Angiotensin II–Stimulated Protein Synthesis in Cultured Vascular Smooth Muscle Cells

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To investigate the role of vasoconstrictor hormones in vascular smooth muscle cell growth we have studied the effects of the potent vasoconstrictor angiotensin II on cell growth in a cultured rat aortic cell model. Angiotensin II was not mitogenic for these cells, as assessed by determining cell number, nor was it synergistic in this regard with 10% calf serum. However, 24-hour exposure to 100 nM angiotensin II caused an 80% increase in protein synthesis (compared with 0.4% increase with serum control) as measured by tritiated leucine incorporation. This was a "hypertrophic" response as indicated by a 30% increase in protein content and a 45% increase in cell volume. Angiotensin II–induced smooth muscle cell hypertrophy was maximal at 100 nM, had an ED50 of 1 nM, and was inhibited by the competitive antagonist [Sar1, Ile8]angiotensin II.

The increase in protein synthesis required continuous presence of angiotensin II for 6 hours and required messenger RNA (mRNA) synthesis as suggested by complete inhibition after exposure to actinomycin D. Angiotensin II–stimulated protein synthesis was dependent on a rise in intracellular Ca2+ concentration evidenced by a 70% decrease in tritiated leucine incorporation after chelation of Ca2+ with 25 μM quin 2-AM. This treatment did not alter protein synthesis induced by 10% calf serum. Decreasing extracellular Na+ to prevent Na+/H+ exchange and intracellular alkalinization did not inhibit the angiotensin II response but decreased the 10% serum–stimulated protein synthesis by 35%. Downregulation of protein kinase C by 24-hour treatment with phorbol 12,13-dibutyrate did not inhibit angiotensin II–induced protein synthesis, while phorbol 12-myristate 13-acetate–stimulated protein synthesis was abolished. These findings suggest that angiotensin II–induced hypertrophy, acting via a Ca2+ mechanism, may play an important role in abnormal vascular smooth muscle cell growth in certain forms of hypertension. (Hypertension 1989;13:305–314)

A fundamental pathogenetic feature of hypertension and atherosclerosis is aberrant vascular smooth muscle cell (VSMC) growth. In hypertensive models such as aortic coarctation and experimental injury models of atherosclerosis, VSMC migration into and proliferation (hyperplasia) in the intima is the most dramatic pathological feature.1,2 In contrast, in both the Goldblatt kidney and spontaneously hypertensive rat (SHR) models of hypertension,3,4 increased medial thickening of the aorta due to increased size of VSMC (hypertrophy) without increase in cell number is the most prominent alteration. Recently Owens et al5 demonstrated in the mesenteric resistance vessels of 107–111-day-old SHR that VSMC proliferation rather than hypertrophy or hyperploidy appeared to account for the increase in VSMC mass.

Several factors have been implicated in growth of VSMC in these models. In experimental injury models, endothelial dysfunction or denudation may result in abnormal interactions between elements in the blood (platelets, polymorphonuclear leukocytes, and monocytes) and the vessel wall that lead to sustained release of a variety of growth factors including platelet-derived growth factor. These growth factors are both chemotactic and mitogenic for VSMC6,7 and thus may contribute to VSMC migration and proliferation in this model. In certain hypertensive models the increased blood pressure may result in altered mechanical stress that stimulates VSMC growth in a manner analogous to the effects of stretch on skeletal muscle protein synthesis.8 This explanation is supported by data that demonstrate a significant correlation between blood pressure and aortic VSMC hypertrophy and polyploidy.6,7 Furthermore, reduction of blood pressure in the SHR model can decrease VSMC polyploidy and hypertrophy.9
Clear differences, however, in the potency of different antihypertensive agents to alter VSMC growth have been demonstrated. In particular, the angiotensin converting enzyme inhibitor captopril prevented VSMC growth to a much greater extent than propranolol or hydralazine for a similar reduction in blood pressure.\textsuperscript{9,10} These data, in addition to several lines of evidence linking vasoconstrictor agonists to growth,\textsuperscript{11-13} suggest that angiotensin II (Ang II) may play a role in modulating VSMC growth responses. We have previously shown in cultured rat aortic VSMC that Ang II stimulates many intracellular signals shared by growth factors including activation of phospholipase C and Na\(^+\)/H\(^+\) exchange.\textsuperscript{14,15} Activation of Na\(^+\)/H\(^+\) exchange with subsequent intracellular alkalization has been proposed as a critical event in initiation of protein and DNA synthesis by mitogens.\textsuperscript{16} We\textsuperscript{12} and others\textsuperscript{17} have recently demonstrated that Ang II induces the messenger RNA (mRNA) for the proto-oncogene c-fos, which is thought to play a role in mediating growth factor responses. Finally, recent work by Geisterfer et al\textsuperscript{18} indicates that Ang II stimulates protein but not DNA synthesis in cultured rat aortic VSMC. These findings in concert suggest that Ang II is likely to be an important regulator of VSMC growth responses.

The purpose of this work was to define a cell culture model that would be useful for studying VSMC growth and to compare mechanisms of growth regulation by classic growth factors such as serum and platelet-derived growth factor with vasoconstrictor agonists such as Ang II. The data reported here demonstrate that Ang II does not significantly affect VSMC proliferation, but potently stimulates protein synthesis and hypertrophy of cultured VSMC. Ang II-stimulated protein synthesis does not require activation of protein kinase C or Na\(^+\)/H\(^+\) exchange, but appears to be related to mobilization of intracellular Ca\(^{2+}\).

**Materials and Methods**

**Cell Culture**

Primary cultures of rat aortic smooth muscle cells were obtained by enzymatic dissociation of thoracic aortae from 250–300 g male Sprague-Dawley rats (Charles River Labs., Inc., Wilmington, Massachusetts) as previously described.\textsuperscript{19} Cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) (GIBCO Labs., Grand Island, New York) supplemented with 10% heat-inactivated calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin. These cultures were harvested twice a week with trypsin-verseine and passed down to a 1:4 ratio in 80-cm\(^2\) flasks. The typical growth experiment was performed with VSMC at passage levels 8–24. Cells were replicate-plated at 5 x 10\(^4\) cells/ml in DMEM supplemented as described above and allowed to grow for 24–48 hours. They were then made "quiescent" by a 48-hour incubation in fresh DMEM supplemented with 0.4% calf serum without antibiotics. Unlike fibroblasts such as 3T3 cells where mitotic indexes near zero are achievable by serum deprivation or prolonged culture postconfluence, rat aortic VSMC do not remain viable under such conditions. Therefore, although progressive decreases in mitotic index and tritiated thymidine incorporation could be attained at serum concentrations below 0.4%, this concentration yielded the best combination of cell viability and quiescence.

**Determination of DNA, Protein, and RNA Synthesis**

The medium from quiescent VSMC grown in 6-well, 35-mm Costar culture dishes (Costar Corp., Cambridge, Massachusetts) was aspirated and replaced with fresh DMEM containing 1 \(\mu\)Ci/ml tritiated thymidine (6.7 Ci/mmol, New England Nuclear, Boston, Massachusetts) plus various additions as described for 24 hours. The medium was then aspirated, cells washed rapidly three times with cold buffer (150 mM NaCl, 10 mM Tris HCl, pH 7.5), washed once with 10% trichloroacetic acid, and incubated at 4\(^\circ\)C for 30 minutes in 1 ml of 10% trichloroacetic acid. The trichloroacetic acid-insoluble material was washed twice with 95% ethanol and the fixed cellular material solubilized in 0.1 N NaOH at 24\(^\circ\)C for 2 hours. A portion was saved for protein determination by the method of Lowry et al,\textsuperscript{20} and tritiated thymidine incorporation was determined by liquid scintillation spectrometry. Triplicate dishes were used for each sample point. For analysis of protein and RNA synthesis, the same procedure was followed substituting 1 \(\mu\)Ci/ml tritiated leucine and tritiated uridine (New England Nuclear), respectively. For cell count determinations, VSMC plated into 24-well Costar culture dishes at 3 x 10\(^4\) cells/ml were made quiescent in 0.4% calf serum and long-term growth was studied after addition of fresh 0.4% calf serum–DMEM supplemented with Ang II (Sigma Chemical Co., St. Louis, Missouri). Counts were performed every 48 hours by hemocytometer measurement after harvesting by gentle trypsinization.

Tritiated thymidine autoradiography was performed by incubating cells for 24 hours in DMEM that contained 1 \(\mu\)Ci/ml tritiated thymidine and agonists as indicated. The dishes were then washed twice with saline solution (150 mM NaCl, 10 mM Tris HCl, pH 7.5), fixed in 2% glutaraldehyde for 5 minutes, dehydrated, and coated with Kodak NTB2 emulsion (Eastman Kodak Co., Rochester, New York) diluted 1:1 with water. Dishes were exposed for 5 days at 4\(^\circ\)C and developed in D-19 (Eastman Kodak Co.), fixed with Rapid-Fix (Eastman Kodak Co.), and stained with hematoxylin. The percentage of cells synthesizing DNA was determined by counting the number of labeled cells (assuming one nuclei per cell) from a total of 400 cells/dish (100 cells/ quadrant). Triplicate dishes were analyzed for each experimental point. The values obtained are likely...
overestimates because of significant polyploidy that may occur in these cells.\textsuperscript{18}

**Cell Volume**

Changes in cell volume were analyzed by flow cytometry using cells grown as described above. Cells were harvested with versene (GIBCO Labs) alone, and cell volume determined electronically using a Becton Dickinson (Sunnyvale, California) FACS \textsuperscript{I} cell analyzer.

**Preparation of Low-Na\textsuperscript{+} Media**

Media containing variable concentrations of Na\textsuperscript{+} were prepared by adding NaCl to Na\textsuperscript{+}-free DMEM (<10 mM Na\textsuperscript{+}) containing HEPES-Tris buffer (20 mM, pH 7.4). Media were made isotonic by adding choline chloride (Calbiochem-Behring Corp., La Jolla, California) to a total osmotic equivalent of 130 mM. Osmolality of all solutions was adjusted to 285±5 mosm with HEPES-Tris. The Na\textsuperscript{+} and K\textsuperscript{+} concentrations of all media were verified by atomic absorption spectrophotometry (model 3030B, Perkin Elmer, Chicago, Illinois). Preliminary experiments indicated that the minimal concentration of NaHCO\textsubscript{3} required for cell viability was 5 mM so all synthetic DMEM contained this concentration.

**Intracellular Electrolyte Content**

Cultures were washed five times with 0.1 M MgCl\textsubscript{2} at 4\textdegree C. After the dishes were dry, 1 ml of Acationex (American Scientific Products, McGraw Park, Illinois) was added to lyse the cells. Na\textsuperscript{+} and K\textsuperscript{+} were measured by atomic absorption spectrophotometry.

**Materials**

\textsuperscript{[sarcosine\textsuperscript{1},isoleucine\textsuperscript{8}]}Angiotensin II ([\textsuperscript{5}Sarc\textsuperscript{1},Ile\textsuperscript{8}] Ang II) was from Peninsula Laboratories, Inc. (Belmont, California). Phenylsepharose-purified platelet-derived growth factor was prepared as described by Raines and Ross\textsuperscript{21} and was a gift from Dr. E. Raines. Tritiated thymidine, tritiated leucine, tritiated uridine, and \textsuperscript{[35S]}cysteine were obtained from New England Nuclear; 5-(N,N-Dimethyl)amiloride (DMA) was prepared as previously described,\textsuperscript{22} and was generously supplied by Dr. E.J. Cragoe, Jr.

**Statistical Analyses**

Values presented are the mean±SD. Student’s \textit{t} test was used in the analysis of paired and unpaired means, with the level of significance chosen at \( p < 0.05 \).

**Results**

**Angiotensin II Effects on Vascular Smooth Muscle Cell Number**

To assay whether Ang II stimulated cell proliferation, quiescent VSMC in 0.4% calf serum were treated with fresh DMEM every 48 hours that contained 100 nM Ang II or 10% calf serum, and cell numbers were determined as shown in Figure 1. Ang II supplementation alone caused no significant increase in cell number after 8 days compared with 0.4% calf serum–DMEM, while addition of 10% calf serum to these quiescent VSMC resulted in a 10-fold increase in cell number after 8 days of treatment. A small increase in cell number was observed in cells given fresh 0.4% calf serum–DMEM every 48 hours. This low level of cell proliferation likely reflects the presence of small quantities of growth factors in 0.4% calf serum–DMEM and the fact that the cells were not totally quiescent after 72 hours in 0.4% calf serum–DMEM. Nonetheless, we chose to use these culture conditions because more consistent growth responses of greater magnitude were observed with this media formulation than in completely serum-free media (unpublished observations).

To ensure that Ang II was not being degraded during these studies, VSMC were supplemented with fresh 100 nM Ang II every 8 hours for a total of 48 hours. No increase in cell number was observed (data not shown). There was also no synergistic effect of 100 nM Ang II on the growth response to 10% calf serum. Thus, Ang II in 0.4% calf serum–DMEM was unable to stimulate proliferation of cultured rat aortic VSMC.

**Angiotensin II Effects on Vascular Smooth Muscle Cell DNA and Protein Synthesis**

To investigate nonmitogenic effects of Ang II on VSMC growth, alterations in the incorporation of tritiated thymidine and tritiated leucine by quiescent VSMC were measured to study changes in DNA and protein synthesis, respectively. No significant difference in tritiated thymidine incorporation was seen in response to 0.1–10 nM Ang II,
FIGURE 2. Line graph showing the effect of angiotensin II (Ang II) on tritiated ([3H]thymidine and [3H]leucine incorporation in cultured vascular smooth muscle cells (VSMC). Cultures of quiescent VSMC were washed two times with fresh 0.4% calf serum–Dulbecco’s Modified Eagle Medium (DMEM) and incubated for 22 hours with Ang II (0.1 to 1,000 nM) that contained 1 μCi/ml [3H]thymidine and [3H]leucine. DNA and RNA synthesis were measured by incorporation into trichloroacetic acid-precipitable material. Shown are the mean±SD of 4–6 experiments.

but a 25±18% (p<0.05, n=6) increase was observed with 100 nM Ang II. Administration of Ang II every 8 hours to the culture media to determine whether peptide degradation was affecting tritiated thymidine incorporation did not alter the results (data not shown). To further study the increase in tritiated thymidine incorporation, autoradiographic analysis was performed. After a 48-hour incubation period in 0.4% calf serum–DMEM, 1 μCi/ml tritiated thymidine and fresh 0.4% calf serum–DMEM supplemented with either 10% calf serum or 100 nM Ang II was added for 24 hours. The percentage of labeled nuclei was 15±6% in 0.4% calf serum, 19±10% in 100 nM Ang II, and 63±14% (n=4, p<0.05) in 10% calf serum. These data support the difference in cell numbers observed above, but do not rule out the possibility that Ang II may have stimulated DNA synthesis without stimulating cell division. The fact that Ang II did not stimulate a significant increase in the percentage of labeled nuclei may be due to the variance in these measurements. Geisterfer et al18 observed that, during a 72-hour incubation with Ang II, a significant increase in DNA content (primarily 4N) was observed, which suggests that the increase in tritiated thymidine incorporation we observed may represent development of polyploidy.

In contrast to the small effect on VSMC and DNA synthesis, Ang II induced a peak 84±15% (n=24) increase in tritiated leucine incorporation as shown in Figure 2. This effect was concentration dependent with a threshold of 0.1 nM, half maximal concentration of 5 nM, and maximal effect at 100 nM. The increase in trichloroacetic acid-precipitable tritiated leucine was not due to an alteration in amino acid pool sizes because it was completely blocked by 10 μM cycloheximide and required continuous exposure to Ang II for at least 3 hours (data not shown). This increase in tritiated leucine incorporation compares with a 141±16% (n=20) increase in response to 10% calf serum and an 86±21% increase in response to 10 ng/ml platelet-derived growth factor (n=3).

[Sar'le5]Ang II (1 μM) was a partial agonist for induction of protein synthesis causing a 30±5% increase in tritiated leucine incorporation (n=6) after 24 hours. In keeping with its partial agonist activity, [Sar'le5]Ang II completely blocked any further increase in tritiated leucine incorporation by 1 or 10 nM Ang II (data not shown).

The time course for Ang II-stimulated increases in tritiated leucine incorporation is shown in Figure 3. Stimulation of tritiated leucine incorporation was significantly different from control after incubation for 6 hours (28% increase) and peaked after exposure to Ang II for 18 hours (76% increase).

Angiotensin II Effects on Vascular Smooth Muscle Cell Volume

Ang II stimulated a maximal 30±8% (n=24) increase in total protein after 24-hour treatment. Similar increases in protein content were observed in response to 10% calf serum (42±6% increase) or 10 ng/ml platelet-derived growth factor (30±7% increase). Ang II causes no change in the rate of [14C]tyrosine protein degradation,18 which indicates...
that the increase in protein content was due to enhanced protein synthesis.

To determine whether this increase in protein synthesis and content resulted in an increase in cell size, cell volumes of quiescent VSMC, treated for 24 hours with 100 nM Ang II or 10% calf serum, were determined by electronic cell volume measurement. As shown in Figure 4, 100 nM Ang II caused a shift of the entire cell population to larger volumes with a change in the mean cell volume from 54 to 78 units (p<0.05, n=4). A similar increase was observed with 10% calf serum and 10 nM Ang II (data not shown). At concentrations of Ang II below 10 nM, significant differences in cell volume were not observed. This 45% increase in Ang II–stimulated cell volume correlates well with the 30% increase in total cell protein. Although some of the increase in cell size may be due to an increase in polyploid cells, the magnitude of the increase in total protein content and synthesis suggests that the majority of the Ang II effect on cell volume is a hypertrophic response.

Effect of Angiotensin II on RNA Synthesis

To investigate whether the Ang II effect involved a significant increase in RNA synthesis, we measured tritiated uridine incorporation. When quiescent VSMC were exposed to 100 nM Ang II, there was a concentration-dependent increase in tritiated uridine incorporation with a maximal 226±204% (n=3) increase compared with a 278±76% (n=3) increase in response to 10% calf serum. This stimulation was significantly inhibited by coinubcation with 1 μM [Sar,Leu]Ang II, although, as was the case for protein synthesis, some partial agonist activity was observed (data not shown).

Addition of 0.1 μg/ml actinomycin D to prevent new RNA synthesis completely abolished the increases in both tritiated uridine (Figure 5A) and tritiated leucine (Figure 5B) incorporation in response to either Ang II or 10% calf serum. This concentration of actinomycin D did not impair other markers of cell function to the same extent (e.g., Ang II–stimulated Ca2+ mobilization measured by fura-2 fluorescence was 70% of baseline, data not shown). These data indicate that in cultured VSMC Ang II and serum-stimulated increases in protein synthesis require RNA synthesis. This requirement is further supported by findings that Ang II induces accumulation of several mRNAs in cultured VSMC, including B-actin as well as the proto-oncogenes c-fos and c-myc (see Reference 12, unpublished observations). Preliminary observations of specific protein synthesis using [35S]cysteine incorporation indicate increased synthesis of several VSMC pro-
centrations. Measurement of intracellular K⁺, which was not significantly affected by extracellular Na⁺ concentration, revealed that 24-hour treatment with 12 mM Na⁺ decreased intracellular K⁺ from 780 μmol/mg protein to 400 μmol/mg protein (data not shown). This decrease is similar to that previously observed in 3T3 cells exposed to 10 mM Na⁺ that was associated with inhibition of serum-stimulated DNA synthesis.

To further investigate the role of Na⁺/H⁺ exchange in mediating the Ang II protein response, the ability of the amiloride derivative DMA to inhibit Ang II-stimulated tritiated leucine incorporation was studied. As shown in Figure 6, addition of 10 μM DMA to 30 mM Na⁺ medium decreased tritiated leucine incorporation after 22-hour incubation with dialyzed 10% calf serum by 58%, but had no effect on the Ang II response. No significant effect of this concentration of DMA was seen in standard DMEM (147 mM Na⁺), which is consistent with the fact that DMA is a competitive inhibitor of the Na⁺/H⁺ exchanger (data not shown). Thus, serum-stimulated tritiated leucine incorporation in cultured VSMC was more strongly dependent on extracellular Na⁺ concentration than Ang II-stimulated tritiated leucine incorporation.

Na⁺ influx via the Na⁺/H⁺ exchange causes intracellular alkalinization, which has been associated with induction of protein synthesis in some systems. The possibility that this mechanism may play a role in Ang II-stimulated protein synthesis was investigated by incubating quiescent VSMC with NH₄Cl, which stimulates a transient rise in intracellular pH. We have previously demonstrated that 10 mM NH₄Cl increases VSMC pH by 0.15, approximately equal to the effect of 100 nM Ang II. Low concentrations of NH₄Cl (0.01-1 mM) caused no significant change in tritiated leucine incorporation, whereas higher concentrations (5-20 mM) were clearly inhibitory (data not shown). Thus, intracellular alkalinization alone is not sufficient to explain Ang II-mediated protein synthesis.

Role of Intracellular Ca²⁺ Mobilization in Mediating Angiotensin II–Stimulated Protein Synthesis

In several cell systems increases in intracellular Ca²⁺ have been demonstrated to stimulate protein synthesis. Binding of Ang II to its VSMC receptor triggers an increase in the free intracellular Ca²⁺ concentration ([Ca²⁺]), mediated by Ca²⁺ release from a nonmitochondrial intracellular source. An increase in [Ca²⁺], has been implicated in skeletal muscle hypertrophy and in epidermal growth factor–mediated activation of S6 kinase, which appears to play a critical role in initiation of protein synthesis. To study the role of intracellular Ca²⁺ in Ang II–stimulated protein synthesis, quiescent VSMC were treated for 8 hours with either 100 nM Ang II or dialyzed 10% calf serum in DMEM in which the [Ca²⁺] was varied as indicated in Figure 7. In the presence of 1.5 mM external Ca²⁺, VSMC...
tritiated leucine was added and then was removed and fresh 0.4% calf serum-

ionomycin in 0.4% calf serum-DMEM to duplicate VSMC were treated for 10 minutes with 1 μM Ca2+ than is serum-stimulated protein synthesis. Thus Ang II-stimulated protein synthesis in VSMC and then were exposed to agonist in DMEM (1.5 mM Ca2+) for 8 minutes to chelate intracellular Ca2+ (approximately 30 mM). This treatment inhibited Ang II-stimulated incorporation of tritiated leucine. Quiescent vascular smooth muscle cells (VSMC) were washed with fresh Dulbecco’s Modified Eagle Medium (DMEM) and preincubated for 15 minutes with the indicated concentrations of quin 2/AM in dialyzed 0.4% calf serum–DMEM. After preincubation, 2 μCi/ml [3H]leucine and 0.4% calf serum (□), 100 nM Ang II (■), or dialyzed 10% calf serum (▲) were added for 8 hours. To chelate extracellular Ca2+ (final concentration <30 μM), 1.5 mM EGTA was added to the DMEM. Incorporation of [3H]leucine was determined as described in Materials and Methods. Results are representative of three experiments.

[Ca2+]i is approximately 90 nM,28 and tritiated leucine incorporation was stimulated by 30% in response to Ang II and 34% in response to 10% calf serum. To prevent mobilization of either intracellular or extracellular Ca2+, VSMC were incubated in DMEM to which both 1.5 mM EGTA and 5 μM quin 2/AM were added (■), 100 nM Ang II (□), or dialyzed 10% calf serum (▲) were added for 8 hours. To chelate extracellular Ca2+ (final concentration <30 μM), 1.5 mM EGTA was added to the DMEM. Incorporation of [3H]leucine was determined as described in Materials and Methods. Results are representative of three experiments.

For 24 hours. Despite a rise in [Ca2+], no stimulation of protein synthesis by ionomycin was observed (102±5% of control). Altering experimental conditions such as a longer incubation (1 μM ionomycin for 1 hour) or a higher concentration (10 μM for 10 minutes) resulted in decreases in tritiated leucine incorporation of 10% and 26%, respectively. Thus, increased [Ca2+], alone is not sufficient to initiate protein synthesis in VSMC. These data indicate that Ang II–stimulated protein synthesis involves at least two signals.

Role of Protein Kinase C in Mediating Angiotensin II–Stimulated Protein Synthesis

Ang II stimulation of Na+/H+ exchange in VSMC is mediated by both protein kinase C-dependent and –independent mechanisms.14 Ang II stimulates protein kinase C in VSMC as demonstrated both by phosphorylation of an 80 kDa acidic protein and by movement of protein kinase C activity to the membrane after Ang II stimulation (T. Tsuda, unpublished results). Regulation of certain Ang II–mediated responses by protein kinase C appears likely based on the finding that phorbol esters inhibit Ang II stimulation of inositol trisphosphate formation and Ca2+ mobilization in VSMC.33

The role of protein kinase C in mediating protein synthesis in cultured VSMC was examined by using the phorbol ester phorbol 12-myristate 13-acetate (PMA) to activate the enzyme. Stimulation of protein kinase C by PMA resulted in a concentration-dependent increase in tritiated leucine incorporation

Table 1. Effect of Phorbol 12,13-Dibutyrate Treatment on Angiotensin II and Phorbol 12-Myristate 13-Acetate–Stimulated Tritiated Leucine Incorporation

<table>
<thead>
<tr>
<th>Ang II (nM)</th>
<th>PMA (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19±26</td>
</tr>
<tr>
<td>10</td>
<td>35±6*</td>
</tr>
<tr>
<td>100</td>
<td>39±12*</td>
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1 μM PDBu for 48 hours, % increase over 0.4% serum

<table>
<thead>
<tr>
<th>Ang II (nM)</th>
<th>PMA (nM)</th>
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<tbody>
<tr>
<td>1</td>
<td>25±34</td>
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<tr>
<td>10</td>
<td>46±5</td>
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<tr>
<td>100</td>
<td>57±21</td>
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Quiescent vascular smooth muscle cells (VSMC) were treated with the indicated concentrations of angiotensin II (Ang II) or phorbol 12-myristate 13-acetate (PMA) in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 0.4% calf serum. After 24 hours, tritiated leucine incorporation was measured. Cells were made quiescent for 48 hours in the presence of 1 μM phorbol 12,13-dibutyrate (PDBu). The medium was aspirated, cells were washed twice with 0.4% calf serum–DMEM, and fresh 0.4% calf serum–DMEM with Ang II or PMA was added for 24 hours. The effect of PDBu treatment on cellular protein kinase C activity was examined by assaying Ca2+-, phosphatidylserine-dependent phosphorylation of H1 histone by DEAE-Sephadex purified protein kinase C (see Reference 34). Results are the mean±SD of five experiments.

*p<0.05 vs. 0.4% calf serum (n=12).

tp<0.05 vs. untreated control (n=12).
(Table 1). Thus a protein kinase C-dependent pathway for protein synthesis is present in these cells. The existence of a protein kinase C-independent pathway was investigated by downregulating the enzyme by using a 48-hour incubation with 1 μM phorbol 12,13-dibutyrate (PDBu). This treatment caused a 50% decrease in protein kinase C activity as measured by Ca2+, phosphatidylserine-dependent phosphorylation of histone III (see legend Table 1). After downregulation of protein kinase C, PMA-induced tritiated leucine incorporation was dramatically inhibited with a 36% decrease relative to 0.4% calf serum. On the other hand, Ang II–stimulated tritiated leucine incorporation was not inhibited and the response to 100 nM Ang II was significantly enhanced from 39±12% to 57±2% (p < 0.05). Thus Ang II–stimulated protein synthesis appeared not to be dependent on protein kinase C activation.

**Discussion**

The results reported here demonstrate that Ang II is a potent stimulator of protein synthesis in cultured VSMC. The increase in protein synthesis was accompanied by increases in cell volume and protein content, but no change in cell number. Ang II also did not potentiate the mitogenic response to 10% calf serum. Thus, Ang II appears to be a "hypertrophic" as opposed to a mitogenic growth factor for VSMC.

The data reported here indicate a predominant effect of Ang II on protein rather than DNA synthesis in VSMC are in complete agreement with the findings of Geisterfer et al. However, Campbell-Boswell and Robertson found increases in both tritiated thymidine incorporation and cell number in human aortic cells in response to Ang II. Our data and those of Geisterfer et al demonstrate that the increase in DNA synthesis in response to Ang II was much smaller relative to 10% calf serum or 10 ng/ml platelet-derived growth factor than was the increase in protein synthesis. The difference between these data and those of Campbell-Boswell and Robertson may be due to species differences, differences in serum used, or the fact that their cells were derived from explants that may have different growth properties than our enzymatically prepared cells. Previous studies in isolated rat atrial and renal tissues found that Ang II induced significant increases in DNA and protein synthesis without a change in total DNA content. These data support the generality of the Ang II–mediated hypertrophic response, but also suggest potential tissue-specific responses.

It is possible that the small increase in tritiated thymidine incorporation in response to 24-hour treatment with 100 nM Ang II may be a reflection of an increase in cells in G2 as was observed in subconfluent cell experiments by Geisterfer et al. They demonstrated that administration of Ang II (1 μM, 4 days) to subconfluent cells caused a 50% increase in the fraction of cells with 4N DNA content, which suggests growth arrest in G2 or development of tetraploidy. Based on these findings one may surmise that Ang II promotes cell cycling through G2 and that its nonproliferative capacity is due to lack of an appropriate signal to allow G2/S transition. It appears reasonable that the failure of Ang II to sustain a proliferative response is due to events late in the cell cycle given the many early signal pathways shared with classic mitogens such as platelet-derived growth factor including increased [Ca2+]i, phospholipase C activity, Na+/H+ exchange activity, protein kinase C activity, and induction of c-fos.

Although Ang II activates a number of cellular events that are stimulated by growth factors, Ang II is unable to stimulate VSMC proliferation. One possible explanation is that elevation of cyclic AMP, which does not occur in VSMC treated with Ang II, is necessary for the mitogenic response. Owen has shown that an increase in cyclic adenosine 3',5'-monophosphate (AMP) may be mitogenic by itself in the VSMC line A-10, and Rosengurt has postulated that there is synergistic growth activity among agonists that elevate cyclic AMP and agonists that increase intracellular Ca2+ and activate protein kinase C. Another possible difference between Ang II and proliferative growth factors is that platelet-derived growth factor and epidermal growth factor activate receptor tyrosine kinases that may directly stimulate mitogenic signals, whereas Ang II may lack such activity.

Protein kinase C has been implicated in initiation of both DNA and protein synthesis in VSMC in response to platelet-derived growth factor and other growth factors. The data in Table 1 indicate that PMA stimulates protein synthesis in quiescent VSMC, and this effect is abolished by protein kinase C downregulation. Similar findings in rabbit VSMC were reported for PMA and platelet-derived growth factor by Kariya et al. Our findings demonstrate that the ability of Ang II to initiate protein synthesis was not affected by protein kinase C downregulation and in fact, appeared to be enhanced. This finding is explained in part by evidence that Ang II–stimulated protein synthesis is dependent on mobilization of intracellular Ca2+ (Figure 7) and activation of protein kinase C inhibits this mobilization. Similar findings have been reported by Huang and Ives for α-thrombin–mediated DNA synthesis in VSMC and by Scott-Burden et al for Ang II–stimulated S6 phosphorylation. These data demonstrate that protein kinase C activation is not essential for Ang II–stimulated protein synthesis. We have previously shown that Ang II stimulates Na+/H+ exchange in cultured VSMC by both protein kinase C–dependent and –independent pathways, findings similar to those reported for fibroblast growth factor in fibroblasts and concanavalin A in lymphocytes. Thus, protein synthesis in VSMC appears to be another cellular event mediated by both protein kinase C–dependent and –independent mechanisms.
Several ionic events have been associated with initiation of DNA and protein synthesis by growth factors in cultured cells. It has become widely accepted that initiation of DNA synthesis in many cultured cells is dependent on Na\(^+\) influx mediated by the amiloride-sensitive Na\(^+\)/H\(^+\) exchange.\(^{16,24,39}\) Because Ang II stimulates amiloride-sensitive Na\(^+\) influx in VSMC,\(^{14,44}\) we studied the dependence of Ang II-stimulated protein synthesis on external Na\(^+\) concentration. As shown in Figure 6, we could demonstrate no effect of altering external Na\(^+\) concentration on Ang II-initiated protein synthesis, although a significant decrease in serum-stimulated synthesis was apparent. These data, in combination with the finding that DMA did not inhibit Ang II-stimulated protein synthesis, indicate that Na\(^+\) influx and intracellular alkalization are unlikely to play a significant role in Ang II-mediated protein synthesis. It should be noted that there was a significant decrease in intracellular K\(^+\) after 24-hour incubation of VSMC in 12 mM external Na\(^+\) that likely accounted for the observed decrease in serum-stimulated protein synthesis as suggested by Burns and Rozengurt.\(^{23}\)

Alterations in extracellular Ca\(^{2+}\) (and by inference [Ca\(^{2+}\)]\(i\)) have been shown to have profound effects on human diploid fibroblast growth with elevated extracellular Ca\(^{2+}\) leading to an enhanced serum proliferative response and cell density,\(^{43}\) while reduced extracellular Ca\(^{2+}\) arrests fibroblasts in G\(_0/G_1\).\(^{46}\) Elevation of intracellular Ca\(^{2+}\) has been associated with initiation of protein synthesis in several systems,\(^{24}\) including skeletal muscle hypertrophy,\(^{29}\) as well as epidermal growth factor activation of S6 kinase, a critical step in initiation of protein synthesis.\(^{30}\) Conversely, depletion of Ca\(^{2+}\) with 1 mM EGTA markedly decreases protein synthesis in C\(_6\) glial cells and adrenal glands.\(^{47}\) Our data suggest that Ang II requires a rise in [Ca\(^{2+}\)]\(i\) to initiate protein synthesis. The mechanism underlying Ca\(^{2+}\)-dependent protein synthesis is unclear at this time, but may involve S6 kinase activation,\(^{30}\) availability of substrate for kinase phosphorylation, and efficiency of mRNA translation. The initial rise in Ca\(^{2+}\) concentration may also be required for induction of new mRNA molecules associated with growth such as c-fos and c-myc, which result in a sustained increase in protein synthesis.

A fundamental pathogenetic feature of hypertension is increased VSMC mass. Owens and Schwartz\(^{3}\) demonstrated that this effect in the aortae of SHR was due to an increase in cell size with development of larger polybomb cells. Thus, a hypertrophic rather than a hyperplastic response appears to be characteristic of hypertension in this model. Conversely, in mesenteric resistance vessels of SHR, increased VSMC mass was not accompanied by hypertrophy or hyperplasia.\(^{5}\) In hypoxic pulmonary hypertension, treatment with the angiotensin converting enzyme inhibitor enalapril (SQ 20881) was associated with inhibition of hypertension-induced changes in protein synthesis of the pulmonary trunk.\(^{13}\) Similarly, administration of captopril causes a greater decrease in arterial hypertrophy for a given decrease in blood pressure than does propranolol or hydralazine in the spontaneously hypertensive rat.\(^{9,10}\) These in vivo findings and the data reported here suggest that Ang II may be one of the mediators involved in the hypertrophic response of aortic smooth muscle associated with development of hypertension. Alterations in intracellular Ca\(^{2+}\) handling have been thought to play a role in the increased vasoreactivity associated with hypertension. Our data now suggest that agonist-mediated increases in VSMC [Ca\(^{2+}\)]\(i\) may also be important in the structural changes of hypertensive vessels.

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References


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B C Berk, V Vekshtein, H M Gordon and T Tsuda

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