serum free control or on mitogenicity induced by FBS. These data indicate that the delayed mitogenic effect of AII on RASM cells is mediated by the AT_1 receptor.

AII-stimulated RASM cell mitogenesis is concentration dependent. Additional experiments were performed to determine if stimulation of delayed DNA synthesis by AII was concentration dependent (Fig. 3). Incorporation of [^{3}H]thymidine doubled at 3 nM and was maximal at 100 nM AII. Although the maximal AII-stimulated response was less than that observed with 10% FBS (usually 15–20 times the serum free control) these results indicate that AII is capable of stimulating DNA synthesis in quiescent RASM cells.

The AT_1 receptor antagonist losartan inhibits AII-stimulated mitogenesis in a dose-dependent manner. In a more detailed dose-response analysis, increasing concentrations of losartan were incubated in the presence of a maximally stimulating concentration of AII (100 nM). As shown in Fig. 4, losartan inhibited AII-stimulated DNA synthesis, with an IC_{50} of 40 nM. These results indicate that losartan may have potential antiproliferative activity in cardiovascular disease models involving AII-mediated smooth muscle cell proliferation.

AII stimulates RASM cell proliferation. In order to determine whether AII is capable of stimulating RASM cell division, cell proliferation assays were conducted. Under control conditions (1% FBS), a small increase in cell number was observed at the end of 4 d (~ 2-fold) (Fig. 5). In contrast, cells maintained in 10% FBS (normal growth conditions) for 4 d multiplied 4.7-fold in comparison to the 1% FBS controls. Daily supplementation of 1% FBS with AII (1 μM) resulted in an increase in cell number after 4 d, equal to that observed with 10% FBS. In separate treatment groups, the effect of AII addition was significantly inhibited by losartan. However, this compound had no effect on RASM cell proliferation in response to 1% FBS or 10% FBS. Taken together, these data support the concept that AII can stimulate RASM cell proliferation.

AII stimulates an increase in the level of TGF-β_1 expression in cultured RASM cells. Previous studies report that AII stimulates the expression of some endogenous growth factor genes, including TGF-β_1 and PDGF, and these factors may act to stimulate cell growth by an autocrine mechanism (12, 13, 23). In order to evaluate the effects of AII on growth factor expression in our RASM cell system, we analyzed total cellular RNA extracted from serum-starved RASM cells or cells stimulated by AII (1 μM) for different times. Extracts of RNA (10 μg) were electrophoretically resolved and electroblotted onto nylon membranes. Blots were hybridized with ^{32}P-labeled cDNA

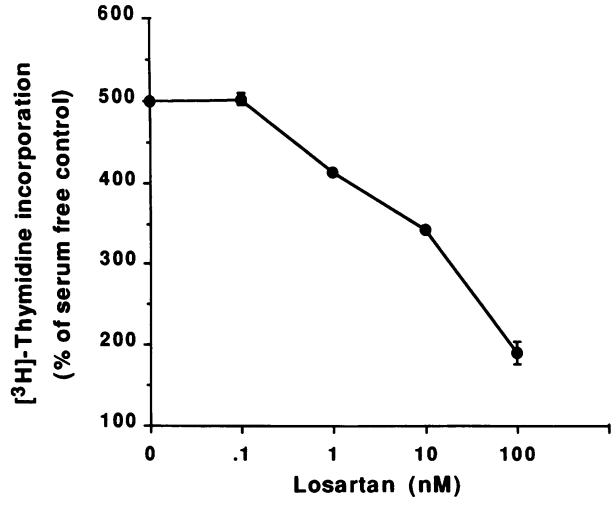

Figure 4. Inhibition of AII-stimulated DNA-synthesis by the AT_1 receptor antagonist losartan is concentration dependent. Cells were pretreated with losartan (5 μM) for 30 min and then incubated with antagonist and [^{3}H]thymidine in the presence of AII (100 nM) for 48 h. Results are expressed as percentage of the unstimulated serum-free control. Baseline [^{3}H]thymidine incorporation was 52,300±6,500 cpm. Points are the mean±SD of three treatment wells from a representative experiment.

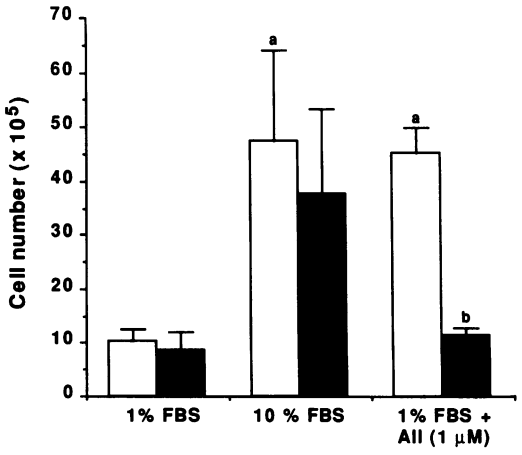

Figure 5. Proliferation of RASM cells is supported by AII. Cells (5 × 10^4 cells per plate) were incubated for 4 d in 1% FBS, 10% FBS, or 1% FBS plus AII (1 μM) without (*open bars*) or with (*solid bars*) losartan (1 μM). Results (cell number) are expressed as the mean±SD of at least three plates per treatment. [a] $P < 0.05$ vs. 1% FBS; [b] $P < 0.05$ vs. 1% FBS + AII (1 μM).

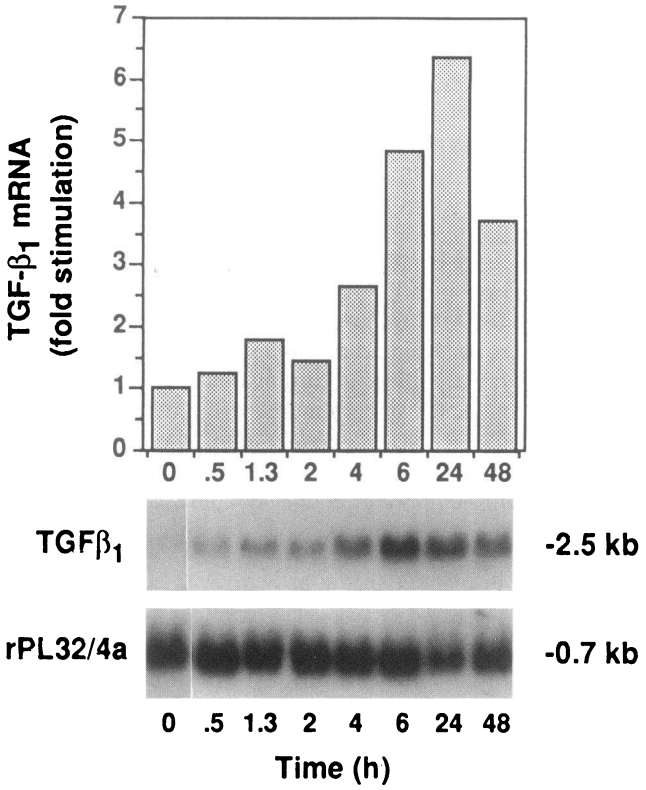

Figure 6. AII stimulates an increase in TGF-β_1 mRNA in cultured RASM cells. Quiescent cells were incubated with AII (1 μM) for the indicated times. Total cellular RNA ($\sim$ 10 μg) was extracted, resolved by agarose gel electrophoresis, blotted, and hybridized with a ^{32}P-labeled rat TGF-β_1 cDNA probe. Relative levels of TGF-β_1 mRNA in each sample, determined by phosphorimaging analysis, were adjusted by comparison to positively hybridized internal control bands for the murine ribosomal protein L32 (rPL32/4a) mRNA. Results are expressed as relative levels of TGF-β_1 mRNA in comparison to serum-starved controls.

probes directed against rat TGF-β_1. Positively hybridized bands were then quantitated by phosphorimaging analysis. As shown in Fig. 6, AII treatment led to a time-dependent increase in expression of TGF-β_1. Relative levels of mRNA increased by a factor of 2 at 4 h after AII addition and maximal levels ($\sim$ 6-fold above control) were observed at 24 h. These results are consistent with a previous report (13), suggesting a role for TGF-β_1 in AII-stimulated cell growth (see below).

AII stimulates the secretion of PDGF activity in RASM cells: detection by anti-P-TYR immunoblot analysis of NIH/3T3 fibroblasts treated with RASM conditioned media. To determine if AII stimulated the expression of functional growth factors, we performed experiments to detect growth factor secretion by RASM cells. For these experiments, conditioned media from untreated (control) and AII (1 μM)-stimulated RASM cells was added to quiescent cultures of NIH/3T3 cells. As a control, NIH/3T3 cultures were treated with AII (1 μM) and recombinant forms of PDGF-AA (10 ng/ml), -BB (10 ng/ml), or basic FGF (10 ng/ml). Protein extracts of NIH/3T3 cells were then analyzed by immunoblotting with anti-P-TYR. By comparing the characteristic patterns of protein tyrosine phosphorylation (i.e., the "biochemical signature") induced by specific growth factors in NIH/3T3 fibroblasts to those observed after stimulation with RASM conditioned media samples, secreted growth factors induced by AII were identified.

As shown in Fig. 7 *A*, the recombinant growth factors induced characteristic patterns of protein tyrosine phosphorylation in the NIH/3T3 cells. For example, both PDGF-AA and -BB stimulated rapid tyrosine phosphorylation of proteins with bands at 180- to 185-, 145-, 120-, and several bands between 30- and 46-kD (Fig. 7 *A*, lanes *3* and *4*). The 180–185-kD bands correspond to activated PDGF receptors, which rapidly undergo tyrosine autophosphorylation upon binding to ligand (32). Basic FGF stimulated tyrosine phosphorylation of a prominent 90-kD protein, thought to be a major substrate of the activated receptor kinase (Fig. 7 *A*, lane *5*) (33). In contrast, NIH/3T3 cells treated with AII did not show any significant change in protein tyrosine phosphorylation (Fig. 7 *A*, lane *2*).

Conditioned media samples from untreated and AII-treated RASM cells stimulated complex patterns of protein tyrosine phosphorylation in the NIH/3T3 fibroblasts (Fig. 7 *B*). Media from untreated, serum-starved RASM cells stimulated tyrosine phosphorylation of proteins at least two proteins, of $\sim$ 150 and 90 kD, which were distinct from the bands observed after growth factor stimulation (Fig. 7 *B*, lane *1*). Conditioned media samples from the RASM cells treated with AII for 3 or 6 h appeared to contain an increased level of this 150- and 90-kD tyrosine-phosphorylation activity (Fig. 7 *B*, lanes *3–6*). The precise nature of this activity remains to be determined, but it may be linked to AII-stimulated mitogenesis (see below).

PDGF activity was detected in conditioned media samples from RASM cells treated with AII for 6–48 h. As shown in Fig. 7 *B*, lanes *4–8*, these samples stimulated tyrosine phosphorylation of a 180–185-kD protein, which migrated at the same position as the autophosphorylated PDGF receptors (Fig. 7 *B*, compare lanes *4–8* to 7 A, lane *4*). To confirm that this activity represented PDGF, conditioned media from RASM cells treated with AII for 24 h was incubated with a PDGF neutralizing antiserum for 30 min before stimulation of the NIH/3T3 cells. The resulting pattern of protein tyrosine phosphorylation lacked the 180/185-kD PDGF receptors, although tyrosine phosphorylation of the 150-kD band was observed (Fig. 7 *B*, lane *9*). Additional support for the induction of PDGF by AII was derived from Northern blot analyses of AII-stimulated RASM cells. Hybridization of these blots using labeled probes for either PDGF-A or PDGF-B chains showed that the RASM cells exclusively expressed the A-chain (data not shown, see below). These data are consistent with previous studies (21). Taken together, our results demonstrate that AII stimulates quiescent RASM cells to produce and secrete significant amounts of PDGF-AA homodimer in a time-dependent manner. PDGF-AA may therefore account, in part, for the delayed mitogenic effects of AII in these cells.

Neutralizing antibodies directed against several growth factors fail to inhibit AII-stimulated mitogenicity in RASM cells. In order to evaluate the possible role of autocrine growth factor induction in AII-stimulated mitogenesis in the RASM cells, we performed mitogenic experiments using several specific growth factor neutralizing antibody preparations. In these studies, serum-starved RASM cells were incubated with a specific growth factor neutralizing antibody for 30 min before addition of growth factors or AII and relative levels of DNA synthesis were determined after 48 h as described in the Methods. As

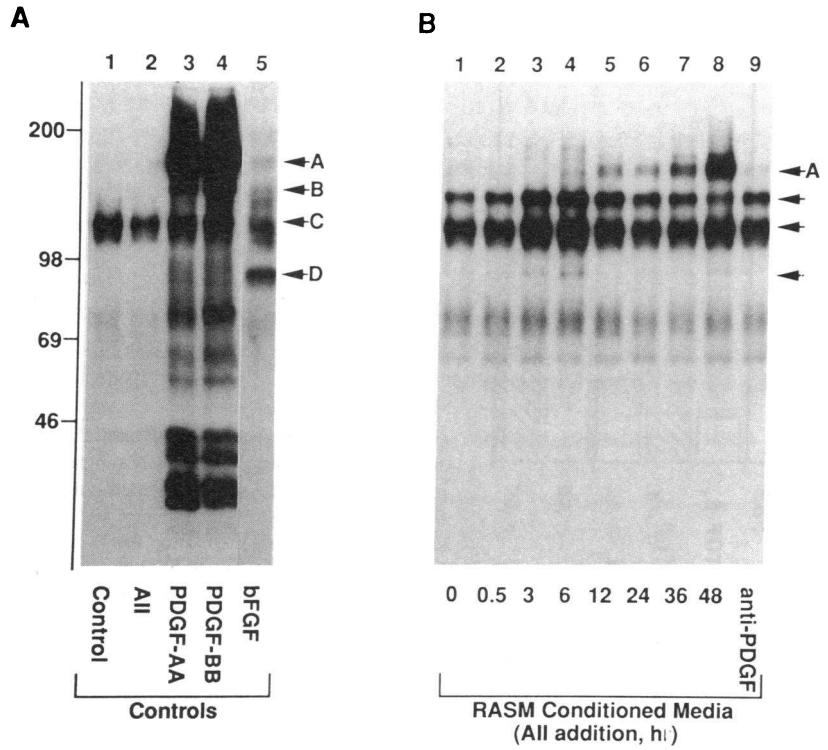

Figure 7. Detection of AII-stimulated growth factors from RASM cell conditioned medium by anti-P-TYR immunoblot analysis of challenged NIH/3T3 fibroblasts. (*A*) Anti-P-TYR immunoblot analysis of NIH/3T3 cell lysates from quiescent cells (lane *1*) and cells stimulated for 10 min with AII (1 μM, lane *2*), PDGF-AA (10 ng/ml, lane *3*), PDGF-BB (10 ng/ml, lane *4*), or basic FGF (10 ng/ml, lane *5*). Arrows indicate the activated PDGF receptors at 180–185 kD (smaller *A*), major tyrosine-phosphorylated proteins of 145 kD (*B*) and 120 kD (*C*), and the basic FGF receptor substrate (90 kD) (*D*). (*B*) Anti-P-TYR immunoblot of anti-P-TYR recovered cell proteins from serum-starved NIH/3T3 cells (lane *1*) and cells stimulated for 10 min with conditioned media samples from AII (1 μM)-treated RASM cells (lanes *2–9*). Individual cell lysate samples (0.5 mg) were first immunoprecipitated with anti-P-TYR, and immunoprecipitated proteins were resolved by SDS-PAGE. Lanes *1–8* list at the bottom the duration of AII treatment used to produce the conditioned media. Note that AII-treated RASM conditioned media stimulated the tyrosine phosphorylation of PDGF receptors (smaller *A*) (lanes *4–8*, compare with panel *A*, lanes *3* and *4*). The ability of 24-h AII-stimulated RASM conditioned media to activate PDGF receptors was blocked by PDGF neutralizing antiserum (lane *9*). Arrows below the PDGF receptor band indicate the 150-, 120-, and 90-kD tyrosine-phosphorylated proteins induced by the RASM conditioned media samples.

shown in Fig. 8 *A*, a PDGF neutralizing antiserum completely inhibited PDGF-BB (25 ng/ml)-stimulated DNA synthesis. Similar results were obtained if PDGF-AA was used as a mitogen (data not shown, see below). In contrast, addition of PDGF neutralizing antiserum had no effect on the level of [^{3}H]thymidine incorporation observed after AII (100 nM) treatment. The PDGF-neutralizing antiserum had no effect on either serum-free or FBS-stimulated DNA synthesis (data not shown). These results suggest that either autocrine expression of PDGF is not responsible for the delayed mitogenic effects of AII or that the interaction of PDGF and its receptors takes place in a cellular compartment that is inaccessible to the antibodies.

We also tested the effect of a TGF-β neutralizing antibody, 1D.11, on RASM mitogenesis (Fig. 8 *B*). This antibody inhibited the mitogenic effects of recombinant human TGF-β_1 on NIH/3T3 cells (data not shown, see also Dasch et al. [24]). As controls, serum-starved RASM cells were treated with recombinant TGF-β_1 (2 ng/ml) in the absence or presence of TGF-β neutralizing antibody, or AII (1 μM). We found that recombinant TGF-β_1 alone did not stimulate significant levels of DNA synthesis in the RASM cells (Fig. 8 *B*). Therefore, no relative change in the levels of DNA synthesis after co-incubation of TGF-β_1 with neutralizing TGF-β antibody was observed (Fig. 8 *B*). Interestingly, we found that AII treatment of cells that had been incubated with TGF-β_1 neutralizing antibodies exhibited increased levels of [^{3}H]thymidine incorporation when compared to AII addition alone (Fig. 8 *B*). Furthermore, addition of recombinant TGF-β_1 to cells treated with AII resulted in small but consistent decreases in the level of DNA synthesis. Taken together, these data indicate that TGF-β_1 is not mitogenic for RASM cells and therefore is unlikely to be involved in AII-stimulated mitogenesis. Rather, these data support recent studies suggesting that autocrine expression of TGF-β_1 may be associated with inhibition of smooth muscle cell growth (13, 34).

In order to confirm our results with TGF-β_1 and evaluate the potential role of other autocrine growth factors in AII-stimulated RASM cell growth, we performed additional experiments using several commercially available growth factor neutralizing antibody preparations (as noted in the Methods). These results are summarized in Table I. Consistent with the results presented above, we found that other TGF-β neutralizing antibodies either caused an apparent enhancement, or had no effect, on AII-stimulated DNA synthesis. However, interpretation of these results was complicated by the observation that these antibodies enhanced the apparent levels of DNA synthesis after treatment of the cells with recombinant TGF-β_1 (data not shown).

In separate experiments, neutralizing antibodies directed against basic FGF or EGF had no significant effect on AII-stimulated DNA synthesis (Table I). It is possible that the specific antibodies utilized may have failed to recognize endogenously expressed rat growth factors. However, excluding this possibility, our results indicate that either (*a*) the major mitogenic factor induced by AII in the RASM cells is distinct from the subset of growth factors assayed or (*b*) the functional autocrine interaction between an endogenously expressed growth factor and its receptor occurs in an inaccessible intracellular compartment in the AII-stimulated RASM cells.

AII-stimulated DNA synthesis in cultured RASM cells is inhibited by the growth factor antagonist compound suramin. Suramin, a polysulfonated naphthylurea (for a review, see La Rocca et al. [35]), has been shown to inhibit PDGF mitogenic activity and induce transient reversion of the v-*sis*–trans-

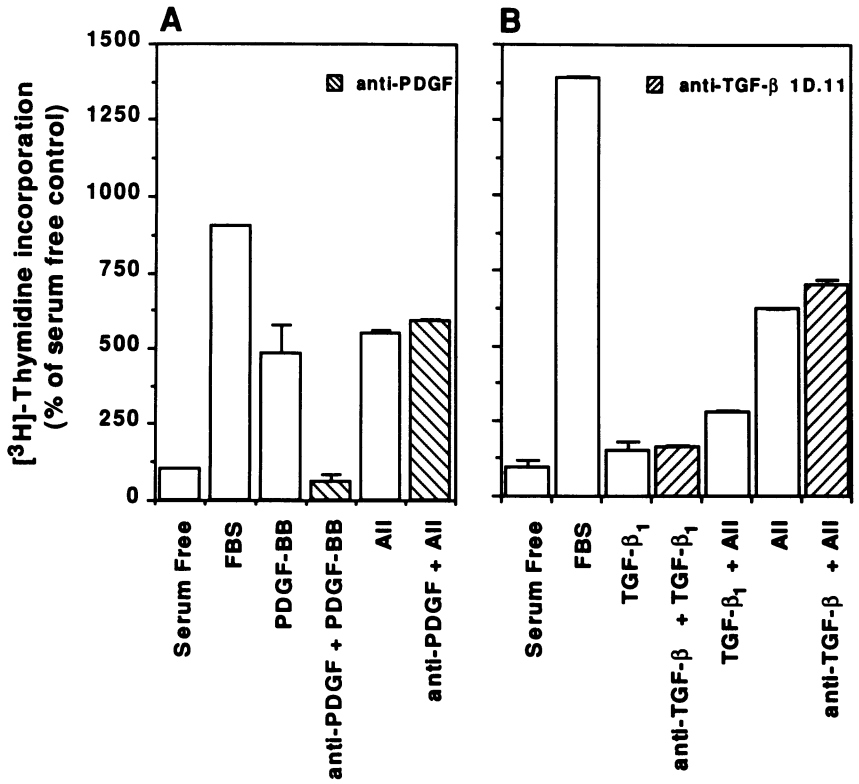

Figure 8. Effect of growth factor neutralizing antibodies on AII-stimulated DNA synthesis in RASM cells. Cells were incubated with antibodies (*hatched bars*) to PDGF (*A*) or TGFβ (*B*) for 30 min before incubation for 48 h with [^{3}H]thymidine and agonists. Results are expressed as percentage of the unstimulated serum-free control. Baseline [^{3}H]thymidine incorporation was 49,800±1600 cpm (*A*), and 40,300±7,800 (*B*). Bars are the mean±SD of three treatment wells from a representative experiment.

formed phenotype in fibroblasts (36, 37). This antiproliferative activity appears to involve binding of suramin to PDGF, as well as displacement of bound PDGF from its receptors (37–39). Suramin also binds other growth factors such as basic FGF, acidic FGF, and IGF-II and interrupts binding to their respective high-affinity surface receptors (40–42). Because suramin is a "broad-spectrum" growth factor inhibitor, and inhibits autocrine transformation under conditions where specific growth factor neutralizing antibodies are relatively ineffective (39), we investigated the possibility that this compound may inhibit AII-stimulated DNA synthesis in cultured RASM cells. For these experiments, serum-starved RASM cells were preincubated with various concentrations of suramin for 30 min and assayed for AII-induced mitogenic activity as described in the Methods. As shown in Fig. 9, suramin inhibited AII-stimulated [^{3}H]thymidine incorporation in a dose-dependent manner in the RASM cells. Furthermore, suramin did not appear to act as a nonspecific cytotoxin as it did not affect the mitogenic response of the RASM cells to FBS, used in these experiments as a positive control (data not shown). For exam-

Table I. Effects of Various Growth Factor Neutralizing Antibodies on AII-stimulated DNA Synthesis in cultured RASM Cells

Neutralizing antibody	[^{3}H]Thymidine incorporation AII (100 nM)	
	No antibody	Plus antibody
	% serum-free control	
Polyclonal rabbit anti-TGF-β_1	623±1.2	972±4.0
Polyclonal turkey anti-TGF-β_1	623±1.2	518±12.6
Polyclonal goat anti-EGF	588±4.2	632±3.4
Polyclonal rabbit anti-EGF	828±20.6	788±7.7
Monoclonal mouse anti-basic FGF	512±7.1	552±12.3

Cells were preincubated (37°C) with the indicated antibody for 30 min, and then incubated for 48 h with antibody and AII (100 nM) in the presence of [^{3}H]thymidine as described in Methods. Results are the mean±SD of three treatment wells from a representative experiment.

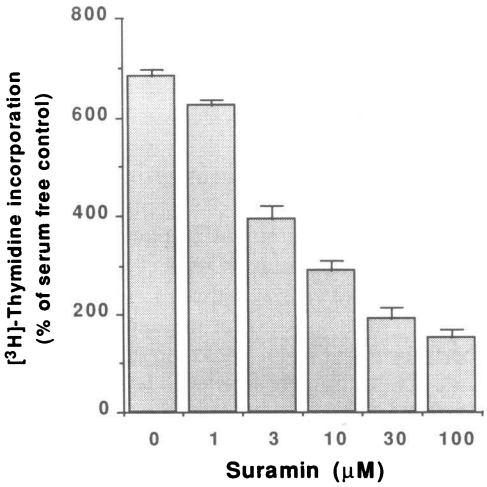

Figure 9. Suramin inhibits AII-stimulated DNA synthesis in a concentration-dependent manner. Quiescent RASM cells were preincubated with different concentrations of suramin for 30 min then incubated with [^{3}H]thymidine and AII (100 nM) for 48 h. Results are expressed as percentage of the serum free control. Serum-free control cells were incubated with [^{3}H]thymidine alone. Baseline [^{3}H]-thymidine incorporation was 15,700±2,500 cpm. Bars are the mean±SD of three treatment wells from a representative experiment.

ple, we found that 200 μM suramin inhibited FBS-stimulated [^{3}H]thymidine incorporation by 30%. In contrast, incubation of 100 μM suramin inhibited the mitogenic response induced by PDGF-BB by 65% (data not shown) and AII by 80% (Fig. 9). The IC_{50} of suramin for the inhibition of AII (100 nM)-stimulated DNA synthesis was determined to be ~ 10 μM.

One possible explanation for the antimitogenic effects of suramin observed in our experiments is that the drug directly inhibited AII binding to the RASM cells. This possibility was tested in binding experiments using the labeled AII receptor ligand [^{125}I]Sar^1, Ile^8-AII. Whole cell binding assays were performed using different concentrations of Sar^1, Ile^8-AII in the absence or presence of 100 μM suramin. In these assays suramin did not significantly inhibit the specific binding of Sar^1, Ile^8-AII to the AT_1 receptor (Fig. 10 *A*).

Suramin is reported to inhibit several intracellular enzymes, including protein kinase C (35, 43). Therefore, it is possible that suramin acts to interfere with early signaling pathways triggered by AII in the RASM cells. In order to exclude this possibility, we evaluated the effect of suramin on the induction of PDGF-A chain mRNA in the RASM cells, a delayed cell response that requires productive intracellular signal transduction. As shown in Fig. 10 *B*, Northern blot analysis using a labeled cDNA probe for PDGF-A chain revealed that cells stimulated with AII for 6 and 18 h exhibited an increase in the expression of three major bands (1.9-, 2.3-, and 2.8-kb) compared to serum-starved control cells. These bands represent specific mRNAs corresponding to PDGF A-chain (44). Pretreatment of the cells with suramin (100 μM) did not inhibit the induction of PDGF A-chain mRNA measured at either 6 or 18 h. Thus, suramin does not interfere with AII-initiated signaling pathways leading to increased expression of PDGF-A mRNA in the RASM cells. These results are consistent with previous reports demonstrating that suramin, at concentrations that cause reversal of autocrine-stimulated cell growth, is not generally cytotoxic, and fails to inhibit cell growth of several normal and tumor-derived cell lines (39, 45). Taken together, these studies support the hypothesis that suramin inhibits an AII-stimulated autocrine loop in RASM cells involving endogenously expressed growth factors.

AII stimulates the secretion of mitogenic factors from RASM cells: detection by NIH/3T3 fibroblast mitogenic assays. In order to provide more direct evidence that AII stimulation of endogenous mitogenic factors may account for AII-stimulated RASM cell growth, we performed experiments to detect mitogenic activity secreted by RASM cells. For these experiments, matched conditioned media samples from serum-starved RASM cells (control) and cells stimulated by AII (1 μM) for different times were added to quiescent cultures of NIH/3T3 cells, and [^{3}H]thymidine incorporation was measured after 24 h. Control NIH/3T3 cells were treated with recombinant forms of PDGF-AA (5 ng/ml) or -BB (20 ng/ml). These concentrations of PDGF were estimated to be at least 10–100-fold greater than those detected in the AII-stimulated RASM cell conditioned media samples (see Fig. 7). In some of the assays, the recombinant growth factors or conditioned media samples were incubated (37°C, 30 min) with either PDGF neutralizing antiserum (50 μg/ml), or suramin (100 μM) before addition to the NIH/3T3 cells.

As shown in Table II, addition of PDGF-AA or PDGF-BB to the quiescent NIH/3T3 cells increased DNA synthesis 43- and 69-fold, respectively. These responses were inhibited by inclusion of PDGF-neutralizing antiserum in the mitogenic

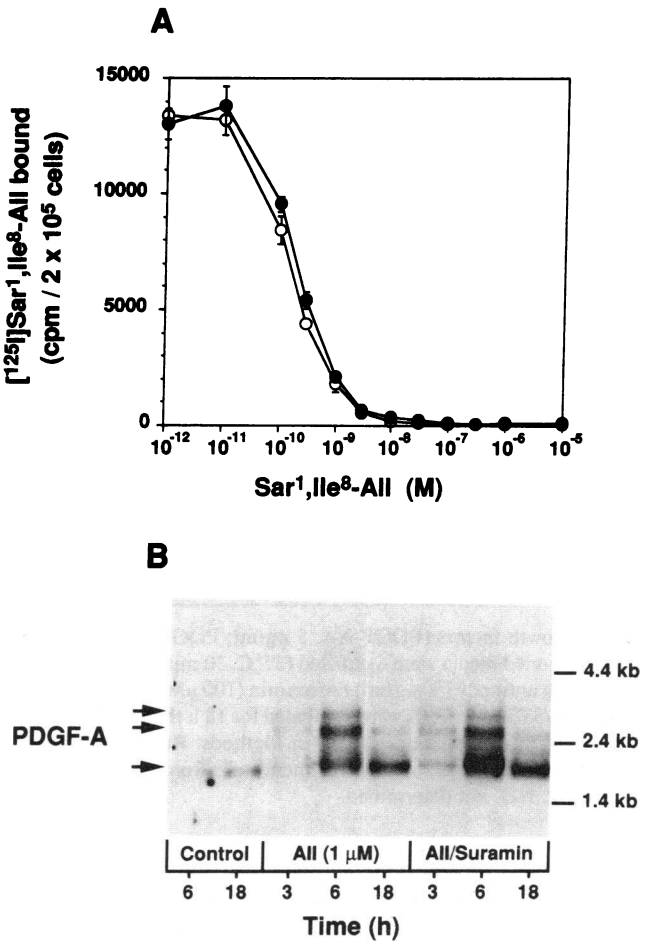

Figure 10. Suramin does not inhibit AII receptor binding or productive intracellular signal transduction in cultured RASM cells. (*A*) Monolayer cultures of RASM cells (5×10^4 cells/cm^2) were incubated (2 h, 37°C, 5% CO_2) in DME + 0.5% BSA supplemented with 0.6 nM of the AII agonist [^{125}I]Sar^1, Ile^8-AII and various concentrations of unlabeled Sar^1, Ile^8-AII in the absence or presence of suramin (100 μM). Results are expressed as ^{125}I-cpm bound/2×10^5 cells. Points are the mean±SD of three treatment wells from a representative experiment. (*B*) Serum-starved RASM cells were either left untreated (control) or stimulated with AII (1 μM) for the indicated times in the absence or presence of suramin (100 μM). Poly (A)$^+$ RNA (2.5 μg) was extracted, resolved by agarose gel electrophoresis, blotted, and hybridized with a ^{32}P-labeled human PDGF-A cDNA probe. Bands corresponding to the PDGF A-chain mRNAs are noted by arrows.

assays. Analysis of RASM conditioned media samples revealed that serum-starved RASM control media (3 h) stimulated a modest increase in DNA synthesis (~ 3-fold), and samples of conditioned media from cells treated for longer periods exhibited even greater mitogenic activity (5–15-fold). Thus, even under serum-starved conditions, RASM cells produce factors that are mitogenic to fibroblasts, and these factors appeared to accumulate in the conditioned media over time.

In parallel assays, conditioned media obtained from RASM cells treated with AII for 3 and 6 h exhibited a 2-fold increase in DNA synthesis in NIH/3T3 cells in comparison to paired controls (Table II). These data imply that AII either stimulates the release of additional mitogens from RASM cells or increases the expression of mitogenic factors that are already secreted at "basal" levels in serum-starved RASM cells. In support of the

Table II. AII-stimulated RASM Cells Secrete Mitogenic Factors

	[^{3}H]Thymidine incorporation		
	Pretreatment		
	None	Anti-PDGF	Suramin
	% of serum free control		
Quiescent NIH 3T3	100±16.0	ND	ND
+PDGF AA	4350±3.7	129±8.5	ND
+PDGF BB	6900±1.5	196±17.3	61±12.9
+RASM conditioned media			
3-h control	395±9.1	435±0.1	189±12.5
3-h AII	779±3.6	1090±10.9	152±16.3
6-h control	602±3.6	884±8.4	244±13.9
6-h AII	1250±4.4	1860±17.3	215±4.5
24-h control	944±4.9	1630±5.0	356±8.7
24-h AII	1060±7.5	1400±11.5	80±29.7
48-h control	1420±18.5	2043±3.0	442±12.7
48-h AII	1070±7.4	913±4.3	95±8.6

Control growth factors (PDGF-AA, 5 ng/ml; PDGF-BB, 20 ng/ml) and conditioned media were incubated (37°C, 30 min) with PDGF neutralizing antibody (50 μg/ml) or suramin (100 μM) before addition to the NIH/3T3 cells. Cells were incubated for 18 h then labeled with [^{3}H]thymidine for 6 h as described in Methods. Results are the mean±SD of two, three, or four treatment wells from a representative experiment. ND, not determined.

latter concept, we observed that while the mitogenic activity of conditioned media remained high at 24 and 48 h (~ 10-fold), there were no longer differences between control and AII-stimulated samples (Table II). Thus, either the accumulation of factors secreted by serum-starved RASM cells was similar to that induced by AII or that degradation of secreted factors occurred between 6 and 48 h.

Of greater significance is that the PDGF-neutralizing antiserum had no effect on mitogenesis induced by any of the RASM conditioned media samples (Table II). In contrast, suramin inhibited the mitogenic activity of all conditioned media samples. Thus, despite the fact that RASM cells secreted functional PDGF A-chain, this factor was not present in sufficient concentration to act as mitogen for the NIH/3T3 cells. These data are also consistent with our previous studies which demonstrate that significant mitogenic effects of PDGF-AA on RASM cells required concentrations > 5 ng/ml (46). Taken together, our results confirm that AII stimulates RASM cells to express and secrete mitogenic factors, distinct from PDGF-A chain, that may be important in the control of smooth muscle cell proliferation. This indirect growth mechanism likely accounts for the delayed mitogenesis observed in RASM cells treated with AII.

Discussion

In the present study, we demonstrate that AII effectively stimulates DNA synthesis and cell proliferation in a well-characterized quiescent RASM cell line. Thus, our findings support recent studies implicating AII in vascular neointimal formation and atherosclerosis. In these studies, ACE inhibitors have an effective antiproliferative effect in rat models of restenosis (6), and primate models of atherosclerosis (47). In addition, direct infusion of AII is reported to stimulate smooth muscle cell proliferation and neointimal formation in normal and balloon-injured arteries in the rat (8).

Although our results support some previous studies (9–11), they differ significantly from others that have examined the growth effects of AII in smooth muscle cell cultures. For example, AII has been reported to induce hypertrophy, but not proliferation, in primary adult rat vascular smooth muscle cell cultures (14, 15). In contrast, a more recent study by Gibbons et al. (13) showed that AII treatment of serum-starved RASM cells for 36 h led to a stimulation of DNA synthesis that was enhanced by co-incubation with TGF-β_1 neutralizing antibodies. The discrepancies noted between these various reports may be partly explained by differences in biological assay conditions. However, they may also be related to phenotypic differences observed between various vascular smooth muscle cell lines. For example, several studies have demonstrated that individual vascular smooth muscle cell isolates display considerable variation in morphology, gene expression, and responsiveness to certain growth factors (48–52), including AII (53). Thus additional experiments, comparing the phenotypes of proliferating AII-stimulated vascular smooth muscle cell cultures with neointimal cells derived from vascular lesion sites in vivo, will be required to establish the exact relevance of this mechanism to hyperproliferative cardiovascular processes.

The cellular mechanisms involved in AII-stimulated mitogenesis are undefined, and may be distinct from other mitogenic agents. For example, our results demonstrate that although AII stimulates acute expression of c-*fos* to a similar degree as mitogenic concentrations of FBS or PDGF-BB, AII-stimulated DNA synthesis is delayed. Since intracellular signaling via AT_1 receptor activation is known to be rapid and involve several biochemical mechanisms including phosphatidylinositol metabolism (19), intracellular calcium mobilization (1), and protein tyrosine phosphorylation (20, 54), our results suggest that all of these pathways are not directly coupled to a prompt mitogenic response in these cells, and may in fact be insufficient for new DNA synthesis.

One possible explanation for the delayed mitogenesis observed after AII stimulation of the RASM cells is that increased expression of new gene products is required before the initiation of DNA synthesis. In support of this concept, we found that AII stimulates the expression of several growth factors, such that one or more may act as "proximal" mitogens that are ultimately responsible for the AII-stimulated cell growth. A similar autocrine growth model is suggested by Naftilan et al. (21) in studies where AII induced the expression of PDGF-A chain in cultured RASM cells. PDGF, therefore, represents one candidate "proximal" mitogen associated with AII-stimulated DNA synthesis in vascular smooth muscle cells. In support of this model, our results indicate that the delayed stimulation of DNA synthesis induced by AII in the RASM cells correlates with the expression of specific endogenous growth factors, including PDGF and TGF-β_1. Furthermore, increased local tissue expression of these factors is described after arterial balloon injury in animal models of restenosis (reviewed in Schwartz et al. [55]). Thus the effects of AII on RASM cell growth in vitro described in this report are similar to animal models of smooth muscle cell proliferation.

A question arises as to the role of the specific AII-stimulated autocrine growth factors in smooth muscle cell proliferation. For example, TGF-β_1 does not appear to have an important growth-promoting role, since exogenously added ligand did not stimulate DNA synthesis in the RASM cells. Furthermore,

TGF-β neutralizing antibodies increased, rather than inhibited, AII-stimulated DNA synthesis. In separate experiments, specific neutralizing antibodies, directed against other growth factors including PDGF, TGF-β_1, EGF, etc., failed to significantly decrease AII-stimulated DNA synthesis. Furthermore, a PDGF neutralizing antiserum did not inhibit the mitogenic activity present in the conditioned media derived from AII-stimulated RASM cells. Taken together, our results suggest that productive autocrine ligand–receptor interactions, if required for AII-stimulated mitogenicity, may occur "intracellularly," i.e., in a compartment inaccessible to neutralizing antibodies. Evidence supporting this type of interaction exists for other growth factors (56–58). Alternatively, these results indicate that the co-mitogenic effects of the growth factors tested in this study do not completely account for AII-stimulated cell growth, implicating other growth factors in this mechanism.

Although our results demonstrate that PDGF-AA secreted from AII-stimulated RASM cells does not contribute to cell proliferation induced by AII, it is possible that this growth factor may have other important biologic functions. For example, increased expression of PDGF-AA may promote smooth muscle cell migration in an autocrine or paracrine fashion (59). This mechanism is particularly relevant in neointimal formation after acute vascular injury in vivo. Thus increased PDGF-AA secretion from proliferating smooth muscle cells in response to AII may provide a chemotactic signal for other cell types involved in vascular tissue repair. This latter model is supported by recent studies in which PDGF-neutralizing antibodies were shown to reduce neointimal formation in the rat carotid balloon-injury model by specifically inhibiting smooth muscle cell migration, but not proliferation (60).

Evidence for the involvement of other growth factors in AII-mediated RASM cell proliferation was derived from the experiments in which the mitogenic effects of conditioned media collected from AII-stimulated cells were measured using NIH/3T3 cells. Analysis of RASM-conditioned media samples for the induction of characteristic protein tyrosine phosphorylation patterns in NIH/3T3 cells revealed novel tyrosine-phosphorylated proteins at ~ 150- and 90-kD that were not inhibited by PDGF neutralizing antibodies. Since this activity roughly correlates with mitogenic activity in AII-stimulated RASM conditioned media, these data indicate that distinct endogenous RASM cell mitogens may be important for AII-stimulated growth. Future experiments, aimed at identifying individual AII-induced mitogenic factors in RASM cell conditioned media, are required to resolve this hypothesis.

In addition, we demonstrate that suramin, a compound previously shown to interfere with autocrine cell transformation, inhibits mitogenesis induced by AII in the RASM cells. Although earlier reports using suramin focus on its role as an inhibitor of PDGF, this compound also binds other growth factors (35, 40, 42). In our experiments, suramin inhibits AII-stimulated mitogenesis but has no apparent effect on AII binding to the RASM cells, or the ability of AII to increase mRNA production for PDGF A-chain. Additional experiments suggest that inhibitory concentrations of suramin have no effect on AII-stimulated phosphoinositide hydrolysis (M. L. Webb and C. J. Molloy, unpublished observations). Suramin is an effective inhibitor of the mitogenic factors secreted by RASM cells in response to AII. These data support an indirect mechanism for suramin inhibition of AII-stimulated smooth muscle cell growth. Thus, it is possible that suramin acts by interrupting the binding of specific AII-induced autocrine growth factors to their receptors. However, the specific mitogen(s) responsible for AII's proliferative effects remains the subject of future investigation.

In summary, we demonstrate that the potent vasoactive peptide AII can stimulate DNA synthesis and promote cellular proliferation in a cultured RASM cell system. AII-stimulated cell growth correlates with the expression of endogenous growth factors, including PDGF and TGF-β_1. Since the effects of AII on the expression of these endogenous growth factors in the RASM cell cultures mimics many similar changes observed in neointimal smooth muscle cells after arterial injury in vivo (48) our results indicate that the autocrine induction of specific growth factors likely accounts for the proliferative effects of AII in cardiovascular diseases. These results predict that inhibition of AII in vivo, through the use of ACE-inhibitors or AII receptor antagonists, may inhibit the localized expression of potent polypeptide growth factors and consequently limit vascular smooth muscle cell proliferation. Furthermore, identification of the precise smooth muscle cell autocrine factor(s) responsible for the proliferative effects of AII may lead to novel targets for drug development for the treatment of hyperproliferative vascular diseases.

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