

α_{1D} -Adrenergic Receptors and Mitogen-Activated Protein Kinase Mediate Increased Protein Synthesis by Arterial Smooth Muscle

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SUMMARY

Catecholamines may influence vascular smooth muscle cell (SMC) growth and vascular hypertrophic diseases. We previously demonstrated that stimulation of α_1 -adrenoceptors (AR) causes hypertrophy of vascular SMCs *in vitro* and *in situ*. Here, we used adult rat aorta SMCs that express α_{1D} - and α_{1B} -ARs (but not α_{1A} -ARs) *in vitro* to examine the mechanisms and α_1 -AR subtypes involved. Norepinephrine (NE) increased protein synthesis and content in a time- and dose-dependent manner. To identify the responsible α_1 -AR subtype, we first documented the selectivity of two α_1 -AR subtype antagonists, BMY 7378 (α_{1D} -AR antagonist) and chloroethylclonidine (CEC; α_{1B} -AR antagonist), using Rat-1 fibroblasts stably transfected with the three different rodent α_1 -AR cDNAs. NE dose-dependently increased protein synthesis in each cell line. In α_{1D} fibroblasts, BMY 7378 inhibited growth and protected α_{1D} -ARs

from CEC alkylation while having little blocking or protecting effect on the growth induced by stimulation of fibroblasts that express α_{1A} - or α_{1B} -ARs. In rat aorta SMCs, pretreatment with CEC in the presence of BMY 7378 to protect α_{1D} -ARs had no effect on NE-induced protein synthesis. BMY 7378 inhibited the SMC growth response with a pK_b of 8.4. NE caused rapid and transient p42-p44 mitogen-activated protein kinase (MAPK) activation that was α_{1D} -AR dependent. Furthermore, NE caused tyrosine phosphorylation of multiple cellular proteins, phosphorylation of Raf-1, and stimulation of *c-fos* mRNA expression in aorta SMCs. The selective MAPK kinase inhibitor PD 98059 inhibited NE-induced protein synthesis and MAPK activation with IC_{50} values of 2.3 and 1.6 μ M, respectively. These data demonstrate that SMC growth induced by NE is mediated by α_{1D} -ARs that couple to activation of the MAPK cascade.

Augmented SMC growth and matrix secretion are key events underlying stenosis after angioplasty and vascular grafting, accelerated atherosclerosis after organ transplantation, and hypertensive wall hypertrophy, as well as normal vessel growth and angiogenesis (1). Thus, the molecular mechanisms regulating SMC growth are under intensive investigation. Accumulating evidence suggests that catecholamines can influence vascular SMC growth. For example, sympathetic denervation decreases medial SMC proliferation during *in vivo* vasculogenesis (2-5). Catecholamine stimulation induces proliferation in subconfluent SMCs in culture (Ref. 6 and references therein) and hypertrophy without hyperplasia in confluent quiescent SMCs (7, 8). This hypertrophy is also evident in SMCs studied *ex vivo* in the intact, pressurized aorta (7) and during chronic NE infusion (9). In addition, elevated plasma NE greatly in-

creases atherosclerotic lesion growth in cholesterol-fed monkeys (10). Also, α_1 -AR blockade reduces aortic intimal hyperplasia induced by balloon angioplasty (11). There also is evidence that α_1 -AR stimulation mediates hypertrophy of cardiac myocytes (12). The hypertrophic response evoked by α_1 -AR stimulation involves increases in cellular protein synthesis and content, RNA content, α -SMC-actin expression (7), and induction of proto-oncogenes such as *c-fos* (13) in vascular SMCs. Catecholamine regulation of SMC growth may provide a physiological means by which vessel wall mass can be coupled to the level of sympathetic stimulation and contractile requirements, and it may also link vessel wall pathological growth processes to enhanced sympathetic activity.

Although it is clear that catecholamine-induced hypertrophy of vascular SMCs is mediated by α_1 -ARs, the specific receptor subtype has not been identified. Both molecular and pharmacological studies have shown that α_1 -ARs are com-

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ABBREVIATIONS: SMC, smooth muscle cell; AR, adrenoceptor; FB, fibroblast; NE, norepinephrine; CEC, chloroethylclonidine; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; SSC, standard saline citrate; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; bp, base pair(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ANOVA, analysis of variance; VC, vena cava; EGF, epidermal growth factor; PKC, protein kinase C; RPA, RNase protection assay; 5-MU, 5-methyl-urapidil; ERK, extracellular signal-regulated kinase; PDGF, platelet-derived growth factor.

posed of three subtypes: α_{1A} , α_{1B} , and α_{1D} . The rat thoracic aorta, which we have used as a model of SMC hypertrophy (7), expresses transcripts and receptors for α_{1B} - and α_{1D} -ARs in intact vessel media and primary SMC culture (7, 14). α_{1A} -AR mRNA is present in much lower levels in media and is undetectable by RPA when these cells are maintained in early passage cell culture.¹ This indicates that at least in rat aorta cultured SMCs, hypertrophy is mediated by α_{1B} - or α_{1D} -ARs or both. Several α_{1D} -AR antagonists with good selectivity (~ 100 -fold) have been recently described, such as BMY 7378 (order of selectivity: $\alpha_{1D} \gg \alpha_{1A} > \alpha_{1B}$) (15). However, other than the irreversible alkylating antagonist, CEC, which has a 5–10-fold higher α_{1B} -AR selectivity (order of potency: $\alpha_{1B} > \alpha_{1A} > \alpha_{1D}$), no selective α_{1B} -AR antagonists are available (16). Recently, CEC was shown to block α_1 -AR increases in SMC hypertrophy (7), hyperplasia (17), and *c-fos* expression (13), leading the authors to suggest an α_{1B} -AR involvement. However, the low selectivity of CEC requires development of strategies and agents not used in these studies to provide a definitive identification of the responsible subtype.

In addition to identification of the α -AR subtype signaling SMC hypertrophy, postreceptor mechanisms coupling α_1 -AR stimulation to increased protein synthesis in these cells have not been examined. MAPK, also known as ERK, and associated upstream effectors and downstream targets of MAPK can be activated by numerous growth factors and G protein-coupled receptors (18, 19). This cascade is a major signal transduction pathway regulating cell growth and differentiation (19). Agonist-evoked phosphorylation of MAPK at both tyrosine and threonine residues within a conserved TEY motif on the enzyme is believed to cause MAPK to translocate to the nucleus, where it initiates increased protein synthesis by phosphorylation of p90^{rsk}, p60^{TCF}, and other proteins involved in the regulation of cell growth (20). For example, recent evidence indicates that phosphorylation of MAPK is required for transactivation of *c-fos*, atrial natriuretic peptide, and myosin light chain-2 promoters in phenylephrine-induced hypertrophy of cardiac myocytes (21). However, the intracellular signals that couple α_1 -AR activation to vascular SMC growth are unclear.

One purpose of the current study was to determine which α_1 -AR subtype mediates increased SMC protein synthesis. To this end, we developed a "protection from alkylation" strategy using CEC and BMY 7378 to distinguish between α_{1B} - and α_{1D} -ARs. Second, we sought to examine whether stimulation of the responsible subtype is coupled to activation of the MAPK cascade. For both purposes, we used a well-characterized rat aorta cell culture model of SMC hypertrophy (7). Our findings demonstrate that NE-stimulated SMC protein synthesis is mediated by the α_{1D} -AR. We also find that this hypertrophic response requires MEK activation and includes tyrosine phosphorylation of multiple SMC proteins, increased phosphorylation of Raf-1 kinase, rapid and transient activation of MAPK, and induction of *c-fos* *trans*-activating proto-oncogene transcription factor.

Experimental Procedures

Cell culture. The methods for culture of rat thoracic aorta SMCs, derived from 200-g male Sprague-Dawley rats, have been described in detail (7). Rat-1 FBs stably transfected with full-length α -AR

subtype cDNAs were grown in Dulbecco's modified Eagle's medium with high glucose, 10% fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 250 μ g/ml G-418 in a humidified atmosphere containing 5% CO₂/95% air. FBs that express either α_{1D} - or α_{1B} -ARs were obtained from D. A. Schwinn (Duke University, Durham, NC), and FBs that express α_{1A} -ARs were provided by A. S. Goetz and D. L. Saussy (Glaxo-Wellcome, Research Triangle Park, NC). These transfected FBs stably express similar densities (1–3 pM/mg of protein) of α_1 -ARs. Before experiments, SMCs and FBs were growth-arrested for 48 hr in serum-free, defined media 1–2 days after reaching confluence. This media consisted of 50% Dulbecco's modified Eagle's medium, 50% F-12 media supplemented with 2.85 mg/liter insulin, 5 mg/liter transferrin, 35.2 mg/liter ascorbic acid, 6 μ g/ml selenium, 100 units/ml penicillin, and 100 μ g/ml streptomycin. SMCs were seeded at 3000–5000/cm² and used at passages 4–6. For experiments with Western and Northern blot assays (see below), SMCs were grown to 85–95% confluence and growth arrested for 48 hr. Viable cell number was determined in duplicate by hemacytometry with trypan blue exclusion.

Protein synthesis and content. [³⁵S]Methionine incorporation and BCA (Pierce Chemical, Rockford, IL) assays, performed in duplicate, were used to examine protein synthesis and protein content, respectively. Growth-arrested SMCs received a change of media containing low methionine (2 mg/liter), 100 μ M ascorbate, 1 μ M propranolol, and various concentrations of NE in the presence or absence of α_1 -AR antagonists. At 4–6 hr before cell harvest, [³⁵S]methionine (1 μ Ci/ml, 1000 Ci/mmol; Amersham, Arlington Heights, IL) was added. Cells were washed twice with 4° PBS and lifted with 0.05% trypsin-EDTA, which was then stopped with serum-containing Medium 199. Pelleted cells were lysed with Nonidet P-40. The supernatant was treated with TCA at a final concentration of 10% in the presence of 100 μ g/ml bovine serum albumen and incubated for 30 min at 4°. TCA-precipitable counts were collected on GF/C filters (Whatman, Clifton, NJ) and counted in Ecoscint H (National Diagnostics, Atlanta, GA). SMCs were pretreated with antagonists for 30 min before the addition of NE (except for specific α -AR antagonist protocol; see below). Total soluble protein was determined using a modified BCA assay (7).

α -AR antagonist protocols. α_1 -AR stimulation was achieved with NE in the presence of 100 μ M ascorbate and 1 μ M propranolol. The blockade of α_2 -ARs was not included because preliminary experiments showed that increases in protein content were similar in the absence of rauwolscine and in our previous study (7). A recent study also reported an absence of effect of α_2 - or β -AR blockade or stimulation on SMC catecholamine hypertrophy (8). In all experiments, time-matched control cells were exposed to vehicle but not to NE. Cells were incubated with competitive antagonists (or vehicles) 30 min before agonist addition or in experiments in which protection of α_{1D} -ARs from CEC alkylation was desired. CEC treatment consisted of incubation of 30 μ M CEC for 30 min at 37°, followed by three washes with warmed PBS and replacement with fresh media.

The apparent equilibrium dissociation constant (K_b) for the α_{1D} -AR antagonist BMY 7378 was determined for NE-induced protein synthesis during 24 hr of exposure to various NE concentrations. The pK_b for BMY 7378 (expressed as $pK_b = -\log K_b$) was obtained according to the equation $K_b = [B]/(\text{concentration ratio} - 1)$, where [B] is the concentration of BMY 7378 (0.3 μ M), and the concentration ratio was derived from the ratio of the NE EC₅₀ values in the absence and presence of BMY 7378.

RPA. Total cellular RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform and standard techniques (7). A 179-bp fragment corresponding to the third intracellular loop of rat α_{1A} -AR cDNA (667–846 bp) was synthesized by PCR with two primers (5'-gaagatgcc GCC AAG AGA GAA AGC CGG-3' and 5'-gggaattcc GCT TTC TTC TCT CGA GAA-3'). The PCR product was subcloned into *Bam*HI/*Eco*RI sites of pBluescript SK⁺ vector. Identity and orienta-

¹ J. E. Faber, X. Xin, N. Yang, and A. D. Eckhart, unpublished observations.

tion of inserts were assessed by restriction enzyme analysis and sequencing. Plasmid DNA containing the fragment was linearized with *Xba*I. *In vitro* transcribed [α - 32 P]CTP-labeled cRNAs were produced by standard techniques with T7 RNA polymerase. The cyclophilin gene, which is ubiquitously expressed in mammalian cells and unresponsive to all stimuli we have examined (22), was used as an internal control for the α_{1A} -AR RPA. A 165-bp linearized fragment of rat cyclophilin cDNA (Ambion, Austin, TX) was transcribed *in vitro* with T7 RNA polymerase. The subsequent RPA protocol was performed as previously described in detail (7). After hybridization and digestion, the hybridized 179-bp fragment of the α_{1A} mRNA and a 103-bp fragment of the cyclophilin mRNA were protected and resolved on an 8 M urea, 6% polyacrylamide gel. Dried gels were exposed to film (Kodak X-OMAT) with intensifying screens at -70° for 12–72 hr.

Western blot analysis. Growth-arrested SMCs treated with α_1 -AR antagonists (same treatment protocol as for protein synthesis measurements) were incubated with or without NE for various times. After incubation, cells were washed twice with ice-cold PBS and lysed in 250 μ l of modified RIPA buffer consisting of 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 50 mM HEPES, pH 7.5, 1.0 mM sodium orthovanadate, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin. Insoluble material was removed by centrifugation (14,000 rpm for 15 min at 4°), and protein concentration was determined using a modified BCA assay. For gel mobility shift assay of MAPK and Raf-1 or immunoblot analysis of tyrosine-phosphorylated proteins, 30–100 μ g of cell lysate protein was electrophoresed in 10–15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked for 1 hr at 25° in Tris-buffered saline/Tween 20 containing 5% nonfat dry milk and probed with anti-MAPK rabbit polyclonal antibody (SC-94, Santa Cruz Biochemicals, Santa Cruz, CA), polyclonal or monoclonal Raf-1 antibodies (C-12, Santa Cruz; and R19120, Transduction Laboratories, Lexington, KY, respectively), or monoclonal antiphosphotyrosine antibody (PY-20, Transduction Laboratories). The immunocomplex was detected using horseradish peroxidase-conjugated protein A visualized by the ECL system (Amersham) after intensive washing of the membranes.

Immunocomplex *in vitro* MAPK assay. Cell lysate protein (300 μ g; obtained as described above) was incubated with 10 μ l each of either ERK-1 (C-16) and ERK-2 (C-14) antiserum (Santa Cruz) and 20 μ l of 50% (w/v) protein A-Sepharose beads for 4 hr at 4° . The immunoprecipitates were washed three times with lysis buffer and once with MAPK assay buffer (50 mM β -glycerophosphate, 1.5 mM EGTA, 0.1 mM sodium orthovanadate, 1.0 mM dithiothreitol, 10 μ M calmidazolium, 10 mM $MgCl_2$, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1.0 mM benzamide). MAPK activity was assayed by resuspending the beads in a total volume of 50 μ l of MAPK assay buffer containing 0.5 mg/ml myelin basic protein, 50 μ M ATP, and 3 μ Ci of [γ - 32 P]ATP (6000 Ci/mmol). Reactions were initiated with the addition of ATP, incubated at 30° for 20 min, and stopped by the addition of cold 20% TCA. Samples (50 μ l) were spotted onto P-81 phosphocellulose filters (Whatman) and washed in 0.5% phosphoric acid four times. The filters were then dried, and the radioactivity that was incorporated into myelin basic protein was determined by liquid scintillation counting.

Northern blot analysis. Equal amounts of total RNA (10 μ g) were denatured and resolved by electrophoresis in a 1.2% agarose gel containing 1.8% formaldehyde. RNA was transferred to Hybond N (Amersham) nylon membrane. The 1.2-kb *Sst*I/*Eco*RI fragment of mouse *c-fos* cDNA and a full-length rat GAPDH cDNA probe (GIBCO BRL, Gaithersburg, MD) were labeled with [α - 32 P]dCTP using a random labeling method according to the manufacturer's instructions. After UV cross-linking of the RNA, the blot was prehybridized in buffer [$5\times$ SSC ($1\times$ SSC = 150 mM NaCl, 15 mM sodium citrate), 0.1% SDS, $5\times$ Denhardt's solution ($1\times$ Denhardt's = 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumen), 5% formamide, and 50 μ g/ml herring sperm DNA] at 42° for 4 hr and

then hybridized in the same buffer with probe (2×10^6 cpm/ml) overnight at 42° . The blot was washed with $2\times$ SSC/0.2% SDS twice at room temperature for 10 min each time and exposed to X-ray film (Kodak X-OMAT; Eastman Kodak, Rochester, NY) with an intensifying screen at -70° for 8–24 hr.

Statistical analysis. Data are given as mean \pm standard error. Differences were analyzed by *t* test and ANOVA, followed by the Bonferroni correction or Dunnett's test for multiple comparisons. Nonlinear regression analysis (InStat, GraphPAD Software, San Diego, CA) was used to determine the pK_b for BMY 7378 and half-maximal inhibitory concentrations for PD 98059. A value of $p < 0.05$ was considered significant. The values for *n* given in figure legends denote the number of independent experiments conducted on different cell lines and/or cell passages.

Results

Prolonged Activation of SMC α_1 -ARs Increases Both Protein Synthesis and Protein Content

We previously reported that α_1 -AR stimulation with NE for 24 hr increases the cellular content of α -actin mRNA and increases per-cell protein synthesis and content in a dose-dependent (0.01–1 μ M) manner for aorta SMCs and intact aorta wall (7). To examine the time dependence of this hypertrophy, growth-arrested SMCs were treated with 1 μ M NE for 8, 12, 24, and 48 hr. As shown in Fig. 1, NE induced a progressive increase in protein synthesis, with a peak at 24 hr (170% of control) and then a decline to 130% at 48 hr. However, protein content per cell continued to increase (125% at 24 hr and 170% at 48 hr). In all NE-treated groups, cell number was not changed, and time-matched vehicle-treated groups demonstrated no change in protein synthesis, content, or cell number (data not shown). These data for protein content at 24 and 48 hr points and protein synthesis

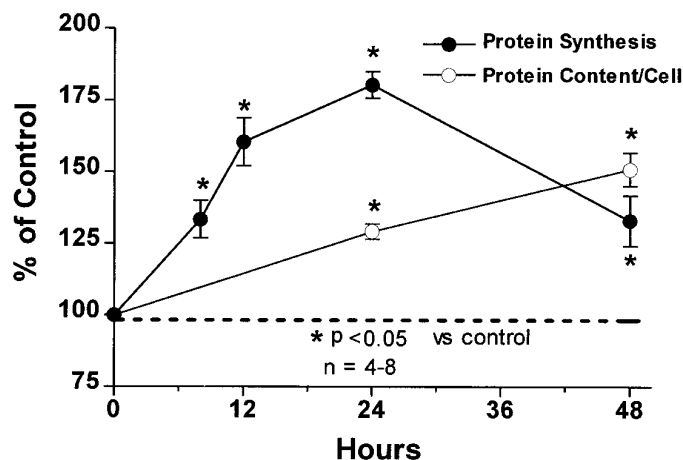


Fig. 1. Prolonged stimulation of α_1 -ARs increases protein synthesis and protein content per cell in a time-dependent manner. Aorta SMCs were exposed to 1 μ M NE in the presence of 100 μ M ascorbate and 1 μ M propranolol (latter two agents were also used with NE in all subsequent figures) for the indicated times. Control group consisted of time-matched cells exposed to ascorbate and propranolol alone. ●, Protein synthesis. Growth-arrested SMCs cultured in six-well plates received a change to low methionine media (2 mg/ml) containing 1 μ M NE (duplicate plates for each experiment). Cells were pulsed with [35 S]methionine (2 μ Ci/well) 6 hr before harvest. [35 S]Methionine incorporation was measured as TCA-precipitable counts/min. ○, Protein content/cell. Total soluble protein was determined by BCA assay. Results in this and all subsequent figures are expressed as mean \pm standard error. *, $p < 0.05$ versus control cells by Dunnett's test. *n* denotes independent experiments.

at 24 hr are virtually identical to values we obtained previously in the presence of 0.5 μ M rauwolscine (7), demonstrating an absence of α_2 -AR involvement. In additional experiments ($n = 4$, data not shown) in which cells were exposed to 10 μ M NE, protein content per cell at 24 and 48 hr was 153 ± 8 and 143 ± 5 , respectively, suggesting that together with the data of Fig. 1, maximal hypertrophy is obtained at ~ 1 μ M NE for 48 hr of exposure.

Characterization of α_1 -AR Subtype Mediating SMC Hypertrophy

Selective alkylation of α_{1B} -ARs and protection of α_{1D} -ARs with CEC in the presence of BMY 7378. The irreversible antagonist CEC is only 5–10-fold selective for α_{1B} over the other α_1 -ARs. This selectivity is critically dependent on CEC concentration, incubation time, and temperature; increasing any of these factors results in progressive alkylation of α_{1A} - and α_{1D} -ARs (23). Therefore, results (7, 13, 17) obtained with CEC alone to differentiate among α_1 -ARs may be inconclusive. We developed a “protection from alkylation” approach using CEC and the highly selective, competitive α_{1D} -AR antagonist BMY 7378 (15) to identify which α_1 -AR mediates SMC growth. To document the selectivity of BMY 7378 against α_1 -AR-mediated growth, we first determined its potency against NE-induced protein synthesis in Rat-1 FBs stably transfected with one of the three different α_1 -AR subtype cDNAs. In other experiments, we have used RPAs to confirm that only one of the cloned α_1 -AR subtypes is expressed in each FB cell line. NE (24 hr) dose-dependently increased protein synthesis to similar degrees in all three FB cell lines that express different α_1 -AR subtypes (Fig. 2A). The inhibitory effects of BMY 7378 on NE-induced (1 μ M, 24 hr) protein synthesis demonstrated the expected selectivity. As shown in Fig. 2B, BMY 7378 inhibited protein synthesis by α_{1D} FBs in a dose-dependent pattern but had little effect against α_{1A} or α_{1B} FBs.

After establishing the selectivity of BMY 7378 against NE-induced protein synthesis, we then tested BMY 7378 for protection of α_{1D} -ARs from CEC alkylation in an effort to increase the selectivity of CEC for α_{1B} -AR inhibition. FBs were first incubated at 37° with 0.3 μ M BMY 7378 for 30 min. Preliminary experiments with different concentrations of BMY 7378 identified that 0.3 μ M afforded optimal selective protection from CEC. After extensive washing, cells were allowed to equilibrate at 37° for 30 min and then incubated with 1 μ M NE for 24 hr. Fig. 2C demonstrates that BMY 7378 dose-dependently protected FBs that express α_{1D} -ARs from CEC alkylation, as demonstrated by BMY 7378 protection of NE-mediated growth. In comparison, BMY 7378 afforded little or no protection of α_{1A} - or α_{1B} -AR FBs from inhibition by CEC. Incubation of all three FB cell lines with CEC alone (30 μ M, 30 min) caused complete inhibition of NE-induced increase in protein synthesis. NE (1 μ M, 24 hr) alone increased protein synthesis in α_{1A} -, α_{1B} -, and α_{1D} -AR FBs by $144 \pm 10\%$, $152 \pm 10\%$, and $139 \pm 3\%$ of control, respectively, in the absence of CEC and by $108 \pm 3\%$, $100 \pm 5\%$, and $98 \pm 6\%$ after treatment with CEC (two or three independent experiments). These data demonstrate the absence of selectivity of CEC under the conditions that were used.

The above scheme for distinguishing among the α_{1D} - and α_{1A}/α_{1B} -ARs was then used to examine NE-induced protein synthesis in SMCs. To minimize BMY 7378 binding to α_{1B} -

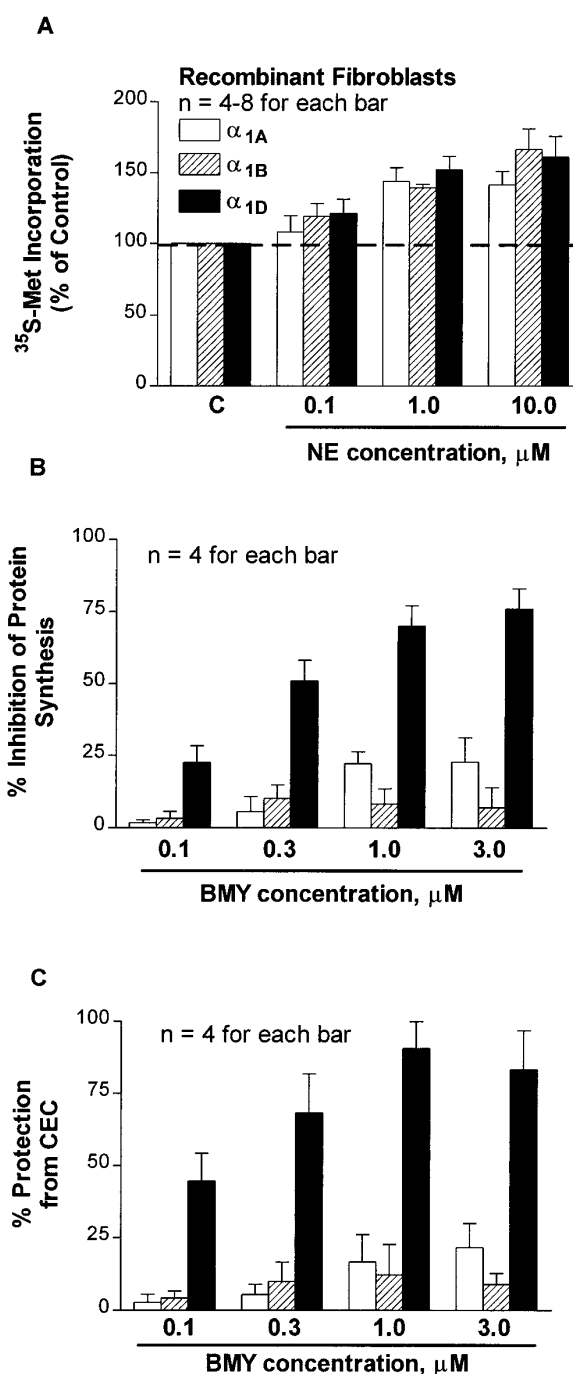


Fig. 2. Use of stably transfected FBs that express different α_1 -AR subtypes to characterize the selectivity of BMY 7378 to antagonize α_{1D} -AR-mediated protein synthesis and to protect α_{1D} -ARs from alkylation by CEC treatment. Growth-arrested FBs were treated with NE or vehicle (propranolol and ascorbate, control cells) for 24 hr in the absence or presence of α_1 -AR antagonists. Protein synthesis was measured as described in Experimental Procedures and legend to Fig. 1. A, Data normalized to time-matched control cells. NE (0.1–10 μ M) dose-dependently increased protein synthesis by each FB cell line. B, BMY 7378 (0.1–3 μ M) selectively inhibited NE-stimulated (1 μ M) protein synthesis by α_{1D} -AR FBs. Responses were normalized as (1 – maximal response with 1 μ M NE). C, BMY 7378 selectively protected α_{1D} -AR FBs from CEC inhibition of NE-stimulated (1 μ M) protein synthesis. FBs were pretreated with BMY 7378 for 30 min, followed by the addition of CEC (30 μ M) at 37° for 30 min. Media were then changed three times and replaced with fresh media containing 1 μ M NE for 24 hr. Responses were normalized as percentage of maximal response with 1 μ M NE. n denotes independent experiments.

ARs in the subsequent SMC studies and because of the lower total α_1 -AR expression in these cells, we used a 10-fold lower BMY 7378 concentration (0.3 μM) that also gave the best selectivity ratios in FBs (see Discussion). As illustrated in Fig. 3, the 160% increase in SMC protein synthesis induced by 1 μM NE at 24 hr (second bar) was completely blocked by 0.3 μM BMY 7378 (fourth bar). Furthermore, use of the same protocol for 0.3 μM BMY 7378 protection from CEC alkylation as in Fig. 2 yielded a similar 60–70% protection of SMCs against CEC inhibition (third bar). Exposure to CEC alone for 30 min, without BMY 7378 protection of α_{1D} -ARs, completely blocked the NE increase in protein synthesis (fifth bar). BMY 7378 and CEC, in the absence of NE stimulation, had no effect themselves on basal protein synthesis (sixth bar). We also used this protocol to examine the effect of BMY 7378 on protein synthesis induced by 1 μM NE exposure for 48 hr and found the same complete inhibition by BMY 7378 of protein synthesis and 80% protection against CEC alkylation (two independent experiments; data not shown).

In other experiments using FBs and SMCs and the above protocols, 5-MU was also tested (0.03, 0.3, and 1 μM). The concentration of 0.3 μM 5-MU inhibited (by 50%) NE-induced protein synthesis by α_{1A} -expressing FBs while having no effect on α_{1B} - or α_{1D} -FBs ($p < 0.05$); the lower concentration was ineffective, and the higher concentration was nonselective. Moreover, 5-MU at these concentrations was ineffective in CEC protection experiments using FBs and yielded equivocal results in SMC protocols, which is consistent with its low selectivity between α_{1B} - and α_{1D} -ARs.

We also examined responses of passage 4–6 rat VC SMCs (five experiments from different cell lines or passages) because in our previous study (7), we saw no effect at 24 hr of

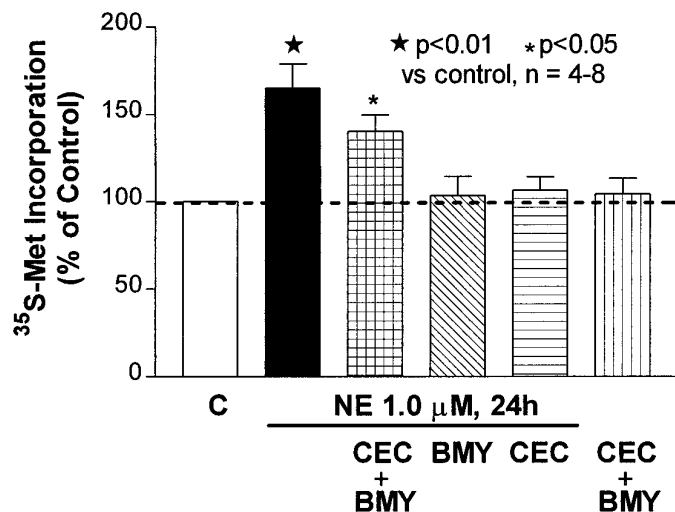


Fig. 3. BMY 7378 inhibits α_1 -AR-mediated protein synthesis of aorta SMCs and protects against CEC inhibition of the response. SMCs were treated with BMY 7378 (0.3 μM) and/or CEC (30 μM) or vehicle before the addition of 1 μM NE or vehicle. In the BMY 7378-plus-CEC group (third bar), SMCs were first incubated at 37° with BMY 7378 for 30 min and then with CEC for an additional 30 min. Cells were then washed three times and received fresh media containing 1 μM NE. In the BMY 7378 group (fourth bar), SMCs were pretreated with BMY 7378 for 30 min before the addition of 1 μM NE. In the CEC group (fifth bar), CEC was added to media for 30 min, cells washed three times, and fresh media containing 1 μM NE were added. The CEC-plus-BMY 7378 group (sixth bar) did not receive NE treatment, vehicle-treated control cells. Analysis by ANOVA and Bonferroni's t test. n denotes independent experiments.

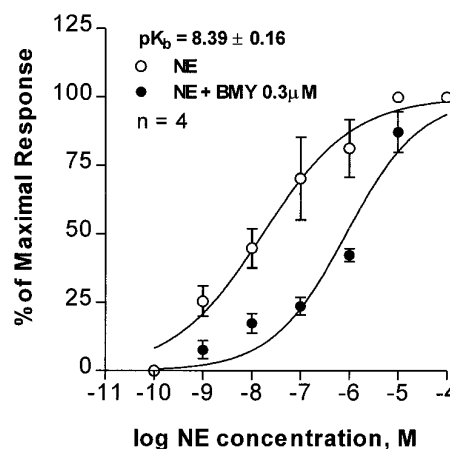


Fig. 4. Determination of apparent equilibrium dissociation constant (pK_b) for BMY 7378 against NE-mediated SMC protein synthesis. Aorta SMCs were treated with or without 0.3 μM BMY 7378, and [^{35}S]methionine incorporation was determined in the presence of different concentrations of NE. Responses were normalized to maximal response for [^{35}S]methionine incorporation stimulated with 100 μM NE in the absence of BMY 7378. n denotes independent experiments.

1 μM NE on VC SMC growth. Exposure to 1 μM NE for 48 hr, to test for a slower response time for VC SMC growth, still had no effect on protein synthesis ($108 \pm 3\%$ of time-matched control cells). However, in the presence of 0.3 μM BMY 7378, NE now increased protein synthesis $30 \pm 5\%$ ($p < 0.05$). A similar increase ($46 \pm 6\%$, $p < 0.05$) was also induced by NE after CEC treatment in the presence of 0.3 μM 5-MU. NE plus 0.3 μM 5-MU had no effect ($100 \pm 10\%$), which is consistent with its absence of selectivity between α_{1B} - and α_{1D} -ARs. Because we also did not detect α_{1A} -AR mRNA in RPAs of VC SMCs (see below), these data suggested that stimulation of α_{1B} - and α_{1D} -ARs on VC SMCs has different effects from those on aorta SMCs. Although differences in relative receptor densities between the two cell types may exist, these data suggest that in contrast to aorta SMCs, stimulation of α_{1D} -ARs inhibits and stimulation of α_{1B} -ARs induces protein synthesis by VC SMCs.

Equilibrium dissociation constant for BMY 7378 against NE-induced SMC growth. To further document the selectivity of BMY 7378 against α_1 -AR mediated aorta SMC hypertrophy, we determined the apparent antagonist dissociation constant (K_b) of BMY 7378. BMY 7378 caused a rightward shift in the NE dose-response curve for SMC protein synthesis (Fig. 4). The pK_b value that we obtained (8.39 ± 0.16) is very similar to K_i values reported for BMY 7378 binding to the cloned α_{1D} -AR (8.2, 8.7, and 9.3; see Refs. 24, 25, and 15, respectively). These data together with those shown in Fig. 3 indicate that α_{1D} -ARs mediate aorta SMC hypertrophy.

Exposure of NE for 24 hr did not induce α_{1A} -AR mRNA expression in SMCs. We have used RPAs to show that both α_{1D} - and α_{1B} -AR (not α_{1A} -AR) transcripts and receptors are expressed in early passage rat SMCs cultured from aorta, VC, superior mesenteric artery, pulmonary artery, and renal arterioles (7, 14)¹ and that exposure of rat aorta or VC SMCs to 1 μM NE for 24 hr does not cause significant change in α_{1B} - and α_{1D} -AR mRNA levels (7). We tested whether prolonged exposure to NE induced α_{1A} -AR mRNA expression, since induction of these receptors by NE

could complicate the interpretation of the data in Figs. 3 and 4. Indeed, in neonatal rat cardiac myocytes, basal levels of α_{1A} -AR mRNA are increased by 24-hr NE treatment (12). However, as shown in Fig. 5, α_{1A} -AR mRNA was not detected by RPAs of a large amount of total RNA ($\leq 100 \mu\text{g}$) that was extracted from postconfluent, growth-arrested SMCs treated with NE for 24 and 48 hr. Expression of the cyclophilin gene was used as an internal assay control. Expression of α_{1A} -AR mRNA by α_{1A} FBs and submaxillary gland provided positive controls, and the absence of expression in liver and α_{2D} -expressing cloned FBs served as negative controls. Identical results were obtained in a second independent experiment. These data demonstrate that the conclusion reached from the previous experiments that α_{1D} -AR mediates aorta SMC growth was not complicated by the possible induction of α_{1A} -AR expression during NE stimulation.

Time- and Dose-Dependent Protein Tyrosine Phosphorylation by NE

Tyrosine phosphorylation of cellular proteins is an immediate response after activation of certain receptor kinases and G protein-coupled receptors. To examine whether NE induces protein tyrosine phosphorylation in aorta SMCs, cell lysates from NE-treated or untreated SMCs were prepared and subject to immunoblot analysis with antiphosphotyrosine antibody. As showed in Fig. 6A, NE stimulated increase in a rapid, transient tyrosine phosphorylation of several proteins with apparent molecular masses of ~ 190 , ~ 145 , ~ 120 , ~ 60 – 90 , and ~ 20 – 55 kDa. Pretreatment with $0.3 \mu\text{M}$ BMY 7378 blocked the increases in tyrosine phosphorylation, implicating the α_{1D} -AR in this response. NE dose-dependently stimulated tyrosine phosphorylation of these proteins (Fig. 6B). In addition, pretreatment with genistein (Calbio-

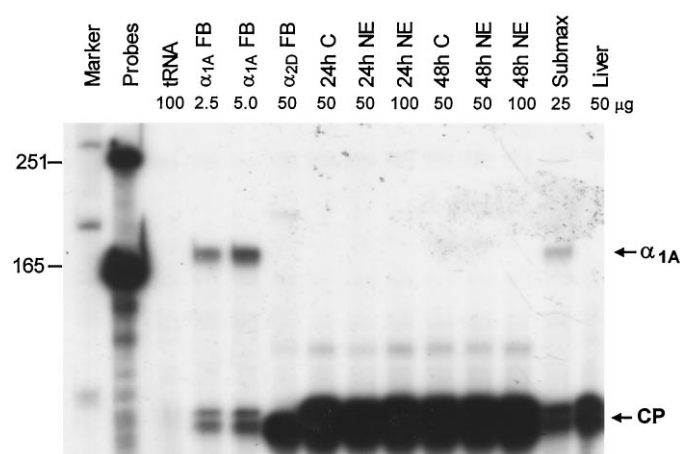


Fig. 5. NE exposure does not induce SMC α_{1A} -AR mRNA expression. Total RNA (amount in μg given above each column) from aorta SMCs exposed to $1 \mu\text{M}$ NE for 24 and 48 hr was assayed for α_{1A} -AR mRNA by RPA. Control groups (C) received only ascorbate and propranolol. Lane 1, marker. Lane 2, probes alone (α_{1A} above cyclophilin). Lane 3, $100 \mu\text{g}$ of tRNA. Lanes 4 and 5, RNA from α_{1A} -AR FBs (positive control). Lane 6, RNA from α_{2D} FBs (negative control). Lane 7, 24-hr control SMCs. Lanes 8 and 9, 24-hr NE. Lane 10, 48-hr control SMCs. Lanes 11 and 12, 48-hr NE. Lane 13, RNA from rat submaxillary gland (positive control). Lane 14, RNA from rat liver (negative control). Lanes 4–8, 10, 11, 13, and 14, balanced to $100 \mu\text{g}$ of RNA with yeast tRNA. After digestion, samples were resolved on an 8 M urea, 6% polyacrylamide gel. The dried gel was exposed to film with intensifying screens at -70° for 72 hr. Results are representative of two independent experiments.

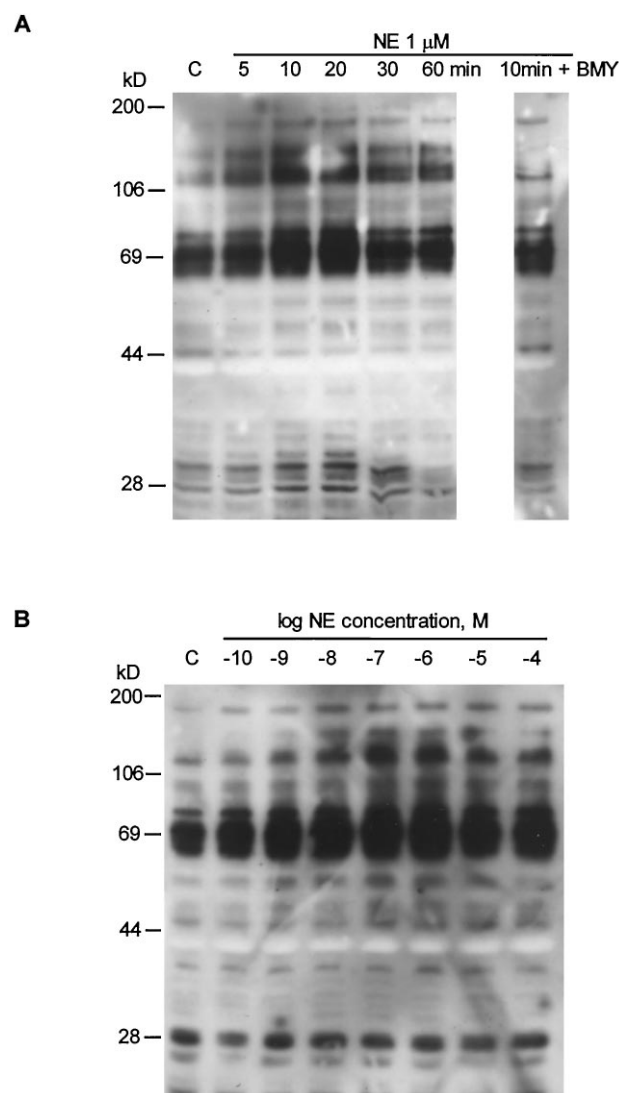


Fig. 6. NE stimulates protein tyrosine phosphorylation in a time- and dose-dependent pattern. Aorta SMCs were treated with NE for indicated time periods, and cell lysates ($100 \mu\text{g}$) were subjected to 10% SDS-polyacrylamide gels. Tyrosine phosphorylation was assessed by immunoblotting with antiphosphotyrosine antibody. C, Vehicle-treated control cells. Protein markers used to denote kDa values. A, NE induced time-dependent tyrosine phosphorylation. Pretreatment with $0.3 \mu\text{M}$ BMY 7378 for 30 min before the addition of NE blocked tyrosine phosphorylation with 10-min NE stimulation. B, Exposure to NE for 10 min caused dose-dependent protein tyrosine phosphorylation. Results are representative for four independent experiments.

chem), a nonselective tyrosine kinase inhibitor, dose-dependently (0.3 – $50 \mu\text{M}$) inhibited NE-mediated protein tyrosine phosphorylation (data not shown). NE increased phosphorylation of a number of proteins with a similar time course, magnitude, and dose-sensitivity (Fig. 6). This likely relates to the expected large number of molecules involved in the contractile and growth pathways activated. Thus, similarities in time course and dose-sensitivity for many of the signals would not be unexpected. Other growth factors (e.g., thrombin, vasopressin, angiotensin II, endothelin) have been shown (36–39) to have similar effects in these and other cells. Proteins that lacked basal phosphorylation that could be detected by this assay under control conditions were not phosphorylated by NE. There were some exceptions to the

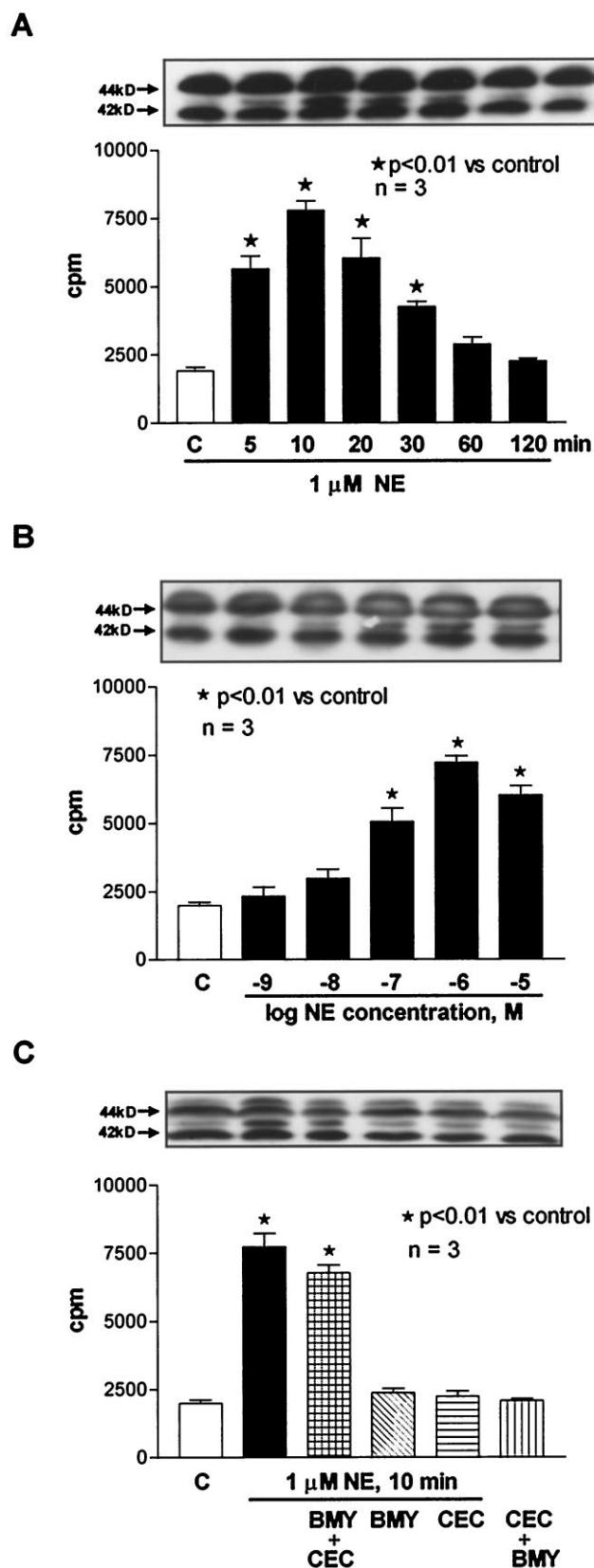


Fig. 7. α_{1D} -AR stimulation activates MAPK. Aorta SMCs were treated with NE and cell lysates were subjected to MAPK assays as described in Experimental Procedures. Results are from a gel shift assay (representative of at least four independent experiments), and data are average values from *in vitro* kinase assays using MBP as substrate ($n = 3$ for each bar graph). C, Control cells exposed to vehicle (ascorbate

and propranolol). As shown in Fig. 6B, the peak response for the 200-kDa band was obtained at a lower NE concentration than for most of the other proteins. NE did not induce additional phosphorylation above control levels for some proteins, especially those between 28 and 65 kDa. Also, not all bands changed intensity with identical time courses (Fig. 6A). For example, the proteins around 28 kDa increase intensity later and decline more quickly. The proteins at 28 and 30 kDa returned to base-line by 60 min, which is in contrast to the 69- and 110-kDa protein clusters.

Role of MAPK Pathway in α_{1D} -AR Mediated SMC Hypertrophy

α_{1D} -AR stimulation activates MAPK. MAPKs are a family of serine/threonine kinases of 42 and 44 kDa that are activated by phosphorylation of specific tyrosine and threonine residues. MAPKs are thought to serve as a convergence point for diverse signaling pathways used by a variety of growth stimuli. To begin to investigate the intracellular pathways through which NE induces growth of aorta SMCs, we tested whether NE activates MAPK. Activation of MAPK was assayed by Western detection of the appearance of their phosphorylated forms, which show reduced mobility in SDS-polyacrylamide gels, and by a direct *in vitro* kinase assay of MAPK immunoprecipitable activity using myelin basic protein as the substrate. As shown in Fig. 7A, both assays revealed that 1 μ M NE caused rapid and time-dependent activation of MAPK (peaked at 10 min), followed by a decline to basal level over a 120-min exposure to NE. When assayed after 10 min of NE stimulation, NE activated MAPK, with the maximal response achieved at the same concentration (1 μ M NE, Fig. 7B) that produced maximal activation of protein synthesis and accumulation (see above). These MAPK data also agree in time and NE sensitivity with the pattern of overall tyrosine phosphorylation (Fig. 6). However, due to lower abundance, p42-p44 MAPKs were not detected with the short film exposure required for the antiphosphotyrosine antibody immunoblot assays (Fig. 6).

To test whether activation of MAPK is through stimulation of α_{1D} -ARs, we used the same "protection from alkylation" method as applied to the protein synthesis experiments (Figs. 2 and 3), except that SMCs were exposed to 1 μ M NE for only 10 min in this experiment. In full agreement with induction of SMC protein synthesis (Fig. 3), 0.3 μ M BMY 7378 or 30 μ M CEC alone completely inhibited activation of MAPK by NE (Fig. 7C, *fourth* and *fifth* bars). Likewise, protection of α_{1D} -ARs by BMY 7378 during exposure to CEC again protected 85% of the MAPK activation from inhibition by CEC (Fig. 7C, *third* bar). In comparison to the control group, BMY 7378 plus CEC treatment alone had no effect on basal MAPK activity (Fig. 7C, *first* and *sixth* bars). These data demonstrate that α_{1D} -AR-mediated aorta SMC protein synthesis is associated with activation of the p42-p44 MAPK pathway.

and propranolol). Analysis by ANOVA and Bonferroni's *t* test. A, time-dependent stimulation of MAPK with 1 μ M NE. B, dose-dependent activation of MAPK with 10 min of NE stimulation. C, 0.3 μ M BMY 7378 abolished NE-stimulated MAPK (*fourth* bar) and protected response from inhibition by CEC (*third* versus *fifth* bars). CEC and BMY 7378 had no effect in the absence of NE (*sixth* bar). Protocol for BMY 7378 and CEC treatment was the same as that for protein synthesis assay as described in Experimental Procedures and in the legend for Fig. 3.

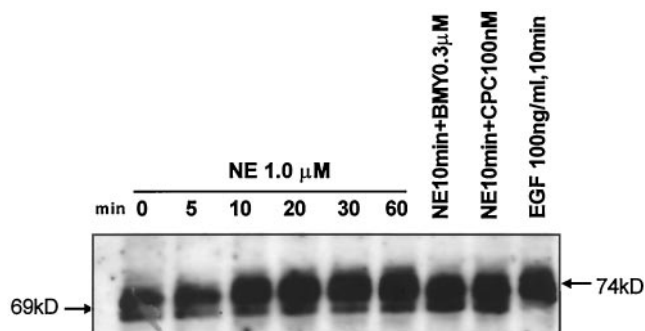


Fig. 8. NE stimulates rapid and sustained phosphorylation of Raf-1. Aorta SMCs were treated with 1 μ M NE or EGF for indicated time periods. The cell lysates (100 μ g) were subjected to 12% SDS-polyacrylamide gels and Western blotting with anti-Raf antibody. Cells were exposed to BMY 7378 or calphostin C (CPC) 30 min before a 10-min exposure to NE. Data are representative of three independent experiments. Raf-1 remained shifted in cells exposed to NE for ≥ 2 hr (data not shown).

α_{1D} -AR stimulation mediates rapid and sustained phosphorylation of Raf-1. Raf-1 is a ubiquitous serine kinase implicated in the signaling cascade upstream to MAPK activation. Activation of Raf-1 kinase is dependent on phosphorylation of multiple serine and threonine residues, which results in retardation of its migration in SDS-polyacrylamide gels. To determine whether α_{1D} -AR stimulation activates Raf-1, aorta SMCs were incubated with 1 μ M NE for various times, and cell lysates containing equal amounts of protein were subjected to immunoblot analysis using anti-Raf-1 polyclonal and monoclonal antibodies. Both antibodies gave similar results. NE caused a time-dependent mobility retardation of Raf-1 to a 74-kDa band compared with the 70-kDa band characteristic of unstimulated Raf-1 (Fig. 8). Raf-1 phosphorylation was sustained for ≥ 2 hr (data not shown). Treatment of cells with EGF for 10 min served as a positive control. Pretreatment with 0.3 μ M BMY 7378 for 30 min followed by 1 μ M NE for 10 min inhibited retardation of Raf-1 mobility. The PKC inhibitor calphostin C (Calbiochem) (100 nM) partially blocked NE activation of Raf-1 (Fig. 8). These data indicated that α_{1D} -AR stimulation of rat aorta SMCs induces rapid and sustained Raf-1 phosphorylation that may involve PKC.

α_{1D} -AR stimulation induces expression of *c-fos* mRNA. Activation of MAPK has been linked to transactivation of various growth-promoting genes, including the *c-fos* proto-oncogene in a variety of cell types, which is partially dependent on tyrosine kinase stimulation (26). We used Northern analysis to test for activation of this distal target in the MAPK pathway. Fig. 9 shows that exposure of aorta SMCs to 1 μ M NE induced time-dependent (30 and 60 min) *c-fos* mRNA expression. Induction was blocked by pretreatment with either 0.3 μ M BMY 7378 or 20 μ M tyrphostin (inhibitor of tyrosine phosphorylation) (Calbiochem). As with Raf-1 activation (Fig. 8), calphostin C partially blocked *c-fos* expression (not shown). These observations strengthen the conclusion that NE-stimulated protein synthesis in rat aorta SMCs is coupled through the α_{1D} -AR to activation of the MAPK cascade. Unlike the data shown in Fig. 9, in the other two replicates of this experiment, GAPDH did not consistently increase with NE or decrease with BMY 7378 or tyrphostin; this is in agreement with the absence of effect of NE on cyclophilin message (Fig. 5).

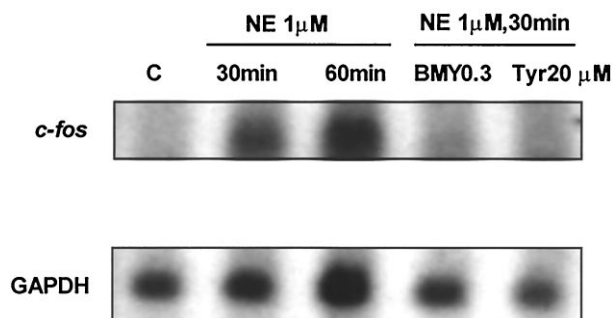


Fig. 9. BMY 7378 and tyrphostin inhibit NE-stimulated *c-fos* mRNA expression. Aorta SMCs were exposed to 1 μ M NE for indicated time periods in the absence and presence of 0.3 μ M BMY 7378 or 20 μ M tyrphostin. Total RNA (10 μ g) was extracted and analyzed by Northern hybridization using a 32 P-labeled *c-fos* cDNA fragment as described in Experimental Procedures. C, Time-matched, vehicle-treated control cells; Tyr, tyrphostin. As a control, blots were stripped and rehybridized with a GAPDH probe. Identical results were observed in three independent experiments.

MEK inhibition with PD 98059 blocks NE-induced protein synthesis and MAPK activation of SMCs. To determine whether activation of the p42-p44 MAPK cascade is required for α_{1D} -AR-induced SMC hypertrophy, aorta SMCs were treated with various concentrations of PD 98059 (0.01–30 μ M), a selective MEK inhibitor (27, 28), or the vehicle Me₂SO (final concentration 0.1%) in the presence of 1 μ M NE for 24 hr. As shown in Fig. 10A, PD 98059 dose-dependently inhibited NE-induced protein synthesis. Inhibition extended over an expected 100–500-fold range of PD 98059, with half-maximal inhibition obtained at ~ 2.3 μ M. However, PD 98059 did not completely inhibit protein synthesis, even at 30 μ M. In contrast, both 10 and 30 μ M genistein completely inhibited NE induction of protein synthesis (data not shown). The selectivity of PD 98059 on MAPK was verified by gel shift and immunocomplex *in vitro* kinase assays. NE activation (1 μ M, 10 min) of MAPK was dose-dependently inhibited by PD 98059 with half-maximal inhibition at 1.6 μ M (Fig. 10B). Furthermore, 0.01–30 μ M PD 98059 had no effect on NE (1 μ M, 10 min)-induced increase in tyrosine phosphorylation of the SMCs cellular proteins identified in the Western blot assay using antiphosphotyrosine antibody (data not shown). In several preliminary experiments, PD 98059, as examined over the full concentration range shown in Fig. 10, had no effect on basal MAPK activity. These data clearly demonstrate the selectivity of PD 98059 at the concentrations used and show that activation of MAPK is required for α_{1D} -AR-induced protein synthesis in SMCs.

Discussion

The subtype or subtypes mediating α_1 -AR growth of aorta SMCs (7, 8) and the signaling pathway have not been identified, in part because SMCs (depending on vessel of origin) can express all three subtypes and because sufficiently selective antagonists, especially for the α_{1B} -ARs, are lacking. SMCs in the intact rat aorta medial layer, which is composed entirely of SMCs (see Refs. 7 and references therein) express α_{1D} - and α_{1B} -ARs (7, 14). It is not clear whether *in vivo* aorta SMCs also express α_{1A} -ARs because previous reports of the presence of this transcript have been for whole aorta and did not distinguish between FB RNA in the adventitial layer

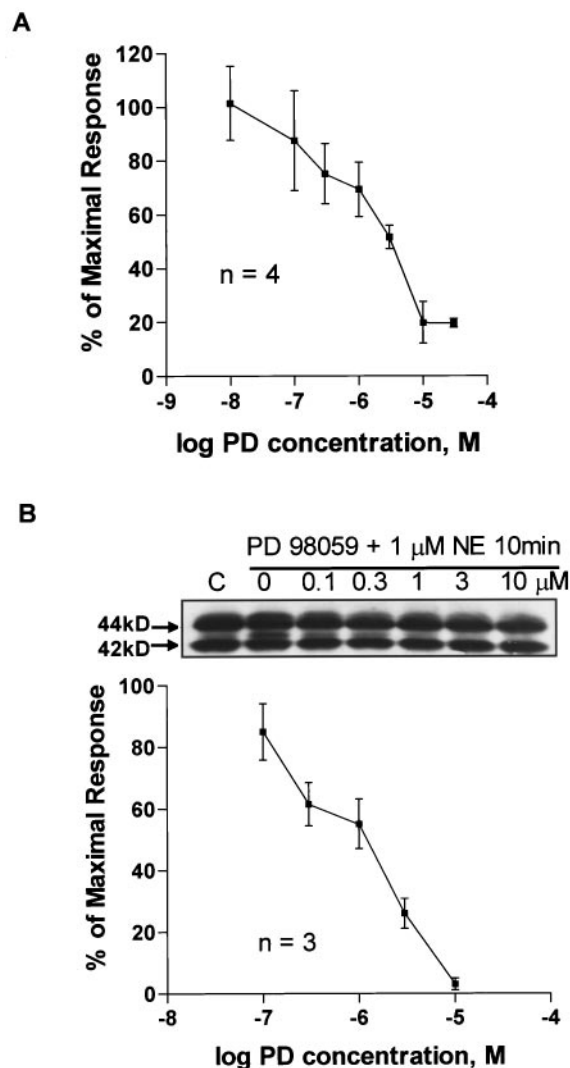


Fig. 10. MEK inhibition with PD 98059 blocks NE-induced protein synthesis and MAPK activation. **A**, Aorta SMCs were treated with various concentrations of PD 98059 in the presence of $1 \mu\text{M}$ NE for 24 hr. Protein synthesis was measured as described in Experimental Procedures. Responses were normalized to a maximal response with $1 \mu\text{M}$ NE in the absence of PD 98059. **B**, SMCs were pretreated with various concentrations of PD 98059 for 30 min, $1 \mu\text{M}$ NE was added for 10 min, and cell lysates were subjected to gel shift assay (film) and immuno-complex *in vitro* kinase assay (line graph) as described in Experimental Procedures. For both assays, control SMCs received Me_2SO alone (0.1% final concentration in media). *n* denoted three or four independent experiments.

which comprise approximately one third of the aorta wall mass. *In vivo* studies are required to confirm the current results and determine whether there is a role for α_{1A} -ARs in SMC growth *in vivo*. In the current study, we found that early passage rat aorta SMCs do not express α_{1A} -AR mRNA detectable by RPAs, nor is expression induced by prolonged exposure to NE. Previously, we demonstrated in cultured rat aorta SMCs the presence of mRNAs for α_{1D} - and α_{1B} -ARs (7, 14) and the presence of CEC-sensitive and -insensitive α_1 -AR binding sites (presumably α_{1B} - and α_{1D} -ARs) using conditions that favor CEC selectivity in radioligand binding assays (14). Thus, the absence of α_{1A} -ARs simplified the pharmacological strategy required to distinguish between α_{1B} - and α_{1D} -ARs for induction of aorta SMC protein synthesis.

We used BMY alone and to protect α_{1D} -ARs during concomitant incubation with CEC as a means of increasing CEC selectivity for α_{1B} -ARs. The validity of this "protection from alkylation" strategy was initially documented using Rat-1 FB cell lines that each express one of the three cloned α_1 -AR subtypes. Stimulation of each subtype with NE induced a similar dose-dependent increase in protein synthesis. This demonstrates that each AR subtype can couple to growth-promoting pathways in these cloned FBs. However, total α_1 -AR density in native vascular SMCs *in situ* or in culture (~ 20 fmol/mg of protein for aorta SMCs) (14) is ≥ 100 -fold lower than for these FBs. Because of potential coupling promiscuity in cells overexpressing transfected receptors, these growth responses in FB cell lines cannot be used to predict that any α_1 -AR subtype natively expressed by SMCs can induce hypertrophy when stimulated. For example, all three α_1 -ARs when overexpressed couple to the same phospholipase C effector system (29) and similarly down-regulate to agonist in Rat-1 FBs (30). However, agonist exposure has a differential effect on down-regulation of α_1 -AR mRNAs in aorta SMCs that express native α_{1B} - and α_{1D} -ARs (7) and in neonatal cardiac myocytes that natively express all three subtypes (12). Also, the endogenous α_{1A} -AR seems to mediate the hypertrophic program in rat cardiac myocytes (12, 31), but overexpression of a constitutively active α_{1B} -AR mutant in mouse heart can also induce hypertrophy (32).

In agreement with previous studies concerning its relative selectivity (15, 24, 25), $3 \mu\text{M}$ BMY 7378 inhibited FB protein synthesis induced by α_{1D} -, α_{1A} -, and α_{1B} -ARs by 75%, 23%, and 5%, respectively, and a similar selectivity for protection from CEC inhibition was obtained (Fig. 2). To minimize BMY 7378 binding to α_{1B} -ARs in the subsequent SMC studies and because of the lower total α_1 -AR expression in these cells, we used a 10-fold lower BMY 7378 concentration ($0.3 \mu\text{M}$) that also gave the best selectivity ratios in FBs. As expected, in α_{1D} -AR FBs, this concentration did not provide complete blockade of NE-induced protein synthesis (50% inhibition) and protection from CEC (69%) (Fig. 2). However, perhaps due to the lower density of α_1 -ARs on SMCs, $0.3 \mu\text{M}$ BMY 7378 completely inhibited the increase in SMC protein synthesis by NE and provided a 65% protection of the response from inhibition by CEC (Fig. 3). The partial inhibition by CEC during BMY 7378 protection likely reflects some alkylation of α_{1D} -ARs. This is consistent with the lack of selectivity of CEC when used without protection of non- α_{1B} -ARs; CEC alone completely abolished protein synthesis in all three FB cell lines and in SMCs. Overall, these data suggest that α_{1D} -ARs on rat aorta SMCs, which comprise ~ 70 – 80% of the total α_1 -AR population, with the remaining being α_{1B} -ARs (14), mediate adrenergic hypertrophy. Although a small α_{1B} -AR contribution cannot be directly ruled out, confirmation of the role of α_{1D} -ARs was provided by the pK_i value obtained with $0.3 \mu\text{M}$ BMY 7378 against NE-induced protein synthesis in aorta SMCs (8.4), which compares well with K_i values reported by others for BMY 7378 (15, 24, 25). And importantly, $0.3 \mu\text{M}$ BMY 7378 completely blocked NE-stimulated SMC protein synthesis, tyrosine phosphorylation, activation of MAPK, Raf-1, and expression of *c-fos* mRNA.

Incubation of SMCs or cloned FBs with $30 \mu\text{M}$ CEC for 30 min, followed by extensive washing, completely eliminated increases in protein synthesis during subsequent exposure to NE for ≤ 48 hr. This suggests that there may be little or no

receptor reserve for α_{1D} -AR-mediated SMC growth and that > 48 hr are required for sufficient replacement of alkylated α_1 -ARs, which is consistent with a relatively slow restoration of α_1 -ARs after alkylation (33, 34). For example, α_1 -AR density in rat aorta SMCs, which was decreased by 83% after a 30-min treatment with 1 μ M phenoxybenzamine, remained decreased by 53% 24 hr later (34). It is also possible that after alkylation with CEC, continuous exposure to NE may further slow replacement of functional receptors due to agonist-induced receptor down-regulation. Thus, in the current study, when α_{1B} -ARs were selectively removed and α_{1D} -ARs were preserved by incubation of SMCs with CEC in the presence of BMY 7378, partial restoration of α_{1B} -ARs may have occurred during the interval before measurement of protein synthesis at 24 and 48 hr. However, the findings that BMY 7378 afforded ~80% preservation of NE induction of protein synthesis at both 24 and 48 hr and a similar 85% protection of MAPK activation when measured within only 1 hr after CEC treatment lend further support to the conclusion that the α_{1D} -AR, and not the α_{1B} -AR, mediates SMC catecholamine hypertrophy.

Protein tyrosine phosphorylation is an essential component in signaling cell growth and mitogenesis (35). The pattern of genistein-sensitive, increased tyrosine phosphorylation produced by α_{1D} -AR stimulation with NE (Fig. 6) seems to exhibit bands both common to and different from other agonists acting on aortic SMCs as reported by others (36–39), although comparisons within the same assay will be needed for confirmation. Comparative immunolabeling studies are also required using antibodies that have identified proteins phosphorylated by vasoactive and growth- or mitogenesis-associated peptides like PDGF-BB and thrombin (36), vasopressin (37), angiotensin II (38), and endothelin (39); these include phospholipase C- γ (145 kDa), p21^{ras}GAP (125 kDa), p125^{fak}, PI3 kinase α subunit (85 kDa), paxillin (76 kDa), caldesmon (70 kDa), p60^{v-src}, p56^{shc}, and p46^{shc}. Such studies are needed to begin to identify common and unique proteins activated by α_{1D} -ARs.

Like many growth factors, NE induced dose-dependent, transient protein tyrosine and MAPK phosphorylation but relatively sustained Raf-1 and *c-fos* activation. The demonstration of NE-elicited Raf-1 phosphorylation (Fig. 8) differs from that produced by EGF in that the intensity of the lower-molecular-weight species does not seem to decline with NE stimulation. However, like EGF, NE clearly leads to hyperphosphorylation and the expected gel retardation of Raf-1. The use of BMY 7378 and CEC in these assays demonstrated their coupling to α_{1D} -ARs. Sustained activation of Raf-1 kinase is consistent with Raf-1 autophosphorylation, which prevents rapid inactivation after release of Ras binding (40). There is evidence that transient rather than sustained activation of MAPK may be a critical determinant of whether certain cells responds with growth or with differentiation (19). Activation of Raf-1 was partially inhibited by the PKC inhibitor calphostin C. Previous studies have demonstrated the activation of *c-fos* by NE in rat aorta SMCs (13, 41). The nonspecific tyrosine kinase inhibitors genistein and tyrphostin inhibited protein tyrosine phosphorylation and abolished *c-fos* increase. Although additional studies are required to confirm these findings, they suggest that α_{1D} -AR stimulation activates both a PKC-dependent and -independent pathway (19). Recent evidence suggests that some G

protein-coupled receptors can activate MAPK through both G_α /PKC/Raf-1- and $G_{\beta\gamma}$ /Ras/Raf-1-dependent pathways (18). The requirement of tyrosine phosphorylation for α_{1D} -AR hypertrophy was suggested by blockade of NE increases in protein synthesis by 10 μ M genistein, which also abolished MAPK activation.

Activation of p42-p44 MAPKs is mediated by phosphorylation of tyrosine and threonine residues by MEK, which is dependent on serine phosphorylation by Raf-1 and possibly by other MEKs (18, 42). There is evidence that α_1 -AR stimulation of rat aorta SMCs activates membrane-bound Ras GTPase (43), which is activated by multiple effectors other than $G_{\beta\gamma}$ and has multiple downstream targets, including Raf-1 (19). Interestingly, α_1 -AR stimulation of aorta SMCs induces coimmunoprecipitation of Ras and PI3 kinase, and the PI3 kinase inhibitor wortmannin reduces NE increases in DNA synthesis (44). Thus, $G_{\beta\gamma}$ activation of PI3 kinase may provide an additional pathway to Ras activation (19, 40, 43, 44), on which α_{1D} -AR SMC hypertrophy may depend. It also remains possible that α_{1D} -AR increases in protein synthesis involve an autocrine growth factor mechanism. Majeski *et al.* (41) demonstrated that constant arterial infusion of phenylephrine or NE over 48 hr (but not endothelin or angiotensin II, which causes similar hypertension) produced 10-fold increase in PDGF-A mRNA in rat aorta but not other tissues examined.

The MEK inhibitor PD 98059 produced dose-dependent (0.1–10 μ M) complete inhibition of MAPK activation and maximal 80% inhibition of the increase in protein synthesis produced by α_{1D} -AR stimulation (Fig. 10). However, PD 98059 had no effect on the pattern of increased tyrosine phosphorylation evident in Fig. 6. These data suggest that MAPK activation is required for the majority of α_{1D} -AR hypertrophy of SMCs. However, although the dose-sensitivity and -specificity we demonstrated for PD 98059 are in agreement with the results of others (27, 28), this conclusion relies on an indirect demonstration of MEK requirement. Interestingly, the PD 98059 data identified a residual MAPK-independent component of the α_{1D} -AR increase in protein synthesis. Additional studies will be required to determine whether this component depends on G protein activation of an additional pathway.

Similar pathways demonstrated in the current study for α_{1D} -AR-mediated SMC hypertrophy may underlie NE-induced SMC proliferation produced by competent vascular SMCs (Ref. 6 and references therein; Ref. 8). In a recent report (17), α_1 -AR stimulation of subconfluent passage 5–15 rat aorta SMC induced a 3-fold maximal increase in DNA synthesis ($EC_{50} \sim 1$ nM). This increase, which was first evident at 12 hr (but not at 8 hr, as in the current study), was accompanied by a 60% increase in cell number over 6 days of stimulation that was comparable to that produced by PDGF and endothelin. Interestingly, increases in MAPK activity reported in that study were similar in magnitude and time course to those that we report. However, as in other previous studies (7, 8), the use of CEC alone by Yu *et al.* (17), which abolished the responses, prevented identification of the responsible α_1 -AR subtype, and no causal relationship was established between MAPK activation and SMC proliferation. Nevertheless, these data and those from the current study suggest that catecholamines may activate a similar signaling cascade for proliferation and hypertrophy.

Several points are noteworthy regarding the potential physiological significance of our results. Pathways activated by both contraction and growth induced by NE as well as other stimuli (e.g., vasopressin, angiotensin II, PDGF, thrombin, endothelin, EGF) and receptor-independent depolarization (45) seem to share many similarities, including activation of the MAPK cascade formerly believed to be induced only by trophic stimuli (46). It is possible that coactivation of a parallel growth-promoting pathway by SMC contraction, which may result in minimal if any trophic changes during short-term contraction, could serve to initiate vascular wall thickening should activation of the trophic pathway be sustained. Coupling of a prolonged contractile stimulus to SMC growth could result in several outcomes. Depending on the amount of increase in lumen pressure and change in lumen diameter, structural increases in wall thickness could favor restoration of normal wall stress. SMC hypertrophy could also increase the mechanical advantage of the vascular wall and amplify the contractile potency and efficacy of the agonist. This could serve to maintain agonist reactivity despite receptor desensitization, an effect that might have both normal adaptive but also potential pathological consequences in vascular disease.

Acknowledgments

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References

- Gibbons, G. H., and V. J. Dzau. Molecular therapies for vascular diseases. *Science (Washington D. C.)* **272**:689–692 (1996).
- Bevan, R. D. Effect of sympathetic denervation on smooth muscle cell proliferation in the growing rabbit ear artery. *Circ. Res.* **37**:14–19 (1975).
- Fronek, K., C. M. Bloor, D. Amiel, and M. Chvapil. Effect of long-term sympathectomy on the arterial wall in rabbits and rats. *Exp. Mol. Pathol.* **28**:279–289 (1978).
- Lee, R. M. K. W., and J. Smeda. Primary versus secondary structural changes of the blood vessels in hypertension. *Can. J. Physiol. Pharmacol.* **63**:392–401 (1985).
- Majesky, M. W., M. A. Reidy, E. P. Benditt, and M. R. Juchau. Focal smooth muscle cell proliferation in the aortic intima produced by an initiation-promotion sequence. *Proc. Natl. Acad. Sci. USA* **82**:3450–3454 (1985).
- Nakaki, T., M. Nakayama, S. Yamamoto, and R. Kato. α_1 -Adrenergic stimulation and β_2 -adrenergic inhibition of DNA synthesis in vascular smooth muscle cells. *Mol. Pharmacol.* **37**:30–36 (1990).
- Chen, L., X. Xin, A. D. Eckhart, N. Yang, and J. E. Faber. Regulation of vascular smooth muscle growth by α_1 -adrenoceptor subtypes in vitro and in situ. *J. Biol. Chem.* **270**:30980–30985 (1995).
- Siwik, D., and R. D. Brown. Regulation of protein synthesis by α_1 -adrenergic receptor subtypes in cultured rabbit aortic vascular smooth muscle cells. *J. Cardiovasc. Pharmacol.* **27**:508–518 (1996).
- Johnson, M. D., A. Grignolo, C. M. Kuha, and S. M. Schanberg. Hypertension and cardiovascular hypertrophy during chronic catecholamine infusion in rats. *Life Sci.* **33**:169–180 (1983).
- Kukregga, R. S., B. N. Datta, and R. N. Chakravarti. Catecholamine-induced aggravation of aortic and coronary atherosclerosis. *Atherosclerosis* **40**:292–298 (1981).
- O'Malley, M. K., E. W. M. McDermott, D. Mehigan, and N. J. O'Higgins. Role of prazosin in reducing the development of rabbit intimal hyperplasia after endothelial denudation. *Br. J. Surg.* **76**:936–938 (1989).
- Rokosh, D. G., A. F. R. Stewart, K. C. Chang, B. A. Bailey, J. S. Karliner, S. A. Camacho, C. S. Long, and P. C. Simpson. α_1 -Adrenergic receptor subtype mRNAs are differentially regulated by α_1 -adrenergic and other hypertrophic stimulation in cardiac myocytes in culture and in vivo. *J. Biol. Chem.* **271**:5839–5843 (1996).
- Okazaki, M., Z. W. Hu, M. Fujinaga, and B. B. Hoffman. Alpha 1 adrenergic receptor-induced c-fos gene expression in rat aorta and cultured vascular smooth muscle cells. *J. Clin. Invest.* **94**:210–218 (1994).
- Eckhart, A. D., Z. Zhu, W. J. Arendshorst, and J. E. Faber. Oxygen modulates α_{1B} -adrenergic receptor gene expression by arterial but not venous vascular smooth muscle. *Am. J. Physiol.* **271**:H1599–H1608 (1996).
- Piascik, M. T., R. D. Guarino, M. S. Smith, E. E. Soltis, D. L. Saussy, and D. M. Perez. The specific contribution of the novel α_{1D} adrenoceptor to the contraction of vascular smooth muscle. *J. Pharmacol. Exp. Ther.* **275**:1583–1589 (1995).
- Perez, D. M., M. T. Piascik, M. R. Gaivin, and R. M. Graham. Cloning, expression, and tissue distribution of the rat homolog of the bovine α_{1C} -adrenergic receptor provide evidence for its classification as the α_{1A} subtype. *Mol. Pharmacol.* **46**:823–831 (1994).
- Yu, S.-M., S.-Y. Tsai, J.-H. Guh, F.-N. Ko, C.-M. Teng, and J. T. Ou. Mechanism of catecholamine-induced proliferation of vascular smooth muscle cells. *Circulation* **94**:547–554 (1996).
- Post, G. R., and J. H. Brown. G protein-coupled receptors and signaling pathways regulating growth responses. *FASEB J.* **10**:741–749 (1996).
- Marshall, C. J. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80**:179–185 (1995).
- Treisman, R. Regulation of transcription by MAP kinase cascade. *Curr. Opin. Cell Biol.* **8**:205–215 (1996).
- Glennon, P. E., S. Kaddoura, E. M. Sale, G. J. Sale, S. J. Fuller, and P. H. Sugden. Depletion of mitogen-activated protein kinase using an antisense oligodeoxynucleotide approach downregulates the phenylephrine-induced hypertrophic response in rat cardiac myocytes. *Circ. Res.* **78**:954–961 (1996).
- Clements, M. L., and J. E. Faber. Mechanical load opposes angiotensin-mediated decrease in vascular α_1 -adrenoceptors. *Hypertension*, in press.
- Michel, M. C., B. Kenny, and D. A. Schwinn. Classification of α_1 -adrenoceptor subtypes. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **352**:1–10 (1995).
- Goetz, A. S., H. K. King, S. D. C. Ward, T. A. True, T. J. Rimele, and D. L. Saussy. BMY 7378 is a selective antagonist of the D subtype of α_1 -adrenoceptors. *Eur. J. Pharmacol.* **272**:R5–R6 (1995).
- Ford, A. P., N. F. Arredondo, D. R. Blue, D. W. Bonhaus, J. Jasper, M. S. Kava, J. Lesnick, J. R. Pfister, I. A. Shieh, R. L. Vimont, T. J. Williams, J. E. McNeal, T. A. Stamey, and D. E. Clarke. RS-17053 (N-[2-(2-cyclopropylmethoxyphenoxy)ethyl]-5-chloro- α , α -dimethyl-1H-indole-3-ethanamine hydrochloride), a selective α_{1A} -adrenoceptor antagonist, displays low affinity for functional α_1 -adrenoceptors in human prostate: implications for adrenoceptor classification. *Mol. Pharmacol.* **49**:209–215 (1996).
- Karin, M. Signal transduction from the cell surface to the nucleus through the phosphorylation of transcription factors. *Curr. Opin. Cell Biol.* **6**:415–424 (1994).
- Servant, M. J., E. Giasson, and S. Meloche. Inhibition of growth factor-induced protein synthesis by a selective MEK inhibitor in aortic smooth muscle cells. *J. Biol. Chem.* **271**:16047–16052 (1996).
- Dudley, D. T., L. Pang, S. J. Decker, A. J. Bridges, and A. R. Saltiel. A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. USA* **92**:7686–7689 (1995).
- Lomasney, J. W., L. F. Allen, and R. J. Lefkowitz. *Molecular Endocrinology: Basic Concepts and Clinical Correlations* (B. D. Weintraub, ed.). Raven Press, Ltd., New York, 115–131 (1995).
- Wise, A., T. W. Lee, D. J. MacEwan, and G. Milligan. Degradation of G_{11} α /Gq α is accelerated by agonist occupancy of α_1A/D , α_1B , and α_1C adrenergic receptors. *J. Biol. Chem.* **270**:17196–17203 (1995).
- Knowlton, K. U., M. C. Michel, M. Itani, H. E. Shubeita, K. Ishihara, J. H. Brown, and K. R. Chien. The α_{1A} -adrenergic receptor subtype mediates biochemical, molecular, and morphologic features of cultured myocardial cell hypertrophy. *J. Biol. Chem.* **268**:15374–15380 (1993).
- Milano, C. A., P. C. Dolber, H. A. Rockman, R. A. Bond, M. E. Venable, L. F. Allen, and R. J. Lefkowitz. Myocardial expression of a constitutively active α_{1B} -adrenergic receptor in transgenic mice induces cardiac hypertrophy. *Proc. Natl. Acad. Sci. USA* **91**:10109–10113 (1994).
- Izzo, N. J., Jr., T. N. Tulenko, and W. S. Colucci. Phorbol esters and norepinephrine destabilize α_{1B} -adrenergic receptor mRNA in vascular smooth muscle cells. *J. Biol. Chem.* **269**:1705–1710 (1994).
- Hu, Z.-W., X.-Y. Shi, M. Okazaki, and B. B. Hoffman. Angiotensin II induces transcription and expression of α_1 -adrenergic receptors in vascular smooth muscle cells. *Am. J. Physiol.* **268**:H1006–H1014 (1995).
- Schlessinger, J., and A. Ullrich. Growth factor signaling by receptor tyrosine kinases. *Neuron* **9**:393–395 (1992).
- Molloy, C. J., J. E. Pawlowski, D. S. Taylor, C. E. Turner, H. Weber, M. Peluso, and S. Seiler. Thrombin receptor activation elicits rapid protein tyrosine phosphorylation and stimulation of the Raf-1/MAP kinase pathway preceding delayed mitogenesis in cultured rat aortic smooth muscle cells. *J. Clin. Invest.* **97**:1173–1183 (1996).
- Tsuda, T., Y. Kawahara, K. Shii, M. Koide, Y. Ishida, and M. Yokoyama. Vasoconstrictor induced protein-tyrosine phosphorylation in cultured vascular smooth muscle cells. *FEBS Lett.* **285**:44–48 (1991).
- Leduc, I., P. Haddad, E. Giasson, and S. Meloche. Involvement of a tyrosine kinase pathway in the growth-promoting effects of angiotensin II on aortic smooth muscle cells. *Mol. Pharmacol.* **48**:582–592 (1995).
- Koide, M., Y. Kawahara, T. Tsuda, Y. Ishida, K. Shii, and M. Tokoyama. Stimulation of protein-tyrosine phosphorylation by endothelin-1 in cultured vascular smooth muscle cells. *Atherosclerosis* **92**:1–7 (1992).

40. Macara, I. G., K. M. Lounsbury, S. A. Richards, C. Mckiernan, and B. Bar-Sagi. The Ras superfamily of GTPase. *FASEB J.* **10**:625–630 (1996).
41. Majesky, M. W., M. J. A. P. Daemen, and S. M. Schwartz. α_1 -Adrenergic stimulation of platelet-derived growth factor A-chain gene expression in rat aorta. *J. Biol. Chem.* **265**:1082–1088 (1990).
42. Cobb, M. H., and E. J. Goldsmith. How MAP kinases are regulated. *J. Biol. Chem.* **270**:14843–14846 (1995).
43. Hu, Z.-W., X.-Y. Shi, R. Z. Lin, and B. B. Hoffman. α_1 Adrenergic receptors activate phosphatidylinositol 3-kinase in human vascular smooth muscle cells. *J. Biol. Chem.* **271**:8977–8982 (1996).
44. Hawes, B. E., L. M. Luttrell, T. van Biesen, and R. J. Lefkowitz. Phosphatidylinositol 3-kinase is an early intermediate in the $G\beta\gamma$ -mediated mitogen-activated protein kinase signaling pathway. *J. Biol. Chem.* **271**:12133–12136 (1996).
45. Katoch, S. S., and R. S. Moreland. Agonist and membrane depolarization induced activation of MAP kinase in the swine carotid artery. *Am. J. Physiol.* **269**:H322–H329 (1995).
46. Hollenberg, M. D. Tyrosine kinase pathways and the regulation of smooth muscle contractility. *Trends Pharmacol. Sci.* **15**:108–114 (1994).

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