Mechanical Strain and Collagen Potentiate Mitogenic Activity of Angiotensin II in Rat Vascular Smooth Muscle Cells

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Abstract

The effects of extracellular matrix proteins and mechanical strain on the mitogenic activity of angiotensins I and II (AI and AII) were examined in cultured rat vascular smooth muscle (VSM) cells. VSM cells on various extracellular matrices were exposed to AII (1 μM) for 48 h. On plastic, AII induced only a 1.6-fold increase in [^{3}H]thymidine incorporation, but on fibronectin- or type I collagen–coated plastic, the response to AII was enhanced from two- to fourfold. On a type I collagen–coated silicone elastomer, to which mechanical strain was applied, [^{3}H]thymidine incorporation dramatically increased to a maximum of 53-fold. Dup 753 (10^{-5} M) blocked the AII-induced increase in DNA synthesis. AI also increased DNA synthesis in VSM cells, and this response was also enhanced by mechanical strain. Mitogenic activity of AI was blocked by ramiprilat (10^{-5} M), indicating that its mitogenic activity was via conversion to AII. The synergy between AII and strain was completely eliminated by neutralizing antibodies to PDGF AB (3 μg/ml). Furthermore, the mitogenic effect of AII in unstrained cells was also synergistic with submaximal concentrations of PDGF AB (1 ng/ml). Thus, the synergy between AII and mechanical strain probably results from synergism between AII and PDGF secreted in response to strain. (*J. Clin. Invest.* 1993. 92:3003–3007.) Key words: vascular smooth muscle • mechanical stress • angiotensin II • platelet-derived growth factor • mitogens

Introduction

Chronically elevated arterial blood pressure is associated with an increase in thickness of the vascular media (1–3). This change is the result of smooth muscle hypertrophy and/or hyperplasia (4, 5), as well as increased quantities of extracellular matrix components (6, 7). These structural vascular changes may contribute to the maintenance of hypertension (8). There is evidence that both increased pressure per se (9), and humoral factors (10) may be involved in smooth muscle proliferation or hypertrophy. One potential humoral factor which may play a role in vascular remodeling is angiotensin II (AII)[1]. Both AII receptor antagonists and angiotensin-converting enzyme (ACE) inhibitors have been shown to prevent the development of vascular hypertrophy in the spontaneously hypertensive rat (SHR) (11–13).

Campbell-Boswell and Robertson (14) showed an increase in both cell size and cell number with AII, whereas Geisterfer et al. (15) reported a hypertrophic response with no mitogenic effect. Such differences may relate to differences in serum concentration in the medium, with hyperplasia occurring in the presence of serum and hypertrophy occurring in the absence, of serum (16). On balance, AII appears to be a relatively weak mitogen for VSM cells, with various studies reporting increases in DNA synthesis ranging from zero- to fourfold (17–21). A limitation of these studies is that they have generally been carried out without potentially important environmental factors such as extracellular matrix and pulsatile mechanical strain. Strain is known to increase cell proliferation in cultured epithelial (22) and endothelial cells (23). We have recently shown that cyclical mechanical strain induces vascular smooth muscle (VSM) proliferation in cultured VSM cells (24) via the autocrine action of PDGF (25). In hypertension, mechanical strain on the vessel wall is increased by as much as 15% (26); the resultant alterations in the cytoplasmic or genomic machinery of the smooth muscle cell may alter the potency of mitogens to which it is exposed.

It has been suggested that the ability of ACE inhibitors to prevent or reverse vascular hypertrophy may be, in part, by inhibition of local vascular ACE (27). Both the vascular endothelium and VSM cells have been shown to have ACE (28, 29). Schiffers et al. reported that despite the presence of ACE accessible to exogenous AI in isolated carotid arteries, inhibition of this enzyme failed to alter basal DNA synthesis in smooth muscle cells from this tissue (30). Thus, the role of vascular ACE, if any, in angiotensin-induced growth remains to be determined.

In the present study, we hypothesized that extracellular matrix proteins and mechanical strain might potentiate the effects of AII in culture. We also studied the roles of angiotensin I (AI) and local ACE as potential mitogenic factors in VSM. Lastly, we examined the interaction between PDGF and AII in the induction of DNA synthesis in VSM cells to explore the mechanism of potentiation of the effect of AII by mechanical strain.

Methods

Materials. All materials were purchased from Sigma Immunochemicals (St. Louis, MO), unless otherwise specified. [^{3}H]Thymidine was purchased from New England Nuclear (Boston, MA). Human α-thrombin was generously supplied by John W. Fenton II (New York State Department of Health, Albany, NY). Neutralizing antibodies to PDGF-AB were goat anti-human PDGF-AB purchased from Upstate Biotechnology (Lake Placid, NY). Upstate Biotechnology has determined that this antibody neutralizes the biological activity of all three forms (AA, AB, and BB) of rodent PDGF. Ramiprilat was supplied by Upjohn Pharmaceuticals (Kalamazoo, MI), and Dup753 (Losartan) was supplied by Du Pont/Merck Pharmaceuticals (Wilmington, DE).

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1. *Abbreviations used in this paper:* AI, angiotensin I; AII, angiotensin II; ACE, angiotensin-converting enzyme; SHR, spontaneously hypersensitive rat; VSM, vascular smooth muscle.

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Cell culture. Primary cultures of VSM cells from newborn rats were established by Peter Jones (University of Southern California) and were selected for significantly greater production of elastin than fibroblasts. From these primary cultures, the R22D cell line was established (31) and generously supplied to us by Dr. Jones at passage 15. The cells were maintained in minimum essential medium with 10% fetal bovine serum, 2% tryptose phosphate broth, penicillin (50 U/ml), and streptomycin (50 U/ml) in a humidified atmosphere of 5% CO_2/95% air at 37°C. Culture medium was changed every other day until cells were confluent. Cells were subcultured with trypsin-versene and 0.2% pancreatin, and cells from passages 16–29 were used for the present study. Cells from passages 16–29 express smooth muscle myosin (Wagdy, H. M., H. P. Reusch, E. Wilson, and H. E. Ives, manuscript in preparation), endothelin (32), and angiotensin II receptors on the basis of intracellular Ca^{2+} response. Before experimentation, cells were plated in 24-well plates, which were uncoated or coated with gelatin, type I collagen (calf skin), or fibronectin (bovine plasma). For gelatin or collagen coating, protein solution was added to dishes at 6 $\mu g/cm^2$, allowed to dry overnight, and rinsed. For fibronectin coating, protein solution was added at the same concentration and plates were air-dried for 45 min at room temperature. Assuming that all of the applied protein is adsorbed, this method yields $\sim 2 \times 10^{13}$ sites/cm^2.

Application of cyclic strain to cultured cells. Cells were grown to confluence in 6-well silicone elastomer–bottomed culture plates with a hydrophilic surface (Flexcell Corp., McKeesport, PA). Cells were subjected to mechanical deformation with a stress unit (Flexcell Corp.). The stress unit is a modification of the unit initially described by Banes et al. (33, 34) and consists of a computer-controlled vacuum unit and a baseplate to hold the culture dishes. Vacuum ($\sim$ 20 kPa) is repetitively applied (1 Hz, 0.5 s on-time) to the rubber-bottomed dishes via the baseplate, which is placed in a humidified incubator with 5% CO_2 at 37°C. The computer system controls the frequency of deformation and the negative pressure applied to the culture plates. Application of the vacuum results in maximal strain of 25–27% to cells at the periphery of the dishes; strain declines towards the center (35). Collagen-coating of the silicone elastomer dishes was by covalent bonding (Flexcell method proprietary), resulting in $\sim 2 \times 10^{14}$ sites/cm^2.

[^{3}H]Thymidine incorporation and cell number. Cells were grown in either the 24-well plastic plates or the six-well Flex plates until confluent and were growth-arrested by placing them in quiescence medium containing 5 μg/ml transferrin and 0.5 mg/ml bovine serum albumin for 72 h. Cells were then treated with the indicated agonists and/or subjected to cyclic strain for a 48-h period. During the last 6 h, 1 μCi/ml of [^{3}H]thymidine was added to the growth medium of each well and incubated at 37°C. Cells were then washed three times with assay medium (containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM Na_2HPO_4, 25 mM glucose, 25 mM Hepes/NaOH (pH 7.20), and 0.5 mg/ml bovine serum albumin, and extracted with 15% trichloroacetic acid at 4°C for 30 min. For plates, the rubber bottom containing the TCA-precipitable material was removed and placed directly into scintillation vials for counting. Cells on plastic plates were washed with distilled water after aspiration of TCA, dried, then 1 M NaOH (0.5 ml) was added for 20 min. 0.5 ml of HCl (1 M) was then added, and the contents of the wells were placed in scintillation vials for counting. Counting efficiency for ^{3}H was 62.4±0.3% in all experiments. In separate plates, cell number was determined in a hemacytometer after trypsin digestion. Cell numbers were $\sim 1.0 \times 10^5$ cells/dish at the beginning of all experiments.

Data presentation and statistics. All data are presented as mean±SEM. Comparisons between two means were made using Student's *t* test. Multiple comparisons were made using a one-way analysis of variance followed by a post hoc Student Neumann Keul's test. The null hypothesis was rejected at $P < 0.05$.

Results

Mitogenic response of VSM to AII on plastic and effect of extracellular matrix proteins. Under conventional culture conditions with plastic dishes, AII (1 μM) induced a 1.6-fold increase in [^{3}H]thymidine incorporation over a 24-h period. This was significantly less than the ninefold effect seen in response to thrombin (0.3 nM, Fig. 1). Exposure of the cells to AII (1 μM) during a 48-h period resulted in no further increase in DNA synthesis.

To examine the effect of extracellular matrix proteins on the mitogenic response to AII, plastic dishes were coated with either gelatin, fibronectin, or type I collagen (Fig. 2). There was no significant increase in the mitogenic response to AII in cells grown on gelatin. However, on fibronectin-coated plastic, AII induced a 2.4-fold increase in DNA synthesis, and in cells plated on type I collagen, AII induced a fourfold increase in DNA synthesis. Lastly, cells plated on type I collagen–coated silicone elastomer (used below for strain experiments), the mitogenic effect of AII increased $\leq$ 12-fold (Fig. 3). The enhanced fold-increase in thymidine incorporation on the collagen–coated surfaces was caused, in part, by a reduction in basal thymidine incorporation on these surfaces (see Fig. 2). The AII-induced increase in thymidine incorporation was almost completely abolished by the selective AII receptor (subtype 1) antagonist Losartan (DuP 753, 10 μM). Since there is debate as to whether AII induces hyperplasia in VSM, we also examined the effect of AII on cell number on collagen–coated dishes. At 48 h, AII (1 μM) induced a significant increase in cell number (32±13%) compared to cells grown on the same substrate (type I collagen–coated plastic) without AII.

Effect of mechanical strain on AII-induced DNA synthesis. Cyclic mechanical strain, to which VSM cells in the arterial wall are subjected, enhances DNA synthesis in VSM cells in culture (24). Therefore, we examined the effect of AII on VSM cells in the presence of mechanical strain. There was some variability in the degree of quiescence obtainable in VSM cells grown on silicone elastomer. This variability was reflected in variability in the fold-stimulation of thymidine incorporation by AII in cells cultured under these conditions. However, in all experiments, AII had significantly greater mitogenic activity in cells exposed to mechanical strain than in cells grown in static culture conditions. Under the best conditions, cyclic mechanical strain (1 Hz) alone caused a 17-fold increase in thymidine incorporation over a 48-h period and the mitogenic effect of AII (1 μM) was synergistic with cyclic mechanical strain, yielding a 53-fold increase in DNA synthesis (Fig. 4). As on static surfaces, this response was significantly reduced by DuP 753. Thus, mechanical strain greatly potentiates the mitogenic activity of AII.

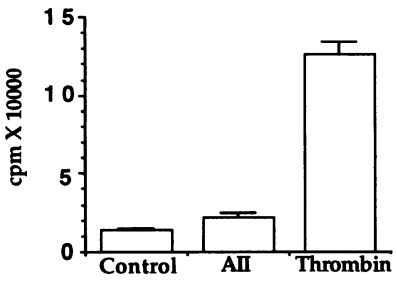

Figure 1. AII is a weak mitogen to VSM cells grown on plastic. Confluent cultures of VSM cells grown in plastic dishes were exposed to serum-free medium for 72 h. The indicated concentrations of AII or α-thrombin were added for 24 h. During the final 6 h, [^{3}H]-thymidine (1 μCi/well) was present. Thymidine incorporation was measured as described in Methods. Experiments were performed in quadruplicate; result shown is representative of three similar experiments. $P < 0.05$, AII vs control; $P < 0.001$, thrombin vs control or AII.

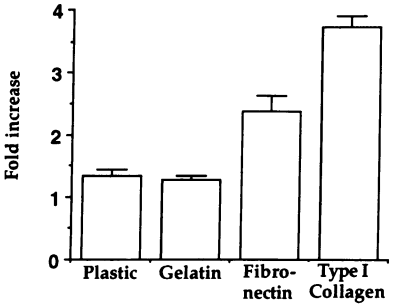

Figure 2. Effect of extracellular matrix proteins on mitogenicity of AII. VSM cells plated on plastic dishes coated with the indicated matrix proteins were exposed to AII (1 μM) for 48 h; for each matrix, thymidine incorporation was measured as in Fig. 1 and compared to basal thymidine incorporation in cells grown on similarly coated dishes not exposed to AII. Data shown is fold-increase in thymidine incorporation induced by AII. Basal thymidine incorporation was 5,595±620 (plastic), 8,602±684 (gelatin), 4,851±301 (fibronectin), and 1,610±147 cpm/dish (collagen, type I). Experiments were performed in quadruplicate; result shown is representative of three similar experiments. $P < 0.01$, fibronectin or type I collagen vs plastic.

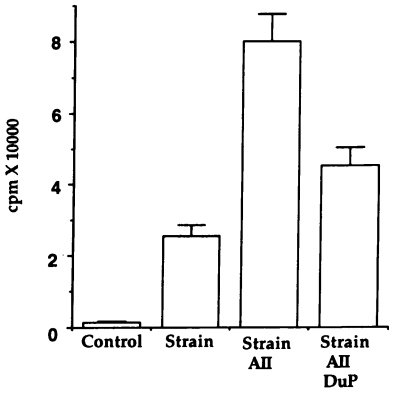

Figure 4. AII and mechanical strain synergistically increase DNA synthesis in VSM. VSM cells plated on collagen-coated silicone elastomer dishes were exposed to AII (1 μM and DuP 753 (10 μM) as indicated. Where indicated, mechanical strain (1 Hz) was produced as described in Methods. Thymidine incorporation was measured as described in Fig. 1. Experiments were done in triplicate; result shown is representative of three similar experiments. $P < 0.01$, strain vs control; $P < 0.001$, strain + AII vs strain alone; $P < 0.005$, strain + AII + DuP 753 vs strain + AII.

Mitogenic response of VSM to AI and inhibition by the ACE inhibitor ramiprilat. Having established conditions under which AII is a potent mitogen, we wished to examine the potential role of endogenous ACE activity on VSM proliferation. Therefore, we determined the effect of AI on thymidine incorporation in VSM. AI (1 μM) significantly increased thymidine incorporation in cells cultured under static conditions and in cells exposed to mechanical strain (Fig. 5). The mitogenic activity of AI was almost completely eliminated by the angiotensin converting enzyme inhibitor ramipril (10^{-5} M), suggesting that the AI-induced increase in DNA synthesis is probably via enzymatic conversion of AI to AII by the VSM cells (Fig. 5).

Mechanism for synergy between AII and mechanical strain on mitogenic response of VSM. We have previously shown that strain induces an increase in DNA synthesis in part via production and secretion of PDGF (25). Therefore, we examined the effect of PDGF AB antibodies (3 μg/ml) on the combined mitogenic effect of AII and mechanical strain. While PDGF AB antibodies had no significant effect on the AII-induced increase in thymidine incorporation, the potentiating effect of mechanical strain on AII-induced proliferation was reduced by 80–90% (Fig. 6).

To show that PDGF produced in response to strain could in fact synergistically enhance the response to AII, we examined the mitogenic effect of AII on VSM cells in the presence of a minimally responsive concentration of PDGF AB (1 ng/ml).

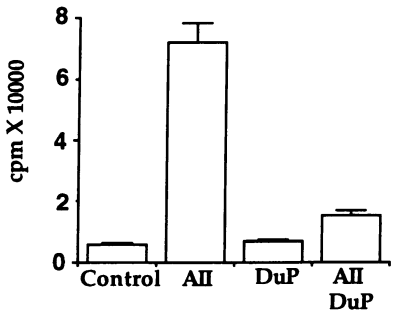

Figure 3. Inhibition of AII-induced DNA synthesis by DuP 753. VSM cells plated on collagen-coated silicone elastomer dishes were exposed to AII (1 μM) and DuP753 (10 μM) for 48 h as indicated; thymidine incorporation was measured as in Fig. 1. Experiments were performed in triplicate; result shown is representative of three similar experiments. $P < 0.001$, AII vs control or AII + DuP 753.

On collagen-coated plastic dishes, 1 ng/ml of PDGF AB caused only a 1.6-fold increase in DNA synthesis, while AII alone caused a fourfold increase. The two growth factors together induced a 10-fold increase in DNA synthesis, greater than the sum of their individual effects (Fig. 7). Taken together with the data presented in Fig. 6, these data suggest that strain enhances the mitogenic response to AII via strain-induced secretion of PDGF AB.

Discussion

Angiotensin II may play an important role in the development of hypertension-associated vascular disease (11, 12). However, previous studies on the mitogenic actions of AII have demonstrated a variable and relatively small effect on the growth of

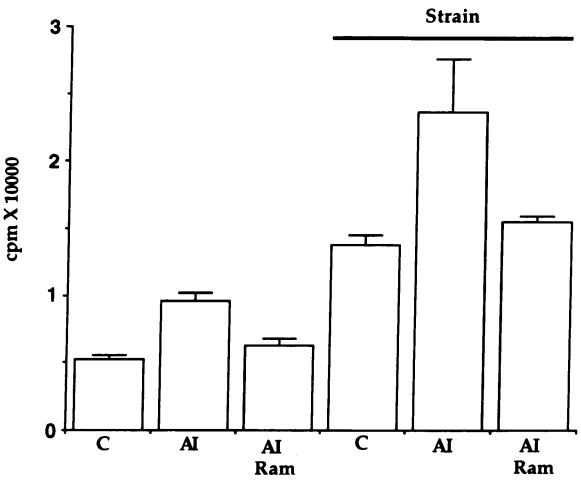

Figure 5. Mitogenic effect of AI is potentiated by mechanical strain and blocked by ACE inhibition. VSM cells plated on collagen-coated silicone elastomer dishes were exposed to AI (1 μM), Ramipril (10 μM), and/or mechanical strain as indicated for 48 h. Thymidine incorporation was measured as described in Fig. 1. Experiments were done in triplicate; result shown is representative of three similar experiments. In unstrained cells $P < 0.05$, AI vs control; $P < 0.05$; AI + Ramipril vs AI. In strained cells $P < 0.005$ AI vs control or AI + Ramipril.

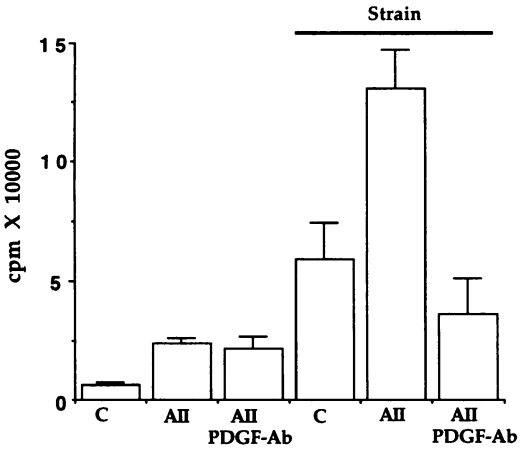

Figure 6. Synergism between AII and mechanical strain is attenuated by antibodies to PDGF AB. VSM cells plated on silicone-elastomer dishes were exposed to AII (1 μM), mechanical strain (1 Hz), or both, with or without antibodies to PDGF AB (3 μg/well). Thymidine incorporation was measured as described in Fig. 1. Experiments were performed in triplicate; result shown is representative of three similar experiments. In unstrained cells AII vs AII + PDGF-Ab, NS. In strained cells $P < 0.001$, AII vs AII + PDGF-Ab.

VSM in culture (17–21). Most of these earlier studies were performed on conventional plastic culture plates. Previous work has shown that extracellular matrix components can alter the phenotype of VSM cells (36), but to our knowledge, the effects of matrix on growth responses to AII has not been explored. Since hypertension is associated with increases in both extracellular matrix proteins (37, 38) and mechanical strain (26), we felt that these factors should be considered in assessing the mitogenic effects of AII. In the present study, we found that certain extracellular matrix proteins, and mechanical strain, strongly potentiate the mitogenic effects of AII on VSM cells. We do not yet know precisely how these factors potentiate the actions of AII, but we are currently exploring the possibility that occupancy of cell surface integrins may provide mitogenic signals that cooperate with the AII receptor.

In addition to an increase in thymidine incorporation, we found a 32% increase in cell number in response to AII on collagen-coated dishes, suggesting that AII causes hyperplasia under these conditions. Since our measurements of thymidine incorporation represent more or less instantaneous rates of DNA synthesis, we cannot determine in these experiments whether the response to AII was primarily hyperplastic or whether hypertrophy and polyploidy also occur.

Cyclic mechanical strain is another factor to which VSM cells are exposed in vivo. In several systems, including cultured bone (39), epithelial cells (22), and endothelial cells (23), strain has been shown to increase cell proliferation. We have recently found that mechanical strain increases thymidine incorporation in VSM cells (24). Preliminary data from our lab suggests that this is caused, at least in part, by autocrine production of PDGF (25). We now find that, under conditions of mechanical strain, the mitogenic response to AII is greatly enhanced. This enhancement of the mitogenic response was almost completely eliminated by neutralizing antibodies to PDGF AB. Furthermore, we found that AII increases DNA synthesis synergistically with submaximal concentrations of PDGF AB. These findings suggest that the modulating effect of strain upon the mitogenic response to AII is mediated by secretion of PDGF.

AII itself has also been shown to induce PDGF A chain expression in VSM cells and PDGF has thus been proposed to serve as an important intermediary in the mitogenic action of AII (40). Since AII was synergistic with submaximal concentrations of PDGF (Fig. 7) and PDGF antibodies did not block the mitogenic response to AII alone (Fig. 6), secretion of PDGF did not appear in our system to be an important intermediary in the mitogenic response to AII when other factors (mechanical strain) were eliminated. The reasons for this difference between systems remains to be resolved.

Our finding that AI, via conversion to AII, is also mitogenic to VSM cells contributes to the ongoing debate on the role of local renin-angiotensin systems in vascular pathophysiology. Other groups have shown that ACE activity is found in the vascular wall (27–30, 41, 42). However, its participation in angiotensin-induced mitogenesis has never been clearly established. In this regard, it is interesting that ACE inhibitors appear to reduce vascular hypertrophy in the spontaneously hypertensive rat (11, 12), a strain that does not exhibit particularly high levels of renin or AII (43). This phenomenon might be caused by abnormally high production of active renin (44) and ultimately AI in the vascular wall of the SHR. Our findings now indicate that AI is a potent mitogen for VSM cells, particularly when they are exposed to mechanical strain. This proliferative effect was virtually eliminated by an ACE inhibitor, ramipril. These data support the notion that ACE inhibitor therapy may help to prevent vascular hypertrophy independently of its effects on blood pressure (45).

In conclusion, AII-induced VSM proliferation in vitro is strongly influenced by the conditions under which the cells are cultured. Since some of the conditions we examined may be altered in hypertension (i.e., extracellular matrix content and mechanical strain), previous in vitro work may have underestimated the importance of AII as a mitogen in hypertension. Therefore, further examination of the potential beneficial ef-

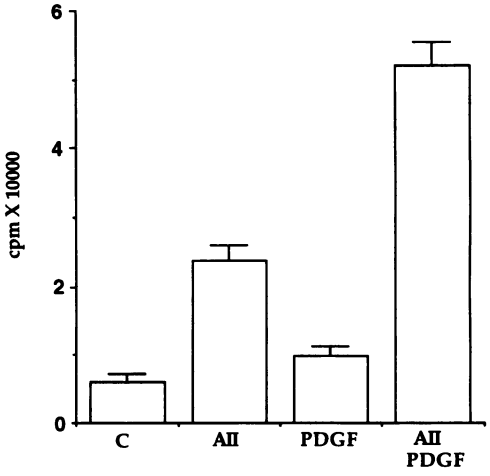

Figure 7. AII and PDGF AB synergistically increase DNA synthesis in VSM. VSM cells plated on collagen-coated silicone elastomer dishes were exposed to AII (1 μM) and/or a submaximal concentration of PDGF AB (1 ng/ml) as indicated for 48 h. Thymidine incorporation was measured as described in Fig. 1. Experiments were done in triplicate; result shown is representative of three similar experiments. $P < 0.001$ AII + PDGF vs control.

fects of inhibitors of the renin-angiotensin system in preventing vascular hypertrophy is warranted.

Acknowledgments

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