Regulation of Vascular Smooth Muscle Growth by \(\alpha_1\)-Adrenoreceptor Subtypes in Vitro and in Situ*

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Rat aorta smooth muscle cells which express all three \(\alpha_1\)-adrenoreceptors (\(\alpha_{1A}\), \(\alpha_{1B}\) and \(\alpha_{1D}\)) were used to determine the effect of stimulation of \(\alpha_1\)-adrenergic receptor subtypes on cell growth. “Combined” \(\alpha_1\)-adrenoreceptor subtype stimulation with norepinephrine alone caused a concentration-dependent, prazosin-sensitive increase in protein content and synthesis; 48 h of stimulation at 1 \(\mu\)M increased cell protein to 216 ± 40% of time-matched controls (\(p = 0.008\)) and RNA to 140 ± 13% (\(p = 0.03\)); protein synthesis increased to 167 ± 13% (\(p < 0.01\)) after 24 h. Stimulation with norepinephrine plus the selective \(\alpha_{1A}/\alpha_{1D}\) antagonist 5-methylurapidil produced greater increases in \(\alpha\)-actin mRNA (270 ± 40% at 8 h; \(p = 0.007\)), total cell protein (220 ± 45% at 24 h; \(p = 0.004\)), and RNA (135 ± 8% at 24 h; \(p = 0.01\)). These effects were prevented by pretreatment with the selective \(\alpha_{1B}\) antagonist chloroethylclonidine. Comparable results were obtained for intact aortae. Stimulation with norepinephrine plus 5-methylurapidil increased (\(p < 0.05\)) tissue protein, RNA, dry weight, and \(\alpha\)-actin mRNA; and as in cultured cells, combined stimulation with norepinephrine alone attenuated these responses. By comparison, adventitia (fibroblasts) was unaffected. Removal of endothelial cells had no effect. \(\alpha_{1D}\) mRNA decreased by 42 ± 12% (\(p = 0.01\)) in cultured cells during combined \(\alpha_1\)-adrenoreceptor stimulation and by 23 ± 8% (\(p = 0.03\)) for intact aorta. \(\alpha_{1D}\) and \(\beta\)-actin mRNA were unchanged in cultured cells, aorta media, and adventitia. These findings suggest that prolonged stimulation of chloroethylclonidine-sensitive, possibly \(\alpha_{1B}\)-adrenoreceptors induces hypertrophy of arterial smooth muscle cells and that stimulation of 5-methylurapidil-sensitive, non-\(\alpha_{1B}\)-adrenoreceptors attenuates this growth response.

Evidence suggests that the sympathetic nervous system and \(\alpha_1\)-adrenoreceptors (AR)\(^{1}\) may exert trophic influences over SMCs during normal development and also contribute to the pathogenesis of vascular hypertrophy and atherosclerosis (1, 2). Hyperinnervation of blood vessels by catecholaminergic fibers in the genetic spontaneously hypertensive rat has been correlated with SMC hypertrophy and hyperplasia in these vessels (3). Also, sympathectomy attenuates normal growth, as well as hypertrophy of the vascular wall in hypertensive animals (4–7). There is considerable evidence that smoking, stress, and hypertension, which are key risk factors for atherosclerosis and hypertrophic vascular disease, are associated with elevated plasma catecholamines (8, 9).

Catecholamines have been shown to initiate not only immediate SMC responses such as contraction of blood vessels, but may also influence proliferation and growth of cultured vascular SMCs (10–13). In nonconfluent, cultured rat and rabbit aortic SMCs, AR stimulation promotes cell proliferation (10, 13). Furthermore, \(\alpha_1\) blockade reduces vascular collagen synthesis in the spontaneous hypertensive rat (14), and inhibits SMC proliferation induced by endothelial denudation (13, 15) and angiotensin infusion (16) in normal rats. In cholesterol-fed monkeys, elevated plasma norepinephrine (NE) greatly increased atherosclerotic lesion growth (17). Infusion of NE over a 2-week period, at a level which did not cause sustained elevation of blood pressure, induced formation of atherosclerotic vascular lesions in rabbit aorta (18). There is also evidence that \(\alpha_1\) ARs mediate growth of myocardial cells. In cultured neonatal rat cardiac myocytes that possess both \(\beta_1\) and \(\alpha_1\) ARs, stimulation of \(\alpha_1\) ARs with NE increased cell protein, RNA, myocyte surface area, and contractile protein expression (19). However, no studies have examined whether \(\alpha_1\) ARs influence proliferation-independent growth of SMCs.

Both molecular cloning and pharmacologic studies have shown that \(\alpha_1\) ARs are comprised of three closely related subtypes (20, 21). We have recently used polymerase chain reaction (22) and RNase protection assays to determine expression of \(\alpha\)AR subtype mRNA by rat vascular SMCs. Freshly isolated and early passage cultured aortic and venous cava SMCs express both \(\alpha_{1B}\) and \(\alpha_{1D}\) mRNA (20), and it appears that both receptors are present on SMCs of rat aorta (24, 25, and Ref. 22 and references therein). Rat aorta has been shown recently to also express \(\alpha_{1A}\) (formerly denoted “\(\alpha_{1C}\”) mRNA (26–29). Given this multiplicity of \(\alpha_1\) AR expression, the purpose of the present study was first to examine both in vitro and in situ, the effects of combined stimulation of the \(\alpha_1\) AR subtypes with NE alone on proliferation-independent growth, and expression of sarcomeric-\(\alpha\)-SMC-actin and cytoskeletal-\(\beta\)-actin mRNAs by arterial and venous SMCs. Second, effects of combined stimulation were compared with those during treatment with NE plus antagonists, 5-methylurapidil (5-MU, selectivity = \(\alpha_{1A} > \alpha_{1D} > \alpha_{1B}\)) to favor \(\alpha_{1B}\) stimulation and after pretreatment with chloroethylclonidine (CEC, selectivity = \(\alpha_{1B} > \alpha_{1D} > \alpha_{1A}\)) to select for stimulation of non-\(\alpha_{1B}\)ARs. The results suggest, in both cultured aorta SMCs and intact aorta media, that stimulation of CEC-sensitive \(\alpha_1\) ARs (possibly \(\alpha_{1D}\)) induces hypertrophy of aorta SMCs and that stimulation of 5-MU-sensitive, non-\(\alpha_{1B}\) ARs antagonizes this response. Thus, alterations in the relative activity of different \(\alpha_1\) AR subtype signaling pathways

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‡The abbreviations used are: AR, adrenergic receptor; SMC, smooth muscle cell; NE, norepinephrine; 5-MU, 5-methylurapidil; CEC, chloroethylclonidine; MOPS, 3-(N-morpholino)propanesulfonic acid; bp, base pair(s); RPA, RNase protection assay; PI, phosphoinositide.

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may participate in both normal vascular growth and remodeling and also in vascular hypertrophic diseases.

MATERIALS AND METHODS

Cell Culture—Primary cultures of vascular SMCs were each obtained from pooled thoracic aortae or vena cavae from eight adult Sprague-Dawley rats (200 g, Sasko, Omaha, NE) by a modification (22) of the method of Turia et al. (30) and maintained in Medium 199 (M199) supplemented with 10% fetal bovine serum, 200 mg/ml l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were passaged at 90–95% confluence with 0.05% trypsin/EDTA every 5–7 days and seeded at a density of 5000 cells/cm² on plastic plates. Viable cell number was determined by hemocytometry with trypan blue exclusion in duplicate. To favor the differentiated phenotype and quiescence, in all experiments cells were exposed to experimental conditions beginning at 4 days after reaching confluence in passage 4. A separate cell line was used in each experimental replicate. These cultures lacked contamination by other cell types as revealed by immunohistochemistry with monoclonal antibodies (Dako A/S, Glostrup, Denmark) against SM-specific α-actin and von Willebrand factor.

Protocols—To examine the effect of combined α1AR stimulation on SMC growth, as reflected by changes in total protein and RNA per cell (19), in the first experiment aorta or vena cava cells were exposed to 8, 24, and 48 h to NE. In this and all subsequent experiments, 100 μM ascorbate was present to prevent NE degradation (19), plus 0.5 μM rauwolscine and 1 μM propranolol to minimize activation of α2- and βARs. Control cells in all experiments were time-matched and exposed to these agents, but not to NE (vehicle groups). In a second set of experiments cells were exposed to 1 μM NE and also to the α1 antagonist prazosin (1 μM), plus 0.5 μM ascorbic acid, 0.5 μM rauwolscine, and 1 μM propranolol, to determine if effects of combined α1AR stimulation were also examined in aorta SMCs maintained in serum-free media after the second day of post-confluence (48 h before start of experiment). Serum-free media consisted of 50% DMEM, 50% F-12 media supplemented with 2.85 mg/ml insulin, 5 μg/ml transferrin, 35.2 μg/ml ascorbic acid, 6 μg/ml selenium, 100 units/ml penicillin, and 100 μg/ml streptomycin. Serum-free media were similar regardless of presence or absence of serum (see "Results") subsequent experiments (below) were conducted in serum-free medium. In a third experiment, aorta SMCs were exposed for 24 h to NE (10⁻¹⁰ to 10⁻³ M) for determination of concentration-response effects. In a fourth experiment aortic SMCs were exposed for 8 and 24 h to 1) vehicle, 2) 10 μM NE, 3) NE plus the α1 antagonist prazosin (1 μM), plus 0.5 μM ascorbic acid, 0.5 μM rauwolscine, and 1 μM propranolol, to determine if effects of combined α1AR stimulation were also examined in serum-free media after the second day of post-confluence (48 h before start of experiment).

RNA and Protein Determination—Total cellular RNA was determined (from duplicate plates or vessel extracts) using standard techniques (36). RNA integrity was assayed, and variations in aliquoted RNA from each RNA preparation were determined by Northern assay (see below), according to film densitometry (UMAX UC630 film scanner and the Image Program (National Institutes of Health)) of 28 and 18 S rRNA bands resolved on ethidium bromide-stained, 1 × MOPS/formaldehyde, 1% agarose gels. RNA quantity and purity were determined spectrophotometrically. Total soluble protein was determined in duplicate using a modified (37, 38) BCA assay (Pierce).

Protein Synthesis—Four-day, post-confluent SMCs maintained in serum-free media received a change of medium containing low methionine (2 mg/liter), 100 μg/ml ascorbate, 1 μM propranolol, and 10⁻⁶ to 10⁻³ M NE (duplicate plates for each NE concentration). Eighteen hours later (50) methionine (5 μCi/ml, 1000 Ci/mmol, Amersham) was added. After 3 h aliquots were washed twice with 4°C phosphate-buffered saline, resuspended in 10 μl of 0.05% trypsin-EDTA, which was then stopped with serum-containing M199. Pelleted cells were lysed with Nonidet P-40 at 4°C. The supernatant was treated with trichloroacetic acid at a final concentration of 10% in the presence of 100 μg/ml bovine serum albumin, and incubated for 30 min at 4°C. Trichloroacetic acid-precipitable counts were collected on Whatman GF/C filters and counted in Ecoscint H (National Diagnostics) after overnight shaking at 20°C.

mRNA Quantification—α-Actin and β-actin plasmids contained, respectively, 191 and 526 base pairs (bp) of the 3′-untranslated region of the cDNA sequences. The 117-bp NeoII BamHI fragment that encodes the third intracellular loop of the α₁AR was subcloned into pGEM-4Z (Promega). The 306-bp BsmHII/PstI fragment that encodes the fourth intracellular transmembrane-spanning regions of the Smad was inserted into pGEM-3Z. Identity and orientation of insertions was assessed by restriction enzyme analysis and sequencing. RNAse protection assays (RPAs) were used to quantitate mRNA levels. In vitro transcribed ([³²P]rCTP-labeled cRNAs (riboprobes) and cold sense RNAs were made according to standard procedures. Each assay consisted of addition of a constant amount of a single labeled probe and total cell RNA corresponding to an equal number of cells or vessel length for control versus treatment groups (instead of constant amounts of RNA) to account for any treatment effects on cell size and number. Because total RNA per cell varied according to treatment groups (i.e. from hypertrophy), it was not appropriate to load a constant amount of cell RNA. Companion samples were used beforehand on ethidium bromide gels for quantitation of the above 28 and 18 S RNA (see above) were used to correct for variations in acutal versus expected amounts of cell RNA loaded. tRNA was added to bring each assay up to a constant amount of total RNA. Known amounts of sense RNA were similarly treated in each RPA to ensure molar excess of probe. Hybridization was performed at 57°C for 16 h and RNAse A (100 μg/ml) and RNAse T1 (250 units/ml, Boehringer Mannheim) + RNaseW₁ (250 units/ml, Boehringer Mannheim; α-actin: RNase One (35 units/ml, Promega)). RNA hybrids were resolved on an 8 M urea, 6% polyacrylamide, 1 × TBE (Tris-borate-EDTA) gel. Dried gels were exposed to film (X-Omat, Kodak) with intensifying screens at −70°C for 3–96 h. Densitometry was performed as described above.

Specificity of RPAs—Riboprobe were tested for specificity using total RNA from liver, kidney, fresh media adventitial and medial layers, and cultured medial SMCs and also with homologous and heterologous
sense RNAs for α₁AR subtypes and actins. In agreement with polymerase chain reaction data (22) and using up to 100 μg of RNA, the α₁D riboprobe only detected mRNA in the aortic medial layer, adventitia, cultured SMCs, and kidney; the α₁B probe only detected mRNA in the medial layer, cultured SMCs, liver, and kidney; the α₁-actin probe detected mRNA in all of these tissues and cells. No cross-reactivity was found among probes and heterologous sense RNAs for expected protected fragments.

**Analysis and Statistics**—Total RNA, protein, and mRNA species were determined on a per cell (SMC cultures) or per millimeter of vessel length (organ culture) basis for NE-treated and time-matched control (vehicle-treated) groups. Data are given as mean ± S.E., means for groups with n sizes ≥ 3 were analyzed using t tests and analysis of variance, followed by Dunnett’s test or the Bonferroni correction for multiple comparisons. A value of p < 0.05 was considered significant. n sizes represent the number of experimental replicates, each obtained from a separate cell culture line or group of pooled aortic maintained in organ culture.

**RESULTS**

**Effect of Combined α₁AR Subtype Stimulation on Aorta SMCs**—Combined stimulation of aorta SMC α₁ARs with 1 μM NE in the presence of α₁AR and βAR blockade did not induce cell proliferation or change cell viability (trypan blue exclusion) (Fig. 1). In both control (vehicle-treated), NE and NE + prazosin groups, viability averaged 97 ± 0.8% (data not shown). Total cell protein and RNA at 0 h in the control group were 713 ± 49 pg/cell and 24 ± 5 pg/cell, respectively, and were unchanged in the NE and NE + prazosin groups. Combined α₁AR stimulation significantly increased cell RNA to 140 ± 19% of control (p = 0.03) at 48 h and increased cell protein to 167 ± 11% (p = 0.004) at 24 h and to 216 ± 40% (p = 0.008) at 48 h (Fig. 1). These increases were prevented by the α₁ antagonist prazosin (Fig. 1). Because cells in defined, serum-free medium (n = 3) exhibited responses for this same protocol that did not differ significantly from cells in the presence of serum (n = 3), the data were combined (Fig. 1).

This growth effect of combined α₁AR subtype stimulation was further characterized by examining α-actin and β-actin mRNA. α₁AR stimulation had no effect on levels of α- or β-actin mRNA, although α-actin tended to increase at the 24-h point (240% of control, p = 0.08) (Fig. 2). In contrast, in the presence of prazosin at the 8-, 24-, and 48-h points, mRNA (in percent of time 0) for α-actin was 85 ± 25, 70 ± 5, and 110 ± 22, respectively, and for β-actin was 92 ± 4, 95 ± 5, and 95 ± 18, respectively. α₁DAR mRNA transiently decreased by 42 ± 12% of control (p = 0.01) at 8 h in serum-containing and by 38 ± 14% (p = 0.02) in serum-free medium and returned to control by 24 h in both groups (Fig. 2, data combined). In contrast, α₁D mRNA evidenced no significant change. In the presence of prazosin, α₁D and α₁B mRNAs were not significantly different from those for control cells whose levels themselves did not change significantly over the 48-h interval. In the presence of NE plus prazosin at the 8-, 24-, and 48-h points, mRNA (in percent of time 0) for α₁D was 107 ± 6, 100 ± 1, and 117 ± 20, respectively, and for α₁B was 88 ± 30, 86 ± 10, and 119 ± 33, respectively. The observation that β-actin mRNA remained constant while total per cell RNA increased provides an internal standard that validates as specific the changes in α₁B and α-actin mRNA.

Concentration-response experiments for per cell protein content and protein synthesis were conducted over 24 h of combined α₁AR stimulation with NE (as in above protocol) in serum-free, defined medium. Combined stimulation caused dose-dependent increases in both protein content and synthesis relative to time-matched, vehicle-treated controls (Fig. 3). As in the preceding experiments, cell number was unchanged.

**Vena Cava Smooth Muscle**—The effect of combined α₁AR stimulation with 1 μM NE was also examined for passage 4, 4-day post-confluent thoracic vena cava SMCs maintained in serum-containing medium (n = 3 each for vehicle, NE, and NE + prazosin groups (data not shown)). There were no significant changes in viable cell number (0 h = 1.7 × 10⁶ ± 0.2 cells/plate), total protein (0 h = 1028 ± 141 pg/cell), total RNA (0 h = 14.1 ± 2.8 pg/cell), or in α₁B, α₁D, α-actin, or β-actin mRNA levels at 8 and 24 h for any group.

**Selective Stimulation of Aorta SMC α₁Adrenoreceptor Subtypes**—To determine the influence of stimulation of α₁AR subtypes on aorta SMC growth, cells maintained in serum-free medium were treated for 8 and 24 h with 10 μM NE alone (combined α₁AR stimulation), NE plus the competitive α₁AD antagonist 5-MU (0.3 μM), or NE after pretreatment with the irreversible α₁B antagonist CEC (30 μM). All groups received rauwolscine, propranolol, and ascorbate as above, as did the vehicle “control groups” (no NE). The control group at time 0 received only these agents, whereas the control groups for the other time periods also received 5-MU and CEC pretreatment (see “Materials and Methods”). In these control groups, as in the control groups for the preceding 48-h experiment, cell number, RNA, protein, and mRNAs for actins and α₁ARs (see below) did not change over the 24-h period, indicating stability of these SMC cultures (Figs. 4 and 5). These control groups also
combined stimulation of the presence of NE had no effect on cell number (Fig. 4). As in the previous experiments, although these responses did not differ significantly. The increase in cytokeratin mRNA that was twice the magnitude exhibited by cells exposed to combined \( \alpha_{1B} \) AR subtype stimulation (NE alone) versus control (Fig. 5). Stimulation with NE + CEC again caused a decrease in \( \alpha_{1B} \) mRNA that was twice the magnitude exhibited by cells exposed to combined \( \alpha_{1B} \) AR subtype stimulation (Figs. 5 and 6). However, during NE + 5-MU stimulation (Figs. 5 and 6). However, during NE + 5-MU augmented these increases by approximately 2-fold more. In the presence of 5-MU, NE increased \( \alpha_{1B} \) mRNA by 270 ± 4% of control (\( p = 0.007 \)), cell protein by 220 ± 45% (\( p = 0.004 \)), and cell RNA by 135 ± 8% (\( p = 0.01 \)) (Fig. 6). Norepinephrine plus CEC pretreatment abolished these increases (Figs. 4–6). In each experiment, additional plates of cells for the CEC and 5-MU groups were exposed to 3-fold higher concentrations of CEC (90 \( \mu \) M during pretreatment, \( n = 5 \)) and 5-MU (1 \( \mu \) M, \( n = 5 \)) to test specificity of the antagonists at the lower concentrations. No significant additional changes were observed relative to values at the lower concentrations.

These data suggest that CEC-sensitive \( \alpha_{1B} \) AR stimulation induces SMC growth, while simultaneous stimulation of 5-MU-sensitive \( \alpha_{1B} \) ARs attenuates this action. This conclusion is supported by analysis of \( \alpha_{1B} \) and \( \alpha_{1D} \) subtype mRNAs for these experiments (Figs. 5 and 6). Stimulation with NE + 5-MU at 8 h induced a significant increase in \( \alpha_{1B} \) mRNA that was almost 3-fold greater than the increase resulting from combined \( \alpha_{1D} \) AR stimulation (NE alone). Both increases were abolished by CEC; \( \alpha_{1D} \) was actually decreased below control in this group, consistent with the growth inhibitory activity of CEC-insensitive \( \alpha_{1D} \) AR stimulation. The increases in \( \alpha_{1D} \) AR induced by 10 \( \mu \) M NE in the NE alone and NE plus 5-MU groups occurred earlier than in the experiments in Figs. 1 and 2 which employed a lower (1 \( \mu \) M) NE concentration. The specificity of these effects on \( \alpha_{1D} \) mRNA is indicated by the absence of change in cytoskeletal \( \beta_{1D} \) AR for any group (Fig. 5).

Stimulation with NE + CEC again caused a decrease in \( \alpha_{1B} \) mRNA that was twice the magnitude exhibited by cells exposed to combined \( \alpha_{1B} \) AR subtype stimulation (Figs. 5 and 6). However, the 20 ± 2% decrease during combined stimulation was less than the 42 ± 12% decrease observed in the Fig. 2 experiments, although these responses did not differ significantly. CEC abolished the decrease in \( \alpha_{1B} \) mRNA. Also in agreement with the first experiment (Fig. 2), \( \alpha_{1B} \) mRNA exhibited no changes during combined stimulation and was unaffected during NE + 5-MU stimulation (Figs. 5 and 6). However, during

verify that CEC pretreatment and 5-MU, alone, had no baseline effect on any parameter. As in the previous experiments, the presence of NE had no effect on cell number (Fig. 4). Combined stimulation of \( \alpha_{1B} \) ARs (NE alone) increased RNA and protein in agreement with the previous experiments. However,
stimulation of non-\(\alpha_{1B}\)ARs (NE + CEC), \(\alpha_{1D}\) mRNA was decreased, possibly due to stimulation of this receptor without concomitant activation of \(\alpha_{1B}\)ARs. Like total RNA and cell protein (above), tripling CEC or 5-MU concentrations had no additional effect \( (n = 5) \). These controls, together with the additional effect (protein (above), tripling CEC or 5-MU concentrations had no stimulation of non-\(\alpha_{1B}\)ARs (NE + CEC), \(\alpha_{1D}\) mRNA was obtained with the chosen drug concentrations. Although some recovery of \(\alpha_{1B}\)AR number may have occurred over the 24-h period following alkylation with CEC, full blockade of the NE-induced increases in cell protein, RNA, \(\alpha\)-actin mRNA and decrease in \(\alpha_{1B}\) mRNA was still evident at 24 h \( (\text{Figs. 4 and 5}) \).

Stimulation of \(\alpha_{1}\)ARs on Aorta SMCs in Situ—To determine if these findings for cultured aorta SMCs correctly predict responses of aorta SMCs \( \text{in situ} \) in the presence of endothelial cells and adventitial fibroblasts, intact aortas were maintained under wall tension in organ culture \( (\text{serum-free medium}) \) for 24 h in the presence or absence of 10 \( \mu \)M NE \( (\text{plus ascorbate, propranolol, and rauwolscine}) \). Adventitia and endothelium were then separated from SMC media \( (\text{see “Materials and Methods”}) \) to avoid contamination of SMC and non-SMC RNA and protein. Combined \(\alpha_{1}\)AR stimulation induced changes in SMC \( (\text{“media”}) \) mRNAs \( (\text{Fig. 7A}) \) that were smaller but qualitatively similar to those obtained in the initial cell culture experiment \( (\text{Fig. 2}) \); \(\alpha_{1B}\) mRNA decreased and \(\alpha\)-actin increased, while \(\alpha_{1D}\) and \(\beta\)-actin mRNA were unaffected \( (\text{Fig. 7A}) \). There was a similar lack of effect on adventitia mRNA for \(\alpha_{1D}\) and \(\beta\)-actin. Riboprobe selectivity is indicated by the absence of detection of \(\alpha_{1B}\) and vascular SMC-specific \(\alpha\)-actin mRNA in adventitia \( (\text{Fig. 7A}) \), findings in agreement with our previous polymerase chain reaction studies \( (22) \).

However, unlike the response of cultured SMCs \( (\text{Fig. 1}) \), combined stimulation of \(\alpha_{1}\)ARs on \( \text{in situ} \) SMCs caused no significant increase in tissue RNA and protein \( (\text{Fig. 7B}) \), indicating the absence of an \(\alpha_{1B}\) growth effect, at least over 24 h of stimulation. Among several possibilities for this difference, the presence of endothelial cells in organ culture and/or the combined activation of stimulatory \(\alpha_{1B}\)ARs plus inhibitory non-\(\alpha_{1B}\)ARs on SMCs may have prevented growth. To test these possibilities, vessels were denuded of endothelium and exposed to either 10 \( \mu \)M NE in the presence of 0.1 \( \mu \)M 5-MU \( (\text{favoring} \alpha_{1B} \text{ stimulation}) \) for 24 h or 5-MU only \( (\text{control group}) \); propranolol, rauwolscine, and ascorbate were present in all four treatment groups. Only total RNA, protein, and \(\alpha\)-actin mRNA for the SMC medial layer were measured so that three, instead of eight, aortae were exposed to each treatment. The experiment with the four treatment groups was repeated three times \( (n = 3) \); protein was 65 \( \pm \) 18 mg/mm for controls and 50 \( \pm \) 17 mg/mm for the denuded group \( (n = 3) \). \(\alpha_{1B}\) stimulation of endothelium-intact vessels in-

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**Fig. 4.** Effect of selective stimulation of \(\alpha\)-adrenoceptor subtypes on cell number, RNA, and protein in aortic SMCs. \( \text{Top panel, viable cell number of post-confluent cells, NE + CEC group pretreated with} 30 \mu\text{M CEC for} 30 \text{min prior to time} 0 \text{in the presence of rauwolscine, propranolol, and} 100 \mu\text{M ascorbate, followed by washes (see “Materials and Methods”; CEC not present in this group during NE exposure). Over the indicated time periods all noncontrol groups were exposed to ascorbate, rauwolscine,} \text{and propranolol and either NE, NE + 5-MU,} \text{or NE after CEC pretreatment. Control cells at time} 0 \text{were exposed to rauwolscine, propranolol, and ascorbate. Control cells at the other time points had these agents and 5-MU present and had been pretreated as above with CEC. Comparison of time points for these control groups indicate that CEC pretreatment and the presence of 5-MU themselves had no effect on any parameters (see also Fig. 5).} \text{n size refers to number of replicate experiments. *}, \( p < 0.05 \) versus to time-matched control cells.

**Fig. 5.** Effect of selective stimulation of \(\alpha\)-adrenoceptor subtypes on \(\alpha_{1}\)AR and \(\alpha_{2}\)AR and \(\alpha\)-actin and \(\beta\)-actin mRNA in aortic SMCs. Groups are as defined in Fig. 4 legend. *}, \( p < 0.05 \) versus time-matched control cells; \( p < 0.05 \) versus time-matched NE alone cells. \( n \) size refers to number of replicate experiments.

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creased RNA by 180 ± 11% of control (p = 0.0003), protein by 191 ± 47% (p = 0.02), and increased α-actin mRNA by 161 ± 35% (p = 0.04) (Fig. 7, C and D). In denuded vessels, α1B stimulation increased RNA by 188 ± 50% of control (p = 0.03), protein by 146 ± 3% (p = 0.0001), and increased α-actin mRNA by 175 ± 29% (p = 0.015). Furthermore, α1B stimulation increased vessel dry weight from 0.45 ± 0.05 to 0.55 ± 0.05 mg/mm (p = 0.0001) in controls and from 0.44 ± 0.06 to 0.54 ±
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0.05 mg/mm³ (p = 0.0002) in denuded vessels (25% increases). These data are consistent with the cell culture experiments.

**DISCUSSION**

The principal findings of this study were that stimulation of aorta SMC α₁ARs over 24–48 h doubled the per cell amount of RNA, protein, and sacromeric α-actin mRNA, while not affecting cytoskeletal β-actin mRNA or inducing cell proliferation. This growth response exhibited a dose dependence, with concentrations as low as 100 nM NE significantly increasing protein synthesis within 24 h. Vena cava SMCs did not display this α₁-mediated “hypertrophy,” nor did they exhibit the decreased α₁B mRNA that was evidenced by aorta SMCs. In contrast, α₁B mRNA was unaffected in both cell types. Incubation of cells with CEC, which preferentially alkylates α₁B ARs, abolished all responses. In contrast, stimulation of non-α₁B ARs appeared to oppose the hypertrophy. This was indicated by the significantly greater increases in protein and α-actin mRNA that occurred with NE exposure during preferential blockade of non-α₁B ARs with 5-MU, compared with NE alone. Importantly, similar findings were also observed in the SMC medial layer of intact aortae maintained in organ culture under tension. Compared with combined α₁AR stimulation, preferential α₁B stimulation (NE plus 5-MU) increased tissue dry weight by 25% and, again, approximately doubled RNA, protein, and α-actin mRNA. And like SMCs, α₁B AR stimulation in intact aorta decreased α₁B mRNA and had no effect on β-actin or α₁D mRNA. Removal of endothelial cells did not influence this SMC growth response. In addition, the fibroblast-rich adventitia, which does not express α₁-like SMCs, α₁B down-regulation, a similar (80%) decrease in α₁B mRNA, while not affect- ing protein synthesis and content and had no effect on cell proliferation or DNA synthesis. These effects were prevented by prazosin, but unaffected by propranolol or the rauwolscine diastereomer, yohimbine.

Unlike arterial SMCs, we did not observe any changes in cultured venous SMCs during combined α₁AR stimulation. There are several possibilities that could underlie this difference. The relative density of α₁AR subtypes present, extent of receptor occupancy at the concentrations of ligands used, and required duration of stimulation may differ between artery and vein cells. Nevertheless, it is interesting that veins do not display hypertrophy in models of arterial hypertension associated with elevated sympathetic state (45), even though hypertrophy of veins occurs in chronic venous hypertension (46). Also, it is well known that compared with arteries, veins are much less susceptible to atherosclerosis (47). While differences in pressure and shear stress could contribute to these differences between arteries and veins, it remains possible that venous SMCs differ in their capacity to respond to certain arterial trophic stimuli, including α₁AR activation.

Previous studies have implicated catecholamines in the regulation of SMC growth. Adrenergic receptor stimulation (NE alone) increases proliferation rate in rat and rabbit cultured aorta SMCs while in log growth phase (10, 13) and induces onconege expression (48–51). Sympathectomy not only impairs normal growth of rabbit ear and rat mesenteric arteries (4, 5) but also attenuates arterial hypertrophy in hypertensive animals (5–7, 52). Furthermore, the degree of hyperinnervation of arteries in the spontaneously hypertensive rat has been associated with the magnitude of smooth muscle hypertrophy and hyperplasia (3). In addition, the ability of antihypertensive drugs to inhibit arterial hypertrophy in several hypertensive models correlates with their efficacy to interfere with sympathetic stimulation of SMCs (e.g. prazosin > reserpine > captopril and calcium channel antagonists) (53). There is also evidence that catecholamines may influence atherogenesis. Besides the associations of stress, smoking, and hypertension with elevated adrenergic activity and with atherosclerosis (8, 9), prolonged elevation of plasma NE in rabbits (18) and choles terol-fed monkeys (17) induces new and greatly exacerbates existing lesion growth, while the α₁ antagonists prazosin and doxazosin oppose it (54, 55) by mechanisms that may not be limited to changes in plasma lipids. It is also known that α₁ blockade decreases vascular collagen synthesis in spontaneously hypertensive rat (14) and SMC proliferation induced by endothelial cell denudation (13, 15) and angiotensin infusion (16). Although the specific α₁AR subtypes underlying these relationships were not examined, it is interesting in light of our
data suggesting a trophic role for the α1BAR, that the reliance on α1A ARs (i.e., CEC-sensitive) for aortic contraction in young, rapidly growing rats is replaced by CEC-insensitive α1AR dominance in adults (24).

α1AR stimulation also induces hypertrophy of cultured neonatal rat myocardial cells (19, 56–58). Treatment with NE over 4 days caused concentration-dependent increases (2-fold maxima at 2–20 μM) in cell protein and RNA which were matched by comparable increases in cell size. α1AR blockade with prazosin abolished the response, while α2 and β blockade had little effect. Consistent with our findings, in these studies cell proliferation and β-actin expression were unaffected, while sarcomeric α-actin gene transcription rate, mRNA and protein were increased. Moreover, α1AR stimulation promoted expression of the fetal/neonatal actin isoform profile. These findings have been confirmed and extended by others (59). Interestingly, in contrast to the CEC-sensitive SMC growth identified in our studies, myocyte hypertrophy was unaffected by CEC, but instead inhibited by 5-MU and (+)-niguldipine, even though CEC-sensitive α1 ARs are present in approximately 2-fold greater abundance (59). Proximal aspects of the signaling pathway include activation of Gαq protein kinase C, and the ras protooncogene (59). Thus, besides short term regulation of intracellular glycosylation and CEC-sensitive α1 ARs mediate long term genetic and morphological features of myocardial hypertrophy. A similar adrenergic growth effect has been reported for rabbit cardiomyocytes (60) and also for adult rat hepatocytes expressing the α1BAR (61). Furthermore, the possibility that α1 ARs promote myocardial cell growth in vivo is supported by evidence that prolonged infusion of NE, at levels which did not cause sustained elevation of arterial pressure, induced cardiac hypertrophy in conscious dogs (62).

The specific second messenger pathways activated by the different α1AR subtypes are undoubtedly central in their capacity to modulate SMC growth differently. In several cell lines and in rat thoracic aorta, CEC-sensitive α1 ARs couple to phosphoinositide (PI) metabolism and protein kinase C, while CEC-insensitive, WB4101-sensitive α1 ARs are linked to influx of extracellular calcium through dihydropyridine-sensitive calcium channels (cf. Ref. 25). Evidence suggests that stimulation of PI metabolism can induce growth of lymphocytes (63) and fibroblasts (64), and transfection of PI-coupled α1A ARs into fibroblasts confers strong NE-induced α1-mediated growth (65). Moreover, transfection of mutant α1B receptors that tonically elevate PI metabolism even in the absence of NE are oncogetic in these cells (65). It is interesting that in myocardial cells CEC-insensitive α1 ARs are coupled to PI metabolism and promote growth, while the non-PI-coupled, CEC-sensitive α1 ARs do not exhibit this capacity (23, 57, 59).

In summary, the present study demonstrated that stimulation of CEC-sensitive, possibly α1A ARs, promoted growth of aorta SMCs regardless of whether they were maintained in cell or organ culture. Stimulation of 5-MU-sensitive α1 ARs (presumably α1B and/or α1A) adrenoreceptors antagonized this response. These effects were not evidenced by vena cava SMCs or aorta adventitial cells. Thus, the sympathetic nervous system may influence normal arterial smooth muscle growth as well as remodeling in hypertrophic vascular disease, depending on the relative activity of these different, opposing α1AR signaling pathways.

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REFERENCES

1. Bauch, H. J., Grunwald, J., Vischer, P., Gerush, U., and Hauss, W. H. (1984) Circulation 69, 441–450

2. Vashishth, R., Siam, M., Franks, P. J., and O’Malleys, M. K. (1992) Br. J. Surg. 79, 1285–1288

3. Head, R. J. (1991) Blood Vessels 28, 173–178

4. Glabe, R. D. and Tsuru, H. (1979) H. K. Vessels 16, 109–112

5. Nyborg, N. C. B., and Mulvany, M. J. (1985) Cardiovasc. Res. 19, 528–536

6. Hart, M. N., Heistad, D. D., and Brody, M. J. (1980) Hypertension 2, 419–423

7. Long, C. S., Ordahl, C. P., and Simpson, P. C. (1989) J. Clin. Invest. 83, 3038–3046
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57. Long, C. S., Kariya, K., Karns, L., and Simpson, P. C. (1991) J. Cardiovasc. Pharmacol. 17, Suppl. 2, S20–S24
58. Bishopric, N. H., Simpson, P. C., and Ordahl, C. P. (1987) J. Clin. Invest. 80, 1194–1199
59. Knowlton, K. U., Michel, M. C., Itani, M., Shubeita, H. E., Ishihara, K., Brown, J. H., and Chien, K. R. (1993) J. Biol. Chem 268, 15374–15380
60. Decker, R. S., Cook, M. G., Behnke-Barclay, M. M., Decker, M. L., Lesch, M., and Samarel, A. M. (1993) Am. J. Physiol. 265, H329–H339
61. Cruise, J. L., Houck, K. A., and Michalopoulos, G. K. (1985) Science 227, 749–751
62. Stewart, J. M., Patel, M. B., Wang, J., Ochoa, M., Gewitz, M., Loud, A. V., Anversa, P., and Hintze, T. H. (1992) Am. J. Physiol. 262, H331–H339
63. Fisher, D. B., and Mueller, G. C. (1971) Biochim. Biophys. Acta 248, 434–448
64. Matsuoka, K., Fukami, K., Osamu, N., Kawai, S., and Takenawa, T. (1988) Science 239, 640–642
65. Allen, L. F., Lefkowitz, R. J., Caron, M. G., and Cotechia, S. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 11354–11358