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Regulation of Vascular Smooth Muscle Growth by α_1-Adrenoreceptor Subtypes *in Vitro* and *in Situ**

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Rat aorta smooth muscle cells which express all three α_1-adrenoreceptors (α_{1A}, α_{1B} and α_{1D}) were used to determine the effect of stimulation of α_1-adrenergic receptor subtypes on cell growth. "Combined" α_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 μM 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 α_{1A}/α_{1D} antagonist 5-methylurapidil produced greater increases in α-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 α_{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 α-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. α_{1B} mRNA decreased by 42 ± 12% ($p = 0.01$) in cultured cells during combined α_1-adrenoreceptor stimulation and by 23 ± 8% ($p = 0.03$) for intact aorta. α_{1D} and β-actin mRNA were unchanged in cultured cells, aorta media, and adventitia. These findings suggest that prolonged stimulation of chloroethylclonidine-sensitive, possibly α_{1B}-adrenoreceptors induces hypertrophy of arterial smooth muscle cells and that stimulation of 5-methylurapidil-sensitive, non-α_{1B}-adrenoreceptors attenuates this growth response.

Evidence suggests that the sympathetic nervous system and α_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, α_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 α_1ARs mediate growth of myocardial cells. In cultured neonatal rat cardiac myocytes that possess both β_1 and α_1ARs, stimulation of α_1ARs with NE increased cell protein, RNA, myocyte surface area, and contractile protein expression (19). However, no studies have examined whether α_1ARs influence proliferation-independent growth of SMCs.

Both molecular cloning and pharmacologic studies have shown that α_1ARs are comprised of three closely related subtypes (20, 21). We have recently used polymerase chain reaction (22) and RNase protection assays[2] to determine expression of αAR subtype mRNA by rat vascular SMCs. Freshly isolated and early passage cultured aortic and vena cava SMCs express both α_{1B} and α_{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 α_{1A} (formerly denoted "α_{1C}") mRNA (26–29). Given this multiplicity of α_1AR expression, the purpose of the present study was first to examine both *in vitro* and *in situ*, the effects of combined stimulation of the α_1AR subtypes with NE alone on proliferation-independent growth, and expression of sarcomeric α-SMC-actin and cytoskeletal β-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 α_{1B} stimulation and after pretreatment with chloroethylclonidine (CEC, selectivity = $\alpha_{1B} > \alpha_{1D} > \alpha_{1A}$) to select for stimulation of non-α_{1B}ARs. The results suggest, in both cultured aorta SMCs and intact aorta media, that stimulation of CEC-sensitive α_1ARs (possibly α_{1B}) induces hypertrophy of aorta SMCs and that stimulation of 5-MU-sensitive, non-α_{1B}ARs antagonizes this response. Thus, alterations in the relative activity of different α_1AR subtype signaling pathways

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[1] 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.

[2] A. E. Eckhart and J. E. Faber, submitted for publication.

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 Turla *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/cm^2 on plastic plates. Viable cell number was determined by hematocytometry 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 SMC-specific α-actin and von Willebrand factor.

Protocols—To examine the effect of combined α_1AR 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 for 8, 24, and 48 h to 1 μM 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 α_2- and βARs. Control cells in all experiments were time-matched and exposed to these agents, but not to NE (vehicle groups). In a second experiment cells were exposed to 1 μM NE and also to the α_1 antagonist prazosin (1 μM). mRNAs for SMC-specific α-actin, β-actin, α_{1D}AR, and α_{1B}ARs were measured (below). Effects of combined α_1AR 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 mg/liter transferrin, 35.2 mg/liter ascorbic acid, 6 μg/ml selenium, 100 units/ml penicillin, and 100 μg/ml streptomycin. Since responses 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^{-9} to 10^{-5} 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 $\alpha_{1A/D}$ antagonist 5-MU (0.3 μM), or 4) NE after pretreatment with the selective α_{1B} antagonist CEC (3 μM) (20, 24, 25, 29, 31). Cells were incubated with CEC for 30 min at 37 °C to alkylate and irreversibly inactivate α_{1B}ARs, followed by several media changes. During this incubation rauwolscine and propranolol were present to minimize alkylation of other ARs. CEC was not present during the subsequent 24-h exposure to NE. Concentrations of all antagonists were selected based on our previous determinations of their efficacy in *in vitro* vascular contractile studies (31, 32) and are in agreement with concentrations used by others (*cf.* Refs. 20, 25, 29, 31 and references therein).

Organ Culture—To examine the effects of α_1AR stimulation on SMC growth and mRNA expression in cells maintained "*in situ*" in a state more closely resembling the normal arterial wall environment than cultured SMC monolayers, an organ culture system was devised for the thoracic aorta with intima, media, and adventitia intact. In rat thoracic aorta the media is composed entirely of SMCs, while the adventitia is ≈ 95% fibroblasts (22, 33). For each experiment, 25-mm lengths of thoracic aortae (1 per 200 g rat) were dissected under sterile conditions from 16 rats after CO_2 asphyxiation and decapitation. Vessels were transferred to 4 °C M199, and loose connective and fatty tissue were carefully removed. Eight aortae were randomly assigned to either the control or treated group in each experiment. Individual aortae were suspended horizontally from a stainless-steel wire in serum-free medium. Circumferential wall tension was provided by a second wire connected to an adjustable weight (0.4 g/mm vessel length). This tension was selected to achieve optimal preload based on contractile studies of isolated rat aortic rings. To examine the effect of combined α_1AR subtype stimulation, in the first experiment vessels were exposed for 24 h to 10 μM NE, 10 μM ascorbic acid, 0.5 μM rauwolscine, and 1 μM propranolol in a 95% air, 5% CO_2 incubator. Control (vehicle) vessels were exposed to these agents but not NE. In a second experiment to examine the effect of endothelial cell presence and of selective α_{1B}AR stimulation on SMC and adventitial growth, both endothelial cell denuded and intact vessels (four groups; $n = 3$ vessels per group) were compared in an experimental design identical to the preceding one, but in the presence of 0.1 μM 5-MU. In these experiments a smaller number of vessels were studied, because only total RNA, protein RNA, α-actin, and vessel dry weight were measured. Endothelial cells were removed from vessels in 4 °C M199 using a nylon wire technique which avoids mechanical damage and proliferative stimulation of the underlying smooth muscle media (34, 35). We have demonstrated that this technique results in removal of greater than 90% of the endothelial cells.[2] In all experiments after 24 h, aortae were incubated in an enzyme solution (Hanks' buffered saline solution (Life Technologies, Inc.), penicillin (100 U/ml), streptomycin (10 μg/ml), 16 mM sodium bicarbonate, 1 mM calcium chloride dihydrate, pH 7.2, 2 mg/ml collagenase, 2 mg/ml soybean trypsin inhibitor, and 13.5 units/ml of elastase (Worthington)) at 37 °C with 5% CO_2, 95% air. Endothelial cells were then removed or sham-removed in the already denuded group by gently rubbing with a cotton tipped applicator, and the media and adventitia were separated with fine forceps using a dissection microscope and a 4 °C tissue bath containing M199. Media and adventitia were washed several times, frozen in liquid nitrogen, powdered, and immediately placed in guanidinium thiocyanate (see below) and stored at −70 °C until RNA extraction. Protein was determined for fresh media and adventitia as described below. Dry weights were determined for 5-mm lengths of intact vessels from each group after baking at 60 °C for 4 h.

RNA and Protein Determination—Total cellular RNA was determined (from duplicate plates or vessel extracts) using standard techniques (36). RNA integrity was assessed, and variations in aliquoted amounts in each RNase protection assay were corrected (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 media containing low methionine (2 mg/liter), 100 μM ascorbate, 1 μM propranolol, and 10^{-9} to 10^{-5} M NE (duplicate plates for each NE concentration). Eighteen hours later [^{35}S]methionine (5 μCi/ml, 1000 Ci/mmol, Amersham) was added. After 6 h, cells were washed twice with 4 °C phosphate-PBS and lifted with 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 albumen, 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 25 °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 *Nco*I/*Bam*HI fragment that encodes the third intracellular loop of the α_{1D}AR was subcloned into pGEM-4Z (Promega). The 306-bp *Bam*HI/*Pst*I fragment that encodes the fourth and fifth transmembrane-spanning regions of the α_{1B}AR was subcloned into pGEM-3Z. Identity and orientation of inserts was assessed by restriction enzyme analysis and sequencing. RNase protection assays (RPAs) were used to quantitate mRNA levels. *In vitro* transcribed [^{32}P]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 run beforehand on ethidium bromide gels for quantitation of 28 and 18 S rRNA (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 digestions at 37 °C for 30 min (α_{1D}, α_{1B}, and β-actin: RNase A (20 mg/ml, Boehringer Mannheim) + RNaseT_1 (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—Riboprobes were tested for specificity using total RNA from liver, kidney, fresh aorta medial and adventitial layers, and cultured medial SMCs and also with homologous and heterologous

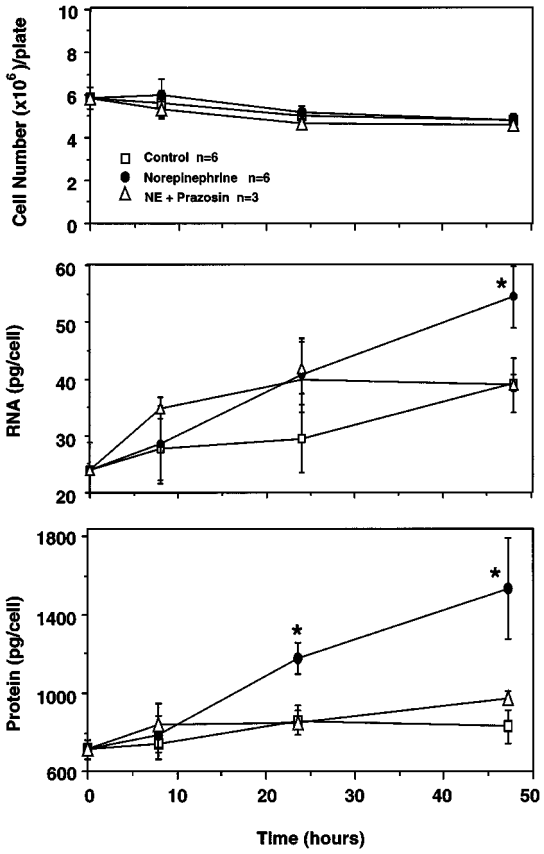

FIG. 1. **Effect of combined stimulation of α_1-adrenoreceptor subtypes on cell number, RNA, and protein in aortic SMCs.** Values in this and all subsequent figures are mean ± S.E. *Top panel*: viable cell number of 4 days post-confluent, passage 4 cells (used in this and all subsequent figures for cultured cells) was determined by hematocytometry and trypan blue exclusion. Exposure to 1 μM NE in the presence of 100 μM ascorbate, plus 0.5 μM rauwolscine and 1 μM propranolol (also used with NE in all subsequent figures) for the indicated times ± 1 μM prazosin. Control (vehicle) group exposed to ascorbate, rauwolscine, and propranolol only. n size refers to number of replicate experiments, each from a separate cell line in this and all subsequent figures for cultured cells. *Middle* and *lower panels*, total RNA and protein content per cell. *, $p < 0.05$ *versus* time-matched control cells.

sense RNAs for α_1AR subtypes and actins. In agreement with polymerase chain reaction data (22) and using up to 100 μg of RNA, the α_{1D} riboprobe only detected mRNA in the aortic medial layer, adventitia, cultured SMCs, and kidney; the α_{1B} probe only detected mRNA in the medial layer, cultured SMCs, liver, and kidney; the α-actin probe detected mRNA only in the medial layer of the aorta and cultured SMCs, and the β-actin probe detected mRNA in all of these tissues and cells. No cross-reactivity was found among riboprobes 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 aortae maintained in organ culture.

RESULTS

Effect of Combined α_1AR Subtype Stimulation on Aorta SMCs—Combined stimulation of aorta SMC α_1ARs with 1 μM NE in the presence of α_2AR 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 α_1AR stimulation significantly increased cell RNA to 140 ± 13% 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 α_1 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 α_1AR subtype stimulation was further characterized by examining α-actin and β-actin mRNA. α_1AR 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. α_{1B}AR 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, α_{1D} mRNA evidenced no significant change. In the presence of prazosin, α_{1D} and α_{1B} 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 α_{1D} was 107 ± 6, 100 ± 1, and 117 ± 20, respectively, and for α_{1B} 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 α_{1B} and α-actin mRNA.

Concentration-response experiments for per cell protein content and protein synthesis were conducted over 24 h of combined α_1AR 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 α_1AR 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 = 1.7×10^6 ± 0.2 cells/plate), total protein (0 h = 1028 ± 141 pg/cell), total RNA (0 h = 14.1 ± 2.8 pg/cell), or in α_{1B}, α_{1D}, α–actin, or β-actin mRNA levels at 8 and 24 h for any group.

Selective Stimulation of Aorta SMC α_1-Adrenoreceptor Subtypes—To determine the influence of stimulation of α_1AR subtypes on aorta SMC growth, cells maintained in serum-free medium were treated for 8 and 24 h with 10 μM NE alone (combined α_1AR stimulation), NE plus the competitive $\alpha_{1A/D}$ antagonist 5-MU (0.3 μM), or NE after pretreatment with the irreversible α_{1B} 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 α_1ARs (see below) did not change over the 24-h period, indicating stability of these SMC cultures (Figs. 4 and 5). These control groups also

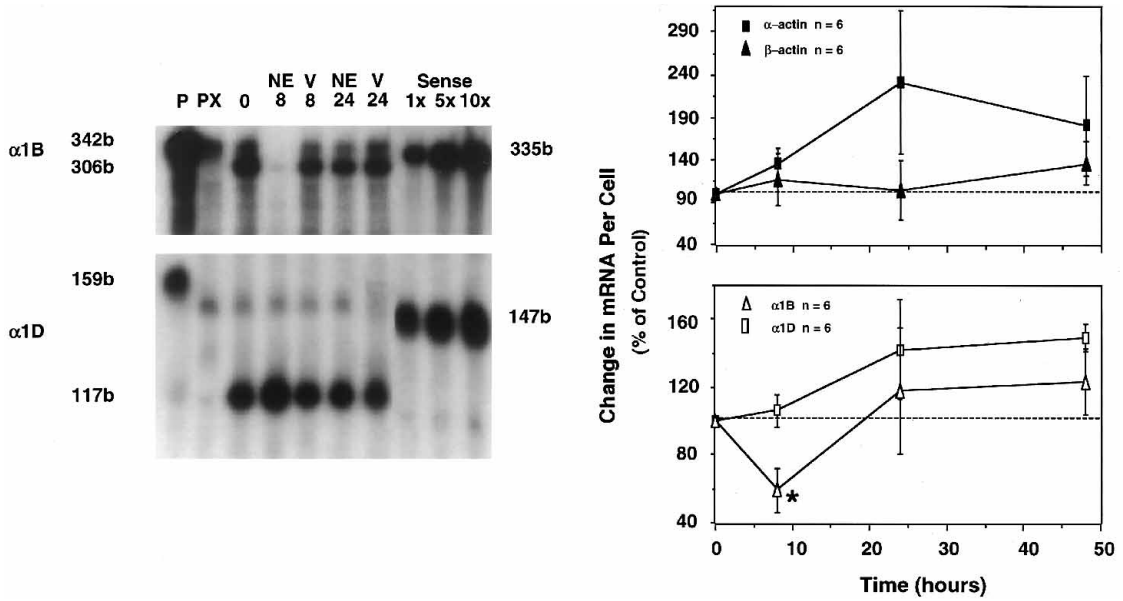

FIG. 2. **Combined stimulation of α_1-adrenoreceptor subtypes decreased α_{1B} and increased α-actin mRNA without altering α_{1D}-adrenoreceptor and β-actin mRNA in aortic SMCs.** *Left panels*, total RNA from 3×10^6 cells exposed to 8 and 24 h of NE ± vehicle (*V*; ascorbate, rauwolscine, and propranolol) was assayed for α_{1D} and α_{1B} mRNA using ribonuclease protection assay (*RPA*). *Lane 1*, probes alone (*P*); *lane 2*, probes + RNases A and T_1 (*PX*); *lane 3*, zero hour (control); *lane 4*, 8-h NE; *lane 5*, 8-h vehicle; *lane 6*, 24-h NE; *lane 7*, 24-h Vehicle; *lanes 8–10*, three amounts of α_{1D} or α_{1B} sense RNA. Total RNA (70–80 μg) was loaded in *lanes 3–7*; *lanes 2, 8, 9*, and *10* balanced to 70–80 mg of RNA with yeast tRNA. *Right panels*, effect of combined stimulation of α_1ARs with 1 μM NE on α_1AR, α-actin, and β-actin mRNA per cell as assessed by RPA. *n* size refers to number of replicate experiments. *, $p < 0.05$ *versus* time-matched control cells.

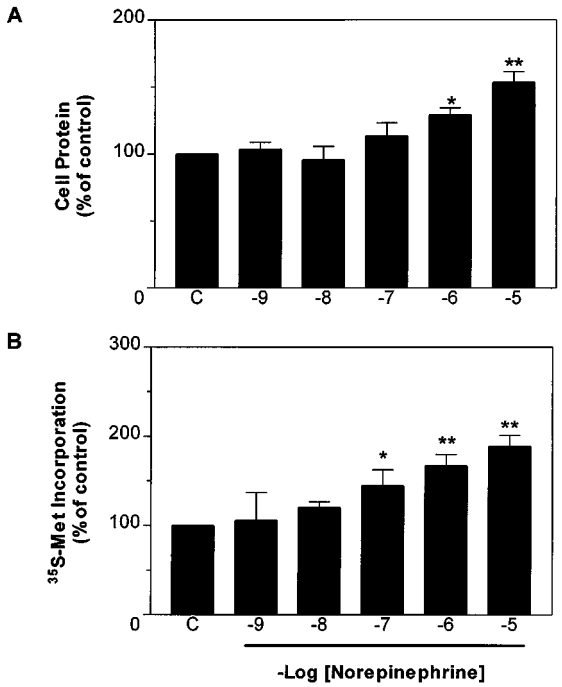

FIG. 3. **Concentration-dependent stimulation of α_1-adrenoreceptor for 24 h increased protein content (*A*) and protein synthesis ([^{35}S]methionine incorporation) (*B*).** *C*, time-matched control cells exposed to vehicle only. * (**), $p < 0.05$ (<0.01) *versus* control (analysis of variance plus Dunnett's test). NE concentrations given in −log molar. *n* sizes (replicate experiments) for all NE concentrations except 10^{-9} molar ($n = 3$) are $n = 4$ for *A* and $n = 5$ for *B*.

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 α_1ARs (NE alone) increased RNA and protein in agreement with the previous experiments. However, NE + 5-MU augmented these increases by approximately 2-fold more. In the presence of 5-MU, NE increased α-actin mRNA by 270 ± 40% 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 μM during pretreatment, $n = 5$) and 5-MU (1 μM, $n = 5$) 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 αAR stimulation induces SMC growth, while simultaneous stimulation of 5-MU-sensitive αARs attenuates this action. This conclusion is supported by analysis of α-actin and α_1AR subtype mRNAs for these experiments (Figs. 5 and 6). Stimulation with NE + 5-MU at 8 h induced a significant increase in α-actin mRNA that was almost 3-fold greater than the increase resulting from combined α_1AR stimulation (NE alone). Both increases were abolished by CEC; α-actin was actually decreased below control in this group, consistent with the growth inhibitory activity of CEC-insensitive αAR stimulation. The increases in α-actin induced by 10 μM 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 μM) NE concentration. The specificity of these effects on α-actin mRNA is indicated by the absence of change in cytoskeletal β-actin for any group (Fig. 5).

Stimulation with NE + CEC again caused a decrease in α_{1B} mRNA that was twice the magnitude exhibited by cells exposed to combined α_1AR 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 α_{1B} mRNA. Also in agreement with the first experiment (Fig. 2), α_{1D} mRNA exhibited no changes during combined stimulation and was unaffected during NE + 5-MU stimulation (Figs. 5 and 6). However, during

stimulation of non-α_{1B}ARs (NE + CEC), α_{1D} mRNA was decreased, possibly due to stimulation of this receptor without concomitant activation of α_{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 changes in α_1AR subtype mRNAs (Figs. 5 and 6), suggest that substantial selectivity of stimulation of α_{1B} and non-α_{1B}ARs was obtained with the chosen drug concentrations. Although some recovery of α_{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, α-actin mRNA and decrease in α_{1B} mRNA was still evident at 24 h (Figs. 4 and 5).

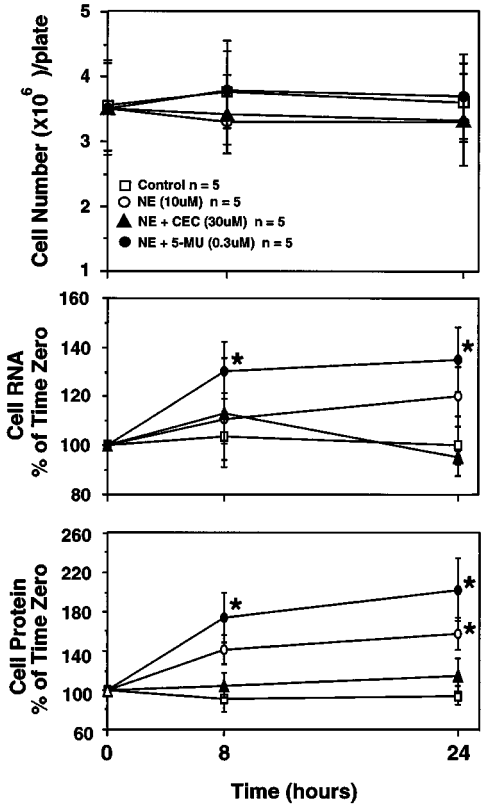

FIG. 4. **Effect of selective stimulation of α_1-adrenoreceptor subtypes on cell number, RNA, and protein in aortic SMCs.** *Top panel*, viable cell number of post-confluent cells. NE + CEC group pretreated with 30 μM CEC for 30 min prior to time 0 in the presence of rauwolscine, propranolol, and 100 μM 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, and propranolol and either NE, NE + 5-MU, or NE after CEC pretreatment. Control cells at time 0 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). n size refers to number of replicate experiments. *, $p < 0.05$ *versus* to time-matched control cells.

Stimulation of α_1ARs on Aorta SMCs in Situ—To determine if these findings for cultured aorta SMCs correctly predict responses of aorta SMCs *in situ* in the presence of endothelial cells and adventitial fibroblasts, intact aortae were maintained under wall tension in organ culture (serum-free medium) for 24 h in the presence or absence of 10 μM NE (plus ascorbate, propranolol, and rauwolscine). Adventitia and endothelium were then separated from SMC media (see "Materials and Methods") to avoid contamination of SMC and non-SMC RNA and protein. Combined α_1AR stimulation induced changes in SMC ("media") mRNAs (Fig. 7*A*) that were smaller but qualitatively similar to those obtained in the initial cell culture experiment (Fig. 2); α_{1B} mRNA decreased and α-actin increased, while α_{1D} and β-actin mRNA were unaffected (Fig. 7*A*). There was a similar lack of effect on adventitia mRNA for α_{1D} and β-actin. Riboprobe selectivity is indicated by the absence of detection of α_{1B} and vascular SMC-specific α-actin mRNA in adventitia (Fig. 7*A*), findings in agreement with our previous polymerase chain reaction studies (22).

However, unlike the response of cultured SMCs (Fig. 1), combined stimulation of α_1ARs on *in situ* SMCs caused no significant increase in tissue RNA and protein (Fig. 7*B*), indicating the absence of an α_{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 α_{1B}ARs plus inhibitory non-α_{1B}ARs on SMCs may have prevented growth. To test these possibilities, vessels were denuded of endothelium and exposed to either 10 μM NE in the presence of 0.1 μM 5-MU (favoring α_{1B} stimulation) for 24 h or 5-MU only (control group); propranolol, rauwolscine, and ascorbate were present in all four treatment groups. Only total RNA, protein, and α-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, Fig. 7, *C* and *D*). Endothelial cell removal, *per se*, in the absence of NE stimulation, had no effect on RNA or protein: RNA was 0.36 ± 0.04 mg/mm for the control group and 0.31 ± 0.1 mg/mm for the denuded group (n = 3); protein was 65 ± 18 mg/mm for controls and 50 ± 17 mg/mm for the denuded group (n = 3). α_{1B} stimulation of endothelium-intact vessels in-

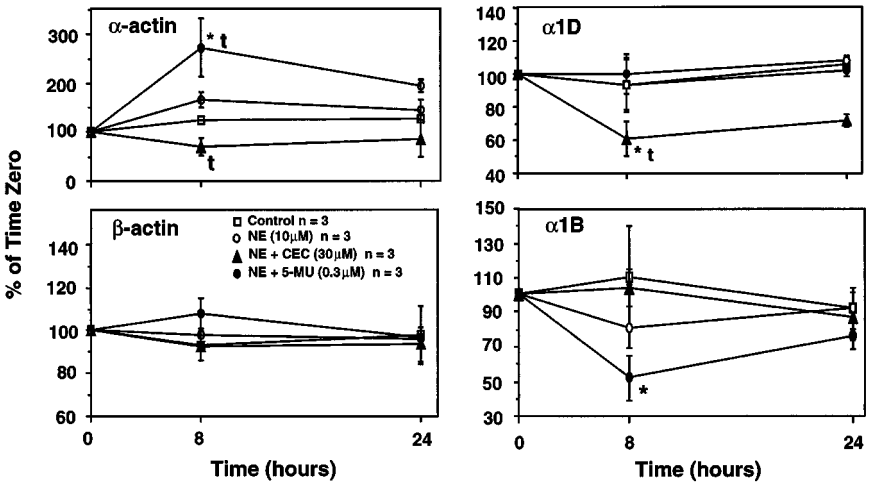

FIG. 5. **Effect of selective stimulation of α_1-adrenoreceptor subtypes on α_{1D} and α_{1B} and α-actin and β-actin mRNA in aortic SMCs.** Groups are as defined in Fig. 4 legend. *, $p < 0.05$ *versus* time-matched control cells; $^t p < 0.05$ *versus* time-matched NE alone cells. n size refers to number of replicate experiments.

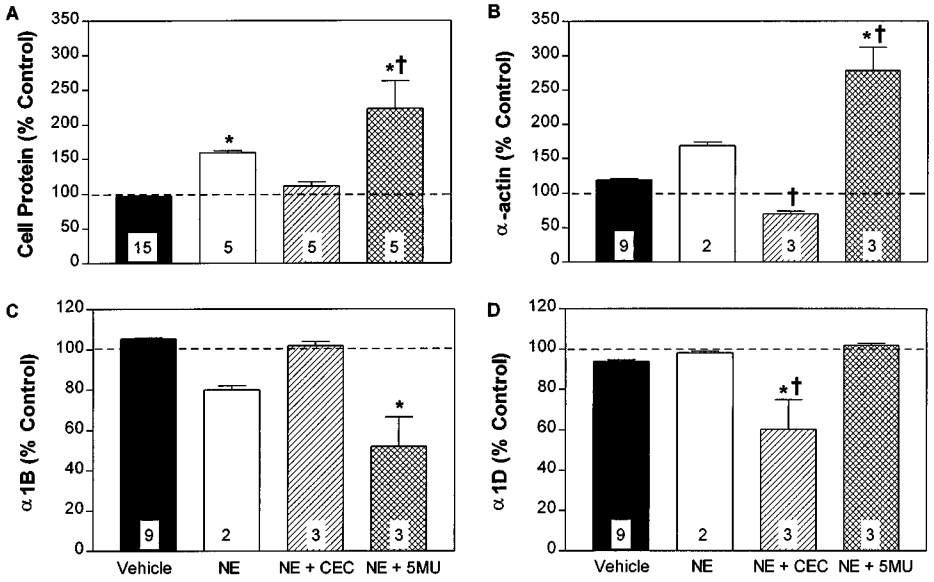

FIG. 6. **Data from Figs. 4 and 5 expressed as a percent of time-matched control (vehicle, *V*) groups.** Cell protein data is for 24 h. All other panels are for mRNA level per cell at 8 h. Groups are as defined in Fig. 4 legend. *, $p < 0.05$ *versus* vehicle; †$p < 0.05$ *versus* NE alone. *n* size refers to number of replicate experiments for each treatment group; vehicle (*V*) group is pooled data for the three control groups at 0, 8, and 24 h time points.

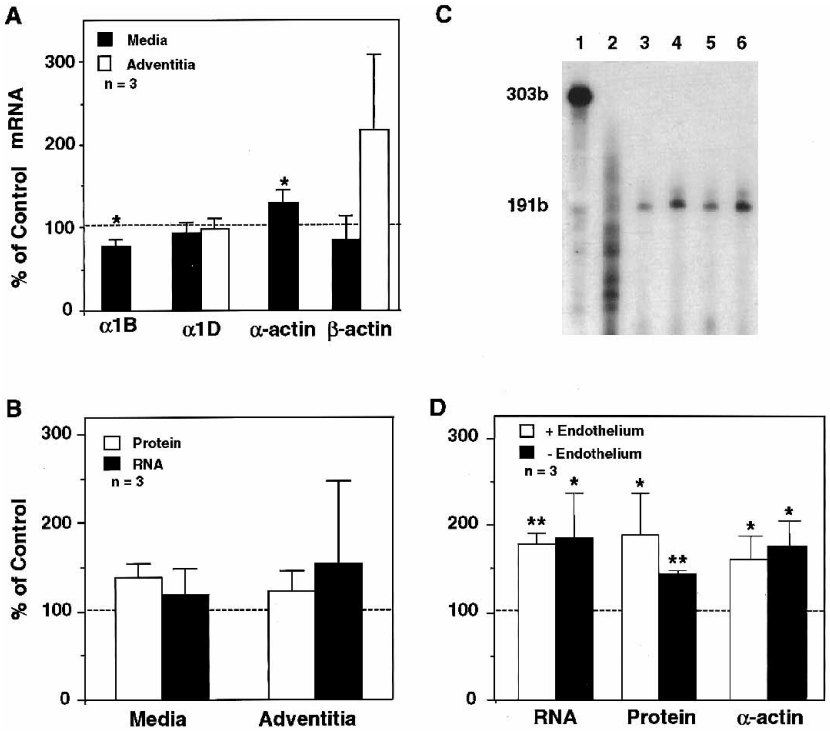

FIG. 7. **Effect of exposure of intact aortae to NE for 24 h in organ culture on RNA, protein, and α-actin, β-actin, α_{1D}, and α_{1B} mRNA levels for the aortic medial layer.** Medial layer (SMCs) and adventitia (predominantly fibroblasts) were separated and endothelial cells were removed as described under "Materials and Methods." *A*, effect of combined stimulation of α_1AR subtypes with 10 μM NE in the presence of 100 μM ascorbate, plus 0.5 μM rauwolscine and 1 μM propranolol on intact aortae in organ culture as assessed by RPA and expressed as percent change from control vessels exposed to these same agents but not NE. *B*, effect of combined stimulation of α_1AR subtypes on protein and RNA content for a constant millimeter length of vessel and expressed as percent change from control vessels. *C*, representative autoradiogram of RPA in organ culture experiments for effect of selective stimulation of α_{1B} (10 μM NE, ascorbate, rauwolscine, propranolol and 0.1 μM 5-MU) on expression of α-actin mRNA. *Lane 1*, α-actin riboprobe (303 bp); *lane 2*, α-actin probe + 5 μg of tRNA; *lane 3*, total RNA from 10 mm of time-matched control (all antagonists, no NE) aorta media (3–5 mg) of sham endothelium denuded group hybridized with α-actin probe (191-bp protected fragment); *lane 4*, 10 mm of aortic media of endothelium intact group exposed to NE (plus all antagonists); *lane 5*, 10 mm of control aortic media from endothelial denuded group; *lane 6*, 10 mm of aortic media from endothelial denuded group exposed to NE. RNase ONE was present in *lanes 2–6*. *D*, effect of selective stimulation of α_{1B} with 10 μM NE, in the presence of ascorbate, rauwolscine, and propranolol plus 0.1 μM 5-MU, on protein, RNA, and α-actin mRNA for a constant millimeter of vessel length (medial layer only) and expressed as percent change from control vessels (no NE) subjected to either endothelium removal or no removal prior to stimulation. Each *n* represents a single determination in a separate organ culture experiment. *, $p < 0.05$ (**, $p < 0.01$) *versus* control vessels.

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, α_{1B} 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, α_{1B} 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 ±

0.05 mg/mm ($p = 0.0002$) 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 α_1ARs over 24–48 h doubled the per cell amount of RNA, protein, and sarcomeric 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 α_1-mediated "hypertrophy," nor did they exhibit the decreased α_{1B}AR mRNA that was evidenced by aorta SMCs. In contrast, α_{1D} mRNA was unaffected in both cell types. Incubation of cells with CEC, which preferentially alkylates α_{1B}ARs, abolished all responses. In contrast, stimulation of non-α_{1B}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-α_{1B}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 α_1AR stimulation, preferential α_{1B} stimulation (NE plus 5-MU) increased tissue dry weight by 25% and, again, approximately doubled RNA, protein, and α-actin mRNA. And like SMCs, α_1AR stimulation in intact aorta decreased α_{1B} mRNA and had no effect on β-actin or α_{1D} mRNA. Removal of endothelial cells did not influence this SMC growth response. In addition, the fibroblast-rich adventitia, which does not express detectable α_{1B} AR mRNA, did not evidence growth. These data suggest the new concept that α_{1B}ARs mediate direct, proliferation-independent hypertrophy of SMCs and that non-α_{1B}ARs (α_{1D} and/or α_{1A}ARs) oppose this effect.

Several methodological aspects of the present study merit discussion. Since αAR number and actin proteins were not measured, additional studies are required to determine if the observed changes in mRNA reflect similar changes in receptors and proteins. However, the purpose and conclusions of the study regarding αAR regulation of SMC growth are predicated on measures of cell proliferation, total RNA and protein content, protein synthesis, and tissue weight. Selective antagonists were used to differentiate among αAR subtypes. The relative affinity of CEC is $\alpha_{1B} > \alpha_{1D} > \alpha_{1A}$ and for 5-MU is $\alpha_{1A} > \alpha_{1D} > \alpha_{1B}$ (20, 25, 26, 28, 29, 31). In the study herein, pretreatment with 30 μM CEC abolished the increases in cell protein, RNA, α-actin mRNA and decrease in α_{1B} mRNA induced by α_1AR stimulation with NE. 5-MU (0.3 mM) augmented the NE-induced hypertrophy and abolished the decrease in α_{1D} mRNA induced by NE + CEC. In each experiment a 3-fold higher concentration of CEC or 5-MU yielded results that were not significantly different from those obtained at the lower concentrations. While the growth-promoting affects we observed can best be ascribed to α_{1B}AR stimulation, the identification of the α_1 subtype(s) that acts to restrain α_{1B} growth remains unclear, since recent reports suggest the rat aorta SMCs may also express α_{1A}ARs (26–28). However, the selectivities of 5-MU and CEC are not sufficient to assure absence of some antagonism of non-α_{1B}ARs by CEC and α_{1B}ARs by 5-MU, and additional experiments are required as more selective antagonists become available.

Recent findings of others are congruent with several of the observations reported herein. The finding that α_{1B} mRNA was decreased by 40–50% at 8 h but returned to control after 24 h of stimulation with 1–10 μM NE has also been reported for rabbit aorta SMCs (39). In that study of the concentration-response relationship for α_{1B} down-regulation, a similar (80%) reduction in α_{1B} mRNA was obtained with 10 μM NE at 4 h of exposure. This occurred even though α_2 and β-ARs were not blocked, in contrast to the present study. These changes were followed by a sustained 60% decrease in α_1AR density at 24 h, despite return of α_{1B} mRNA levels to control. This transient decrease in α_{1B} mRNA and sustained down-regulation of α_1ARs appears to involve protein kinase C (40, 41). Similar findings have been reported for the DDT_1-MF-2 smooth muscle cell line, where stimulation of α_{1B}ARs that are coupled to phospholipase C decreased α_{1B} mRNA by 50% at 48 h, by reducing gene transcription but not mRNA stability (42). In addition, Okazaki *et al.* (43) reported that stimulation of α_1ARs induced transient expression of c-*fos*, c-*jun*, and c-*myc* in cultured rat aorta SMCs (and in intact aorta media for c-*jun*) that was completely blocked by CEC. Our finding that α_1AR stimulation induces hypertrophy of rat aorta SMCs is supported by a recent preliminary report in confluent, quiescent rabbit aorta SMCs (44). Over a time course similar to the present study, phenylephrine dose-dependently ($EC_{50} = 0.2$ μM) increased 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 α_1AR stimulation. There are several possibilities that could underlie this difference. The relative density of α_1AR 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 α_1AR 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 oncogene 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 cholesterol-fed monkeys (17) induces new and greatly exacerbates existing lesion growth, while the α_1 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 α_1 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 αAR subtypes underlying these relationships were not examined, it is interesting in light of our

data suggesting a trophic role for the α_{1B}AR, that the reliance on α_{1B}ARs (*i.e.* CEC-sensitive) for aortic contraction in young, rapidly growing rats is replaced by CEC-insensitive α_1AR dominance in adults (24).

α_1AR 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. α_1AR blockade with prazosin abolished the response, while α_2 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, α_1AR 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 α_1ARs are present in approximately 2-fold greater abundance (59). Proximal aspects of the signaling pathway include activation of G_q, protein kinase C, and the *ras* protooncogene (59). Thus, besides short term regulation of inotropy and glycogenolysis, CEC-insensitive αARs 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 α_{1B}AR (61). Furthermore, the possibility that α_1ARs 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 α_1AR subtypes are undoubtedly central in their capacity to modulate SMC growth differently. In several cell lines and in rat thoracic aorta, CEC-sensitive α_1ARs couple to phosphoinositide (PI) metabolism and protein kinase C, while CEC-insensitive, WB4101-sensitive α_1ARs 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 α_{1B}ARs into fibroblasts confers strong NE-induced α_1-mediated growth (65). Moreover, transfection of mutant α_{1B} receptors that tonically elevate PI metabolism even in the absence of NE are oncogenic in these cells (65). It is interesting that in myocardial cells CEC-insensitive α_1ARs are coupled to PI metabolism and promote growth, while the non-PI-coupled, CEC-sensitive α_1ARs do not exhibit this capacity (23, 57, 59).

In summary, the present study demonstrated that stimulation of CEC-sensitive, possibly α_{1B}ARs, promoted growth of aorta SMCs regardless of whether they were maintained in cell or organ culture. Stimulation of 5-MU-sensitive α_1ARs (presumably α_{1D} and/or α_{1A}) 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 α_1AR signaling pathways.

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