Angiotensin II Induces Hypertrophy, not Hyperplasia, of Cultured Rat Aortic Smooth Muscle Cells

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We have explored the hypothesis that contractile agonists are important regulators of smooth muscle cell growth by examining the effects of one potent contractile agonist, angiotensin II (All), on both cell proliferation and cellular hypertrophy. All neither stimulated proliferation of cells made quiescent in a defined serum-free media nor augmented cell proliferation induced by serum or platelet-derived growth factor. However, All did induce cellular hypertrophy of postconfluent quiescent cultures following 4 days of treatment, increasing smooth muscle cell protein content by 20% as compared with vehicle-treated controls. All-induced hypertrophy was maximal at 1 μM, had an ED₅₀ of 5 nM, and was blocked by the specific All receptor antagonist Sar²Ile² All. The cellular hypertrophy was due to an increase in protein synthesis, which was elevated within 6–9 hours following All treatment, while no changes in protein degradation were apparent. All was even more effective in inducing hypertrophy of subconfluent cultures, causing a 38% increase in protein content after 4 days of treatment (1 μM) and showing a maximal response at concentrations as low as 0.1 nM. Interestingly, in subconfluent cultures, All treatment (1 μM, 4 days) was associated with a 50% increase in the fraction of cells with 4C DNA content with the virtual absence of cells in S-phase of the cell cycle, consistent with either arrest of cells in the G₁ phase of the cell cycle or development of tetraploidy. These studies show that All is a potent hypertrophic agent but has no detectable mitogenic activity in cultured rat aortic smooth muscle cells and describe an in vitro model that should be extremely valuable in exploring the cellular controls of smooth muscle cell hypertrophy. (Circulation Research 1988;62:749–756)

There is considerable interest in the cellular mechanisms involved in the control of vascular smooth muscle growth and its role in the etiology of hypertension and atherosclerosis. It has been demonstrated that aortic smooth muscle cells (SMC) are capable of two distinct cellular growth responses in vivo, depending on the mode of growth stimulation. Coarctation models and experimental injury models of atherosclerosis are characterized by extensive proliferation and intimal migration of aortic SMC. In contrast, in Goldblatt and spontaneously hypertensive rats, the increase in aortic smooth muscle mass can be accounted for primarily by enlargement of existing cells, or cellular hypertrophy, rather than cellular proliferation. Interestingly, SMC hypertrophy is often accompanied by increases in ploidy. However, SMC hypertrophy is not dependent on development of polyploidy because diploid cells from hypertensive animals are also hypertrophied with respect to diploid cells from normotensive controls. There is indirect evidence that SMC hypertrophy may represent a response to increased blood pressure. We consistently have observed a high degree of correlation between blood pressure and SMC hypertrophy and hyperploidy. Furthermore, pharmacological lowering of blood pressure can prevent development of SMC hypertrophy and hyperploidy and can reverse cellular hypertrophy that already has occurred in spontaneously hypertensive rats. However, these data must be interpreted with caution because there is no direct evidence that these effects on growth are due to lowering of blood pressure as opposed to some other direct or indirect effect of the drugs on cellular DNA and protein synthesis. In fact, recent drug intervention studies in this and other laboratories have demonstrated dissociation of blood pressure changes and SMC hypertrophy and hyperploidy in spontaneously hypertensive rats treated with different antihypertensive drugs. Results suggested that medial SMC hypertrophy was not simply a response to increased blood pressure and implicated a direct role of specific contractile agonists in control of SMC growth. For example, we observed that captopril, a converting enzyme inhibitor, was more effective than hydralazine or propranolol in preventing hypertrophy of aortic SMC in spontaneously hypertensive rats than was predicted based on the magnitude of the decrease in blood pressure, implicating a role for angiotensin (All). Similarly, Yamori and coworkers have presented indirect evidence that catecholamines might be involved in regulation of SMC growth. The hypothesis that contractile agonists might play some direct role in mediation of SMC growth is of interest because it may explain how an SMC might alter its mass in response to an induced change in its workload. It is worth noting that contractile agonists share many common intracellular signaling mechanisms.
with growth factors. However, very few studies have been done to explore the possible growth properties of contractile agonists in SMC, and no studies have specifically explored the potential role of contractile agonists in mediation of cellular hypertrophy. Thus, in the present studies, we have addressed the possible role of one contractile agonist, All, in regulation of SMC growth by studying the effect of All on both proliferation and hypertrophy in cultured rat aortic SMC.

Materials and Methods

Cell Culture

Rat thoracic aortic smooth muscle cells were isolated and cultured by the procedure described previously with the following changes: 150-175-g rats were used instead of 200-225-g rats, and the following enzymes in Hanks’ Balanced Salt solution were used for cell isolations: 1 mg/ml collagenase (type II, 158 µg/mg, Cooper Biomedical, Freehold, New Jersey), 0.25 mg/ml elastase (type I, 3 µg/mg, Cooper Biomedical), 1 mg/ml soybean trypsin inhibitor (Cooper Biomedical).

Cells were harvested for passaging at confluency (approximate 5-day intervals) with trypsin-EDTA (0.05% trypsin, 0.02% EDTA, GIBCO, Grand Island, New York) solution and plated at 2–5 × 10⁵ cells/cm². Passaged cells were grown in a one-to-one mixture of Dulbecco’s Modified Eagle Medium (GIBCO) and Ham’s F12 medium (GIBCO), containing either 10% fetal bovine serum (FBS, Hyclone, Logan, Utah), L-glutamine (0.68 mM, Sigma Chemical, St. Louis, Missouri), penicillin (100 U/ml) and streptomycin (100 µg/ml) (designated DF10) or insulin (5 × 10⁻⁷ M, Sigma Chemical), transferrin (5 µg/ml, Sigma Chemical), ascorbate (0.2 mM, Sigma Chemical), L-glutamine, and antibiotics (designated serum-free medium, SFM). This SFM has been shown to maintain SMC in a quiescent, noncatabolic state for extended periods of time and to promote expression of smooth muscle specific contractile proteins. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂-95% air with media changes every 2-3 days.

Protein Synthesis

Cultures were washed with PBS and harvested using either trypsin-EDTA or 1 mg/ml collagenase, 0.25 mg/ml elastase, and trypsin (0.25%, GIBCO) in Hanks’ Balanced Salt solution. Samples were divided for protein and DNA analysis and then centrifuged (113g, 6 minutes). For the protein staining, cells were fixed in 75% methanol/25% PBS on ice for 30 minutes, centrifuged (113g, 6 minutes), stained with FITC (75 ng/ml fluorescein isothiocyanate, Sigma Chemical; 0.5 M NaHCO₃, pH 8) on ice for 30 minutes, and then centrifuged (113g, 6 minutes) and resuspended in 0.85% NaCl. For measurement of DNA, cells were stained with either propidium iodide (Sigma Chemical) or Hoechst 33258 (Sigma Chemical). For propidium iodide staining, cells were resuspended in a propidium iodide staining solution (propidium iodide 50 ng/ml, NaCl 150 mM, Tris base 100 mM, CaCl₂ 1 mM, MgSO₄ 0.54 mM, MgCl₂ 103 mM, bovine serum albumin [BSA, Fraction V, Sigma Chemical] 2 mg/ml, concentrated HCl 0.7% vol/vol, NP40 0.6% vol/vol), kept on ice 5 minutes, centrifuged (113g, 6 minutes), and resuspended in the propidium iodide staining solution. For the Hoechst staining, cells were fixed for 30 minutes on ice in 75% methanol/25% PBS, centrifuged (113g, 6 minutes), and resuspended in a Hoechst 33258 staining solution (Hoechst 33258 0.58 µg/ml, NaCl 145 mM, Tris 100 mM). All samples for protein and DNA measurements were kept on ice until analyzed on a Coulter Epics V fluorescence activated cell sorter (Hialeah, Florida). Cell clumping was less than 1% based on analysis of peak versus integrated fluorescence.

Protein Synthesis

Cultures were treated with either All or vehicle (control) in SFM. Beginning at time of treatment and then every 3 hours, cultures were pulsed with [³⁵S]methionine (2.0 µCi/ml, 1,100 Ci/mmol, New England Nuclear). Cold L-methionine (1 mM, Sigma Chemical) was added to reduce possible pool effects on protein determination, as recommended by Rannels et al. At the end of the 3-hour interval, the culture was washed with PBS, and the cells were enzymatically harvested into tubes containing 10 µg BSA in solution. Trichloroacetic acid (TCA) was added to each tube for a final concentration of 10%, and samples were kept on ice for a minimum of 20 minutes. TCA-precipitable counts were collected on Whatman GF/F filters (Maidstone, England) and counted in 5 ml Scintiverse II (Fisher Scientific, Springfield, New Jersey) in a
Beckman LS8100 liquid scintillation counter (Irvine, California).

Protein Degradation
Protein degradation assays were performed as described by Libby and O'Brien. Briefly, cells were loaded with $^{[14C]}$tyrosine (0.2 $\mu$Ci/ml, 500 mCi/mmol, New England Nuclear) in SFM for 3 days. Cultures were then washed and incubated twice for 1-hour intervals with SFM plus 5 mg/ml BSA to remove short-lived proteins. Dishes were then fed with SFM plus 1 mM tyrosine and either All, vehicle (control), or 10% FBS. At 12 hours, the medium was removed and replaced with fresh medium. At 24 hours, the medium was removed, and perchloric acid-insoluble material was collected from the dish. Medium removed at 12 and 24 hours was treated with perchloric acid (final concentration 0.2 M) and BSA (final concentration 2 mg/ml) and centrifuged in a table-top microfuge, and the supernatant was counted. Acid-insoluble material from each dish was collected in 1% sodium dodecyl sulfate in 5 mM NaOH after a 0.2 M perchloric acid treatment and two washes with Hanks' Balanced Salt solution.

The fraction of cell protein degraded during the time interval was calculated by dividing the counts obtained during a specific time interval by the total counts obtained by summing all time intervals plus the counts obtained from the acid-insoluble material collected at the end of the experiment.

Statistics
All results were analyzed by either Student's $t$ test or an analysis of variance combined with Newman-Keuls multiple range test for intergroup comparisons. Probabilities of 0.05 or less were interpreted as being statistically significant. Values reported in the text are mean ± SEM.

Results

Effects of Angiotensin II on Smooth Muscle Cell Proliferation
To determine whether All would induce proliferation of rat aortic SMC, cell counts were obtained from cultures that were maintained in SFM for 3 days to induce quiescence and were then treated with All (1 $\mu$M) or vehicle in either 10% FBS, partially purified platelet-derived growth factor (PDGF, 2.5 ng/ml; a gift from R. Ross) in SFM, or SFM alone (Figure 1).

Results demonstrated that All was not mitogenic by itself nor did it augment the growth response to serum (p>0.05). The failure to see an enhanced growth response in the presence of serum was unlikely due to degradation of All because no response was seen with Sar$^1$ All, a more stable analogue of All (data not shown). In addition, All did not enhance the growth

![FIGURE 1. Influence of angiotensin II (All) on smooth muscle cell cultures fed either serum free medium (circles), 10% fetal bovine serum (squares), or serum free medium + partially purified platelet-derived growth factor (2.5 ng/ml, triangles) and treated with either All (1 $\mu$M, solid symbols) or vehicle (open symbols). Cell counts were performed at time zero and at 2-day intervals thereafter using an Electrozone Celloscope. Each sample was counted at least three times, and triplicate wells were evaluated for each group at each time point. Values are expressed as mean ± SEM, n = 3. All-treated groups were not significantly different from respective control (p>0.05). Results similar to these were obtained in four independent experiments.](image-url)
response to a submaximal dose of a defined competence factor, PDGF (p > 0.05). Consistent with cell number data, AI had no effect on the fraction of cells undergoing DNA replication in either postconfluent or subconfluent SMC as assessed by [3H]thymidine autoradiography (Table 1). In fact, both SFM control and AI-treated cultures had extremely low [3H]thymidine labeling indexes throughout the treatment period. Likewise, flow microfluorimetric analysis of cell cycle of postconfluent cultures demonstrated that by far the majority of cells were in G0/G1, with few cells in S + G2, and no differences were observed between the AI-treated and control groups (Figure 2).

Effects of Angiotensin II on Cellular Protein Content

Although AI was not mitogenic for rat aortic SMC, it did induce cellular hypertrophy (Figure 3). We chose to assess cellular hypertrophy, defined as an increase in mass or size of a cell, by flow microfluorimetric analysis of cellular protein content because greater than 50% of the dry mass of the cell is made up of protein and nucleic acids and the remaining components of the cell are largely dependent on them.1 The mean protein content of AI-treated cells (1 µM AI in SFM, 4 days) was increased by approximately 20% in postconfluent cultures as compared with the vehicle-treated control cultures (Figure 4). Cultures in 10% FBS treated with either AI or Sar1-Ile8 AI showed no increase in protein content as compared with the control cultures (data not shown). The AI-induced increase in protein content in quiescent cultures (i.e., those maintained in SFM) was concentration-dependent with the maximal response seen at 1 µM in postconfluent cultures (Figure 5).

Receptor Dependence of the Angiotensin II Hypertrophic Response

Cultures were treated daily for 4 days with equimolar concentrations of AI and either Sar1,Ile8 AI (an AI receptor antagonist) or Des-Phe8 AI (a biologically inactive AI analogue). Cellular hypertrophy was then analyzed by flow microfluorimetric analysis of cellular protein content. Sar1,Ile8 AI completely blocked the AI-induced hypertrophy, while Des-Phe8 AI had no effect (Figure 6). When used alone, neither analogue exerted any effect on protein content. While the observation that AI-induced hypertrophy in postconfluent cultures occurred over several log orders of concentration is of some concern, the fact that hypertrophy was blocked by the highly specific AI antagonist, Sar1,Ile8 AI, strongly indicates that the hypertrophic response is receptor mediated. Likewise, the observation that Des-Phe8 AI did not effect the AI-induced hypertrophy indicates that the response to AI did not involve some non-receptor-mediated effect of AI. It is likely that the gradual slope of the concentration-dependence curve in postconfluent cultures is due to degradation of AI in these high cell density cultures and that the true concentration of AI is less than that calculated based on what was added.

Effects of Angiotensin II on Protein Synthesis and Degradation

The increase in protein content observed in AI-treated cultures could reflect either an increase in protein synthesis or a decrease in protein degradation.
Response of Subconfluent Cultures to Angiotensin II

To determine whether cell density or contact inhibition of growth might be an important component of All-induced SMC hypertrophy, whether All could induce cellular hypertrophy in subconfluent cells made quiescent by maintenance in SFM was explored. All (1 μM) induced a 38% increase in cellular protein content in subconfluent cultures treated continuously with the peptide for 4 days (Figure 9). All was extremely potent in inducing hypertrophy under these conditions in that a maximum response was obtained at a concentration of 1 μM or greater (Figure 10) or [3H]thymidine autoradiography (Table 1). The mean fraction of cells in G₀ in the All-treated (1 μM) and control cultures was 25.1 ± 0.6% and 16.7% ± 1.1, respectively, following 4 days of treatment.

Discussion

In the present study, we have explored the hypothesis that contractile agonists are involved in the mediation of growth of cultured vascular SMC by examining the effects of one potent contractile agonist, All, on SMC hypertrophy and cell proliferation. Under the conditions examined, All neither was mitogenic nor potentiated the proliferative responses to FBS or PDGF. The failure of All to stimulate proliferation of rat aortic SMC in the present study contrasts with results of Campbell-Boswell and Robertson, who found that All increased growth rates of subconfluent human aortic SMC in the presence of 10% FBS. The differences in the results of our study and that of Campbell-Boswell and Robertson may reflect either a species difference or differences in serum. The failure of All to enhance growth of rat aortic SMC in 10% FBS probably was not because the SMC were already maximally stimulated since All also did not augment the replication induced by submaximal doses of PDGF. Alternately, the differences may relate to differences in the differentiaional status of the rat SMC used in the present study as compared with the human SMC used by Campbell-Boswell and Robertson. We have previously demonstrated that under the conditions used in these studies, our cells express high levels of smooth muscle–specific contractile proteins, while the explant-derived SMC used by Campbell-Boswell and Robertson were most likely highly modulated and expressed little, if any, of these differentiated proteins.

Although All was not mitogenic for rat aortic SMC in these studies, we found that it was extremely potent in inducing receptor-dependent hypertrophy of both subconfluent and postconfluent quiescent SMC. It is of interest that the degree of hypertrophy observed in these cultured SMC (i.e., 20% and 38% increases in protein content in postconfluent and subconfluent cells, respectively) is comparable to that observed in aortic SMC in vivo in the spontaneously hypertensive rat following development of chronic hypertension, where the protein content of diploid aortic SMC is increased.
between 20% and 50%. An extremely interesting observation in subconfluent cells was that All induced a 50% increase in the fraction of cells in G2 without a detectable increase in the fraction of cells in S-phase of the cell cycle. Whether this represents induction of true tetraploidy (i.e., doubling of chromosome number in quiescent cells) as has been shown to occur in vivo in conjunction with SMC hypertrophy or whether it represents arrest of cells in the G2 phase of the cell cycle will require extensive further investigation. Nevertheless, results indicate that the SMC hypertrophy induced in vitro resembles hypertrophy that occurs in vivo in many respects and that this model will be very useful in exploring the underlying mechanisms involved in SMC hypertrophy. Indeed, these studies lend support for a role of All in the in vivo hypertrophic response, as initially suggested by our drug intervention studies. However, we caution that these studies provide no direct evidence as to the factors that mediate SMC hypertrophy in vivo in models such as the spontaneously hypertensive rat.

The mechanism whereby All induces SMC hypertrophy is not known. All may increase protein synthesis by stimulation of ribosomal protein S6 phosphorylation, which is believed to play a role in control of protein synthesis. This could occur through All-stimulated Na+-H+ exchange, which has been implicated in control of S6 phosphorylation. Alternatively, All may have a direct effect on ribosomal genes. Robertson and Khairallah have shown that All, or a rapidly formed degradation product, is found in the perinuclear space in coronary smooth muscles and cardiac myocytes within 45 seconds after injection of All into the vena cava. Also, Re et al have shown that All binds with high affinity to nuclear membranes. Another possibility is that All could increase protein synthesis via increased intracellular Ca2+, which has been implicated in skeletal muscle hypertrophy. One other possibility that must be considered is that the increased protein synthesis may be induced by mechanical forces, although it is unclear whether All causes active stress development in these cells and how this might result in increased protein synthesis.

It is interesting to note that contractile agonists and growth factors have many properties in common. Both types of agents have been reported to mobilize intracellular calcium, increase inositol trisphosphate turnover, activate the sodium-proton antiport, and have overlapping protein phosphorylation profiles. Also, PDGF and other growth factors cause contraction of aortic rings. Thus, the response of a cell to a contractile agonist involves similar intracellular calcium-dependent signaling pathways.

**FIGURE 6.** Receptor dependence of angiotensin II (All)-induced hypertrophy of postconfluent smooth muscle cells treated daily for 4 days with All and equimolar concentrations of either Sar1-Ile8-All (a specific All receptor antagonist) or Des-Phe8-All (a biologically inactive All analogue). Mean (protein contents) ± SEM, n = 3. Only the All and Des-Phe8-All + All groups are significantly different from control (p < 0.05). Results similar to these were obtained in an independent experiment.
of cell fusion or by selective replication of a preexisting tetraploid population.

In summary, results of the present study demonstrate that All is a potent hypertrophic agent for cultured rat aortic SMC. Findings are consistent with the hypothesis that contractile agonists may modulate SMC hypertrophy and thereby provide a mechanism for SMC to regulate its mass in response to its workload. Further studies are required to determine the cellular mechanisms responsible for All-induced hypertrophy, whether the hypertrophy observed with All is also a property of other contractile agonists, and whether such mechanisms are important in mediation of smooth muscle hypertrophy in vivo.

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Figure 8. Influence of angiotensin II on the rate of protein degradation. Cells were plated and fed DF10 until confluency. Cells were then maintained in serum free medium for 3 days to induce quiescence. Cultures were prelabeled with "C]tyrosine in serum free medium for 2 days, washed with serum free medium, and then treated with either angiotensin II (1 μM, - - -), vehicle (control, - - ), or 10% fetal bovine serum (---). Percentage of total protein degraded was calculated by dividing trichloroacetic acid-soluble counts released into media at 0–12 hours or 12–24 hours by total of trichloroacetic acid-soluble counts and trichloroacetic acid-insoluble counts (collected from dish at 24 hours). Values are mean ± SEM, n = 3. At both 12 and 24 hours, the 10% fetal bovine serum group is significantly different from control (p < 0.05). Results similar to these were obtained in one other independent experiment. Note that the vertical axis is truncated.

Figure 9. Influence of angiotensin II (All) on subconfluent quiescent cultures of smooth muscle cells. Cells were plated in DF10 at 3 × 10⁶ cells/cm². Medium was changed to serum free medium 24–48 hours later. After 3–5 days, cultures were treated with All for 4 days. Protein content was determined as shown in Figure 3. Results are mean ± SEM of three independent experiments. The All-treated group is significantly greater than control (p < 0.05).

Figure 10. Cell cycle histogram obtained from subconfluent cultures using Hoechst 33258 stain. Staining cultures were maintained in serum free medium for 3 days and then treated with either angiotensin II (1 μM, - - -) or vehicle (control, - - ) for 4 days.
References


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