Expression of α-Adrenoceptor Subtypes by Smooth Muscle Cells and Adventitial Fibroblasts in Rat Aorta and in Cell Culture

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ABSTRACT

Previous radioligand binding reports of vascular α-adrenoceptor (AR) density have been limited to total α₁- or α₂-ARs. Studies using whole blood vessel homogenates have not differentiated among receptor or mRNA expression by medial smooth muscle cells (SMCs) versus adventitial fibroblasts (AFBs). Therefore, we used quantitative reverse transcription-polymerase chain reaction and radioligand binding to measure α₁-AR subtype mRNA levels and receptor(s) mediating SMC contraction for control of blood flow, pressure, and vascular wall compliance. These receptors may also regulate growth of vascular wall cells, although much less is known about this possibility. Determination of which α-AR subtypes are expressed and mediate constriction of different vessels was recently reviewed (Piascik et al., 1996; Docherty, 1998). Among the three α₁-AR subtypes, α₁A-, α₁B-, and α₁D-AR subtypes in media were 19 ± 2, 26 ± 4, and 55 ± 2%, and in adventitia were 44 ± 3, 37 ± 5, and 19 ± 2%. No α₁B- or α₁D-AR transcripts were detected in either layer or in cultured SMCs or AFBs. Total α₁-AR densities in cultured SMCs and AFBs (Bmax = 111 ± 4 and 48 ± 6 fmol/mg of protein, respectively) were similar to media and adventitia, with α₁B- and α₁D-AR transcript levels and receptors largely sustained. However, α₁A- and α₁D-AR expression in cultured SMCs and AFBs was strongly reduced, compared with media and adventitia, an effect not prevented by 30 different culture conditions. Like SMCs, exposure of AFBs to norepinephrine induced protein synthesis and proliferation of AFBs. This is the first study to quantitate α-AR subtype expression in media and adventitia and in cultured SMCs and AFBs. In addition, we report the intriguing finding that AFBs express α₁-ARs in similar abundance as medial SMCs and that norepinephrine induced them to proliferate.

Vascular α-adrenoceptors (ARs) mediate sympathetic regulation of smooth muscle cell (SMC) contraction for control of blood flow, pressure, and vascular wall compliance. These receptors may also regulate growth of vascular wall cells, although much less is known about this possibility. Determination of which α-AR subtypes are expressed and mediate constriction of different vessels was recently reviewed (Piascik et al., 1996; Docherty, 1998). Among the three α₁-AR subtypes, α₁A-, α₁B-, and α₁D-AR subtypes in media were 19 ± 2, 26 ± 4, and 55 ± 2%, and in adventitia were 44 ± 3, 37 ± 5, and 19 ± 2%. No α₁B- or α₁D-AR transcripts were detected in either layer or in cultured SMCs or AFBs. Total α₁-AR densities in cultured SMCs and AFBs (Bmax = 111 ± 4 and 48 ± 6 fmol/mg of protein, respectively) were similar to media and adventitia, with α₁B- and α₁D-AR transcript levels and receptors largely sustained. However, α₁A- and α₁D-AR expression in cultured SMCs and AFBs was strongly reduced, compared with media and adventitia, an effect not prevented by 30 different culture conditions. Like SMCs, exposure of AFBs to norepinephrine induced protein synthesis and proliferation of AFBs. This is the first study to quantitate α-AR subtype expression in media and adventitia and in cultured SMCs and AFBs. In addition, we report the intriguing finding that AFBs express α₁-ARs in similar abundance as medial SMCs and that norepinephrine induced them to proliferate.

ABBRiEVATIONS: AR, adrenoreceptor; SMC, smooth muscle cell; AFB, adventitial fibroblast; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate-buffered saline; FBS, fetal bovine serum; FSP, fibroblast-specific protein; bp, base pair; RPA, RNase protection assay; α-SM, α-smooth muscle; BMY, BMY 7378; KMD, KMD 3213.
bodies are not available with sufficient selectivity to quantify receptor abundance in blood vessels (Hrometz et al., 1999; Shen et al., 2000). Reports of mRNA levels have been limited mostly to qualitative determinations (Price et al., 1994; Rokosh et al., 1994; Piascik et al., 1996; Xu and Han, 1996; Phillips et al., 1997; Docherty, 1998; Handy et al., 1998; McNeill et al., 1999). In addition, previous reports of α-AR transcript and receptor abundance have been derived from whole vessel homogenates and used to infer expression by SMCs. However, the arterial wall is composed of three layers of different cell types: the intima is composed of a single layer of endothelial cells (plus dispersed “stellate” cells in mammals larger than rodents), the media is composed of SMCs (and some non-SMC-like cells in mammals larger than rodents) (Bochaton-Piallat et al., 1996: Faggin et al., 1999; Frid et al., 1999), and the adventitia is composed of more than 99% adventitial fibroblasts (AFBs) (cf. Chen et al., 1995; Faggin et al., 1999) plus nerve endings and occasional mast cells and macrophages (and a vasa vasorum that can extend into the outer media in animals larger than the rabbit). Endothelial cells possess α1- and possibly α2-ARs (Bockman et al., 1996; Docherty, 1998), although the small number of these cells, compared with other vascular wall cells, suggest they contribute minimally to RNA or membrane preparations from the intact vessel wall. However, in many arteries, the adventitia can be as thick as the media, with the AFBs comprising a large fraction of the total vascular wall cells.

Perhaps because fibroblasts are regarded as structural cells and are not known to express ARs, the possibility that AFBs might possess ARs has not been questioned. Recently, however, we found that both media and adventitia of the rat aorta express mRNAs for multiple α1- and α2-ARs (Yang et al., 1999). However, quantitative mRNA transcript levels and receptor proteins were not determined. In addition, little is known about the effect of cell culture on α-AR expression, despite its importance for study of vascular α-ARs.

Therefore, the purpose of this study was to develop quantitative RT-PCR and radioligand binding assays to determine α-AR subtype expression in small tissue samples, i.e., rat aorta media and adventitia, and to examine the effect of culture conditions on α-AR expression by SMCs and AFBs derived from these layers. A surprising finding was that AFBs express the same four α-ARs as media and with the same total α1-AR density, but where the proportion of AR subtypes differs greatly between the two layers. Moreover, AFBs respond to norepinephrine with proliferation and protein synthesis.

Materials and Methods

Tissue Collection. As detailed elsewhere (Yang et al., 1999), thoracic aortae of 300-g male Sprague-Dawley rats were isolated without the use of a pool of 4°C phosphate-buffered saline (PBS), after gently opening the parietal pleura, separating away loose connective tissue/ fat, and transecting the segmental arteries at their origins. Aortae were incubated for 25 min in a 37°C, 5% CO2 incubator with 1 mg/ml collagenase, 1 mg/ml soybean trypsin inhibitor, and 13.5 units/ml elastase (Warington Biochemicals, Freehold, NJ) to loosen endothelial cells and the external elastic lamina. Adventitial and medial layers were then separated with fine forceps in 4°C PBS, which contained 10 mM vanadyl ribonucleoside complex (Life Technologies, Gaithersburg, MD) when layers were being isolated for RNA extraction or the proteinase inhibitors: 30 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate (Sigma, St. Louis, MO) when layers were being isolated for protein extraction. Endothelial cells were removed by two strokes of a cotton-tipped applicator after opening the media longitudinally. En-face staining with silver nitrate verified removal of >99% of the endothelial cells. Layers were frozen in liquid nitrogen and stored at −80°C.

Smooth Muscle and Adventitial Fibroblast Cell Cultures. Each SMC and AFB primary culture was obtained from eight thoracic aorta media and sterile-isolated adventitia as above from 200-g rats. SMCs were dispersed from the media with the above enzymes, followed by differential plating (the SMCs, but not the AFBs, readily adhere to plastic culture dishes) to prevent contamination of SMC cultures with any adherent AFBs (Eckhart et al., 1996). To obtain AFBs, adventitia was minced into 1-mm2 pieces and incubated for 30 min at 37°C without shaking in 2.4 units/ml neutral protease II (Roche Molecular Biochemicals,Summerville, NJ). After gentle trituration, cells were placed in M199 culture media with 20% fetal bovine serum (FBS) on ice to arrest protease activity. After repeating this dispersion procedure three times, pooled cells were gently re-suspended in M199 plus FBS and sieved (38 μm, no. 400) to separate the smaller AFBs from occasional non-AFBs present in adventitia (SMC-like cells, mast cells, macrophages, and adipocytes). SMCs and AFBs (20,000 cells/cm2) were grown in M199 + 10% FBS, 200 mg/l L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin passed at −95% confluence with 0.10% trypsin/EDTA every 3 (AFBs) or 5 days (SMCs), and seeded at a density of 5000 cells/cm2. Unless noted otherwise, cells were used in passages 3 to 5, carried 2 days beyond confluence, and then growth-arrested for 2 days in serum-free defined medium, consisting of 50% Dulbecco’s modified Eagle’s medium-high glucose, 50% F12, 2.85 mg/l insulin, 5 mg/l transferrin, 35.2 mg/l ascorbic acid, 6 mg/l selenium, 100 units/ml penicillin, and 100 μg/ml streptomycin. As tests of culture homogeneity, absence of endothelial cell contamination was confirmed with morphology and anti-von Willebrand factor immunohistochemistry (Chen et al., 1995; Eckhart et al., 1996). As well, macrophages, lymphocytes, and endothelial cells cannot proliferate in the above culture conditions sufficiently to survive first passage (Babij et al., 1993). Also, we do not detect the SMC marker proteins, metavinculin, and SM1 in AFB cultures that do, however, express fibroblast specific protein-1 (FSP-1); likewise, we do not detect FSP-1 in our SMC cultures (N. Yang and J. E. Faber, unpublished data). Rat 1 fibroblasts stably transfected with α1A-, α1B-, α1D-ARs, and NIH 3T3 fibroblasts stably transfected with α2D-ARs, were maintained in Dulbecco’s modified Eagle’s medium-high glucose, 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 μg/ml G-418.

RNA Preparation. Frozen tissue was pulverized under liquid nitrogen, and total RNA from tissues and cell cultures was homogenized and extracted in acid guanidinium thiocyanate-phenol-chloroform (Eckhart et al., 1996). Genomic DNA was digested with RNase-free DNase (1 unit per 50 μg of RNA) for 45 min at 37°C, and the absence of contamination was confirmed in each RT-PCR assay by inclusion of a no-reverse transcriptase tube. RNA concentration was determined spectrophotometrically at A260. Purity was assessed according to an A260/A280 ratio of >1.8, and quality was checked by electrophoresis.

Oligonucleotide Primers. The following oligonucleotides were used as sense (A) and antisense (B) primers for RT-PCR. α1A-AR: A, 5′-CGAGTCTACGTAGTAGC-3′, B, 5′-CTCGGGACGCTTTC-3′; α1B-AR: A, 5′-ATCGTGGCAAGAGGACC-3′, B, 5′-TGGCTGTCTTTTC-3′; α1D-AR: A, 5′-CGGCTGTAAGTGGTGAC-3′, B, 5′-CTTGCCAGCTTTTTTC-3′; α2D-AR: A, 5′-GAGAACGCTTACACGTTG-3′, B, 5′-TCGTTAAGCAGCAAGCAGCCG-3′; α3B-AR: A, 5′-CCCGATCCGCTGC-3′, B, 5′-GTTGGCAGGCTGACAGC-3′, A, 5′-GGAGACGCCGCTGTCGC-3′, B, 5′-TCGGCAGCCGGCCG-3′, A, 5′-TCCGGCGCGCGCAGGACG-3′, B, 5′-AGTGAGAACAGAGTTAC-3′, which amplify 204, 201-, 218-, 271-, 583-, 557-, and 340-bp fragments of the respective gene transcripts. All primers
were synthesized commercially by Life Technologies. Amplification efficiency was determined in Fig. 1 or as described under Results. Primer pairs amplified fragments of similar size and location. Sequences of primers for the α2R- and α3C-AR were as noted by Richman and Regan (1998), and were used for qualitative but not quantitative RT-PCR, since no product was detected in any fresh or cultured vascular tissue or cell type.

**Construction and Synthesis of Mutant cRNAs.** For quantitative RT-PCR, α1-AR mutants were made with an inserted EcoRI site, whereas a BamHI site was inserted for the α2D-AR mutant. These point mutations were made at similar locations in the 3rd intracellular loop sequences with the following sense (A) and antisense (B) primer pairs: α1A-AR: A, 5′-AGACTCGAGAGATATCCGCTC-CCG3′, B, 5′-TGCGAAGACATTTGCTCCTCACTCGT3′; α1B-AR: A, 5′-TGACCCCTGAGAATTCCTGGAATC-3′; α1D-AR: A, 5′-GTGTTCTCGAGTTAAGTCTCCTCAAC3′, B, 5′-GCGGACGTGGATTCGCAAGAC3′; and α2D-AR: A, 5′-TCTTGGTTGCAATGGGCAGC-3′, B, 5′-GCTTGGTGCGAGATGCGAAC3′. The T3 primer was 5′-AATTTTGCTCTGCTGCTGCTGCTG-3′ and the T7 primer was 5′-GTAATACCCTCACTAATAGGG3′. The T7 primer was 5′-GATAATACGACTCACTATAGGG3′. To generate mutant constructs, cDNA fragments of α1A, α1B, and α1D, and α2D-AR were amplified by RT-PCR of RQ1 RNase-free DNase-treated RNA from tissues abundant in the target RNA (rat submaxillary gland for α1A-AR, rat liver for α1B-AR, and rat cerebral cortex for α1D-AR and α2D-AR) using the above primer pairs prepared with BamHI and EcoRI linkers (α1A- and α2D-AR) and EcoRI and Smal linkers (α1B- and α3C-AR). The resultant PCR products were restricted with these enzymes and subcloned into pBluescript SK− vector. Each subclone served as a template to generate a site-directed mutant fragment by PCR using two pairs of primers [T3 primer and specific antisense (α1A- or α2D-AR) template or α1B- or α1D-AR] oligonucleotide with an EcoRI or BamHI restriction site in the middle of the primer; T7 primer and the complementary sequence of the antisense or sense oligonucleotide primer. The resultant two PCR products were mixed and re-amplified with T3/T7 primers. The final PCR product was digested with XbaI/HindIII, and then cloned into pBluescript SK− vector at XbaI/HindIII sites. All primary clones and mutant clones were confirmed by sequencing.

Mutant α-AR plasmid constructs, whose products served as competitive templates for competitive RT-PCR, were linearized with either HindIII (α1A- and α2D-AR) or XbaI (α1B-, α1D-, and α2D-AR), purified with phenol-chloroform extraction, and precipitated by ethanol. Mutant cDNAs were transcribed by T3 (α1A- and α2D-AR) or T7 (α1B- and α1D-AR) RNA polymerase, in the absence of [32P]CTP, as described previously (Yang et al., 1999). Transcription products were digested with RQ1 RNase-free DNase and extracted, precipitated, dissolved in diethyl pyrocarbonate-treated water, and stored in small aliquots at ~80°C after determination of RNA concentration in triplicate. Mutant products were differentiated from endogenous PCR products by restriction enzyme digestion and electrophoresis.

**Quantitative Competitive RT-PCR.** Single-tube RT-PCR using rT3A DNA polymerase (Promega, Madison, WI) (Chiocchia and Smith, 1997) gave superior results when compared with conventional RT-PCR. Total RNA from tissue or cell culture (amounts given under Results) was mixed with known amounts of mutant cRNA competitor, and reverse-transcribed in a 20-μl reaction containing 200 μM dNTPs, 1 mM MnCl2, 10 mM Tris-HCl, pH 8.3, 90 mM KCl, 75 μM antisense primer, and 5 units of rT3A DNA polymerase. The reactions were performed in a GeneAmp 2400 thermocycler using thin-walled MicroAmp reaction tubes (PerkinElmer, Norwalk, CT) without mineral oil overlay. Reverse transcription was allowed to proceed for 20 min at 60°C and stopped on ice. PCR amplification was then carried out in the same tube in a 100-μl volume containing 1.5 mM MgCl2, 10 mM Tris-HCl, pH 8.3, 100 mM KCl, 0.05% (w/v) Tween 20, 0.75 mM EDTA, 5% (v/v) glycerol, 0.15 μM sense primer, and 1 μCi of [32P]dCTP. Initial denaturation at 95°C for 60 s was followed by 35 cycles each of denaturation at 95°C for 15 s and annealing and extension at 62°C for 30 s, with a final extension at 62°C for 8 min. Forty cycles were performed for α2D- and α3C-AR RT-PCR using both single-tube and standard RT-PCR assays. Thirty cycles were performed for cyclophilin detection.

One of the completed PCR reaction mixture was digested with EcoRI (α2D-AR digested with BamHI) for 2 h at 37°C to permit differentiation of target RNA from competitor RNA, followed by electrophoresis on 1.5% agarose containing 0.5 μg/ml ethidium bromide. Complete digestion of PCR products arising from amplification of the mutant cRNAs was confirmed in each assay. Ethidium bromide-stained DNA bands corresponding to target or competitor products were excised under UV light, dispersed from the excised bands, and counted (Wallac Microbeta 1450 LSC). RT-PCR for cyclophilin, which like α-AR mRNAs, is in low abundance in many tissues, including arteries (Yang et al., 1999), was run in parallel in each assay, and excised bands were counted for correction of intra-assay variability among RT-PCR reactions. The log of the ratio of the radioactivity of target PCR product to competitive mutant product was plotted against the log of the competing mutant cRNA template concentration added to each reaction (Prism, GraphPad Software, Inc., San Diego, CA). Concentration of target RNA (in molecules per nanogram of sample RNA) was obtained by interpolation of the resultant linear regression to the equivalence point, where the amount of target mRNA present in the sample equals the amount of competitor RNA (Chiocchia and Smith, 1997). All RT-PCR products, when first tested against positive control tissues and vascular cells/tissue, were sequenced for identity.

**RNP Protection Assay (RPA).** RPA for α-smooth muscle (α-SM)-actin mRNA was performed as described previously (Yang et al., 1999). For RPA of α2D-AR, the 339-bp SpeI/RpnI (1245–1584) fragment of the α2D-AR cDNA was cloned into pBluescript SK− vector digested with SpeI and RpnI. A 380-bp cRNA probe was synthesized after linearization with XbaI. The 179-bp cyclophilin probe construct was obtained from Ambion (Austin, TX). The identity of products for these assays has been determined by sequencing (Yang et al., 1999).

**Immunohistochemistry.** Thoracic aorta media and adventitia were examined for α-SM-actin. Thoracic aortas were perfusion cleared with PBS and fixed at 100 mm Hg with 4% paraformaldehyde in PBS. Paraffin-embedded, 5-μm sections were subjected to standard immunohistochemistry using biotinylated anti-mouse α-SM-actin monoclonal antibody (Dako Lab, 1:50 dilution; DAKO, Carpenteria, CA) and diaminobenzidine visualization. Adjacent sections were also stained with Masson’s trichrome, nuclear fast red, and nonimmune mouse IgG.

**Membrane Preparation and Radioligand Binding Assay.** For each binding experiment, purified membrane protein (microsomal preparation) from 12 aorta media and adventitia (72 rats total) was obtained according to modifications of Deng et al. (1986). Briefly, frozen tissue was pulverized in liquid nitrogen, and homogenized in 4°C buffer [25 mM Tris-HCl, 1 mM EDTA, 2 mM MgCl2, and 100 mM KCl, pH 7.4, plus the proteinase inhibitors identified above (see Tissue Collection) and 10 μg/ml leupeptin, trypsin inhibitor, and chymostatin] with a homogenizer (model TH, Omni, Atlanta, GA) at maximal speed (5 × 10 s). All procedures below were carried out at 4°C. The homogenate received 3 strokes in a Polytron, treated with 10 mmol/l ethidium bromide in PBS. Purified membranes were subjected to standard immunohistochemistry using biotinylated anti-mouse α-SM-actin monoclonal antibody (Dako Lab, 1:50 dilution; DAKO, Carpenteria, CA) and diaminobenzidine visualization. Adjacent sections were also stained with Masson’s trichrome, nuclear fast red, and nonimmune mouse IgG.
20 min, and the pellet was resuspended with homogenization buffer, rehomogenized, and recentrifuged at 25,000g for 20 min. The final pellet was resuspended in incubation buffer. The concentration of membrane protein was determined by bicinchoninic acid assay (Pierce, Rockford, IL).

Saturation and competition binding assays were performed in duplicate as described previously (Eckhart et al., 1996) in 250-μl total reaction volumes at 25°C with shaking, and repeated at least three times on independent membrane preparations. Protein samples assayed were 20 μg of crude protein for cloned fibroblasts, 50 μg of crude protein for kidney, and 50 μg of purified protein for media, adventitia, SMCs, and AFBS. Competition assays used the α1D-AR antagonist BMY 7378 (BMY) (RBI, Natick, MA) or α1A-AR antagonist KMD 32213 (KMD) (Kissei Pharm Co, Matsumoto-City, Japan), and a [3H]prazosin concentration (85 Ci/mmol, Amersham Pharmacia Biotech, Arlington Heights, IL) (see Results) equal to its KD determined in prior saturation binding assays. Nonspecific binding was defined by 10 μM phentolamine HCl (RBI) and was <10% for cultured cell membranes and ~20% for tissue membranes. Data were analyzed with Prism (GraphPad Software, Inc., San Diego, CA), including ANOVA (p < 0.05) and fitting to one-site versus two-site models.

Protein Synthesis. Protein synthesis in confluent AFBs that had been growth-arrested for 24 h in serum-free defined media was determined during the last 6 h of a subsequent 24-h interval of continued exposure to norepinephrine or vehicle, using [35S]methionine incorporation (1000 Ci/mmol, Amersham) as described previously (Xin et al., 1997). Cell number was determined by hemocytometry. Protein content was determined by bicinchoninic acid assay.

Each "n" represents an independent experiment derived from different tissue extraction pools for RNA or protein, and from different cell lines for cultured cell experiments.

Results
Validation of RT-PCR. α2B- and α2C-AR mRNAs were not detected in aorta media, adventitia, or cells cultured from these tissues (see below), thus competitive RT-PCR assays for these receptors were not developed. Pilot studies screened various primer pairs and competitors for the α2D- and the α1-ARs. Efficiencies were assessed by obtaining the cycle number-amplification product curve for each target mRNA, permitting identification of the total amount of RNA and cycle number required to yield a product amount near the midpoint on the linear portion of each curve. Primer pairs found to amplify with similar efficiencies (sequences given under Materials and Methods) were then tested to identify the amounts of total RNA required, depending on α-AR subtype assayed, that would permit the approximate midpoint of each curve to be obtained by the same number of cycles for all four α-AR mRNAs (35 cycles) and by 30 cycles for the more abundant cyclophilin. In assays of tissue, 100 ng of RNA was used for α1A-, α1B-, α1D-, and α2D-AR detection. In assays of cultured cells, 100 and 400 ng of RNA were used for α1B/α1D-AR and α1A-α2D-AR, respectively. Ten nanograms of RNA were used for assay of cyclophilin in tissue and cells.

The efficiency curve for the α1B-AR assay against 100 ng of SMC RNA is shown in Fig. 1, left. Thirty-five cycles yielded an α1B-AR product that fell near the midpoint of a semilog plot of yield against cycle number (Fig. 1, right). Similar curves were obtained for the other α-ARs and cyclophilin. In competition assays, the midpoint product amount at each competitor concentration was visualized on ethidium bromide-stained agarose gels, permitting band excision and [α-32P]dCTP scintillation counting. Examples of resultant competition curves are shown in Fig. 2 for the α1-AR primer pairs and competitor cRNAs against RNA from tissues with moderate abundance for each subtype. To determine assay sensitivities, different known amounts of in vitro transcribed cRNA for α1D-, α1B-, α1A-, α2D-AR, and cyclophilin (in the context of 100 ng for α1B/α1D-AR, 400 ng for α1A-α2D-AR, or 10 ng for cyclophilin) of yeast carrier tRNA, respectively] were amplified with 35 (α-ARs) or 30 cycles (cyclophilin) against different ranges of competitor cRNA. Message levels ≥0.05 molecule of target mRNA/ng of total RNA could be consistently detected.

Aorta Media and Adventitia Express mRNAs for All Three α1-ARs and the α2D-AR. mRNA transcript levels were significantly different for each α-AR subtype between the two layers (p < 0.05) (Table 1). Aorta media expressed transcripts for α1A-, α1B-, α1D-, and α2D-AR of (in fold differences, where α1-AR = 1) 1, 6, 115, and 1. Aorta adventitia also expressed α-ARs, where compared with media, mRNAs

![Graph 1](https://via.placeholder.com/150)

**Fig. 1.** Left, efficiency of RT-PCR detection of α1B-AR mRNA from 100 ng of total RNA per reaction. Product shown as a representative ethidium bromide-stained gel, and [3H]dCTP incorporation is shown in the graph in counts per minute (cpm). Values are mean ± S.E.M. for determinations from two separate SMC culture lines, each done in duplicate. Amounts of total RNA assayed in vascular cells/tissues were optimized (see Results) so that 35 cycles yielded product located at approximately the midpoint on the linear portion of the curve for all α-AR subtype assays, whereas 30 cycles yielded the approximate midpoint for cyclophilin. Right, α1A-AR mRNA in adventitia of thoracic aorta. Assay variability of cpm of product yield among reactions was corrected by using cyclophilin cpm variability. Values are mean ± S.E.M. for n = 3 samples of RNA, each extracted from adventitia pooled from two to three rats, yielding a level of 1770 ± 161 molecules of α1C-AR mRNA/ng of total RNA. Average r² given for linear regression.
were 10- and 21-fold higher for \(\alpha_{1A}\)- and \(\alpha_{2D}\)-AR, and 15- and 7-fold fold lower for \(\alpha_{1B}\)- and \(\alpha_{1D}\)-AR.

**\(\alpha_{1A}\)- and \(\alpha_{2D}\)-AR mRNAs Are Reduced in Cultured SMCs and AFBs.** Adult rat aorta media is composed entirely of SMCs [i.e., \(\alpha\)-SM-actin positive cells (cf. Chen et al., 1995; Fagggin et al., 1999)], so that homogeneous SMC cultures were derived from it using differential plating. However, cells other than AFBs can occasionally be found in adventitia of rodent arteries (mast cells, macrophages, leukocytes, adipocytes, and several \(\alpha\)-SM-actin positive cells sometimes present just outside of the external elastic lamina). These cells are larger than AFBs, permitting their exclusion by sieving after enzymatic dispersion. Also, we have found that non-AFBs make up less than 1% of rat aorta adventitial cells, as determined by hemocytometry of dispersed adventitial cells during primary isolation, and with anti-\(\alpha\)-SM-actin immunohistochemistry. However, with the exception of activated mast cells in atheromatous plaques that appear to express \(\alpha_{2D}\)-ARs (Handy et al., 1998), there is no evidence that the few non-AFB cells of the adventitia normally express \(\alpha\)-ARs.

In early passage 3 to 5 primary cultures of aorta SMCs and AFBs grown to 2 days beyond confluence plus 2 days in serum-free defined medium (quiescent phenotype), levels of \(\alpha_{1D}\)- and \(\alpha_{1B}\)-AR mRNAs were not detected. Average \(r^2\) values for linear regression of RT-PCR competition are given in parentheses. Receptor values are percentages of \(\alpha_{1}\)-ARs (where 100% is the total \(\alpha_{1}\)-AR population) from the binding studies of Figs. 9 and 10 (see Results for details). Total \(\alpha_{1}\)-AR densities (fmol/mg of protein) for media, SMCs, adventitia, and AFBs were 101 ± 10, 111 ± 4, 96 ± 16, and 48 ± 6, respectively.

**TABLE 1**

<table>
<thead>
<tr>
<th>Vascular Wall</th>
<th>(\alpha_{1A})-AR</th>
<th>(\alpha_{1B})-AR</th>
<th>(\alpha_{1D})-AR</th>
<th>(\alpha_{2D})-AR</th>
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<tr>
<td>Media</td>
<td>106 ± 2(^a)</td>
<td>19 ± 2(^a)</td>
<td>564 ± 44(^a)</td>
<td>26 ± 4(^a)</td>
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<td>(0.99)</td>
<td>(0.94)</td>
<td>(0.96)</td>
<td>(0.98)</td>
<td>(0.97)</td>
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<tr>
<td>Cultured SMCs</td>
<td>0.11 ± 0.03</td>
<td>N.D.</td>
<td>410 ± 36(^a)</td>
<td>66 ± 5</td>
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<td>(0.97)</td>
<td>(0.98)</td>
<td>(0.97)</td>
<td>(0.97)</td>
<td>(0.94)</td>
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<tr>
<td>Adventitia</td>
<td>1064 ± 98(^d)</td>
<td>44 ± 3</td>
<td>39 ± 8</td>
<td>37 ± 5(^d)</td>
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<td>(0.98)</td>
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<td>(0.95)</td>
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<tr>
<td>Cultured AFBs</td>
<td>0.23 ± 0.05</td>
<td>N.D.</td>
<td>16 ± 4</td>
<td>82 ± 6</td>
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<td>(0.97)</td>
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</table>

N.D., not detected. Statistical comparisons by ANOVA followed by Bonferroni protected \(t\) tests. Comparisons that revealed significant differences were: \(^a\)media vs. cultured SMCs, \(^b\)media vs. adventitia, \(^c\)cultured SMCs vs. cultured AFBs, and \(^d\)adventitia vs. cultured AFBs. To obviate complexity, statistics for horizontal comparisons were not added to the table.
SMCs and AFBs, \(a_{1A}\) and \(a_{2D}\)-AR levels in both cell types were reduced 200- to 7000-fold below in vivo levels, as determined by competitive RT-PCR (Table 1, \(p < 0.05\)). This marked reduction in mRNA (and receptors—see below) occurred regardless of aorta SMC cell culture conditions. These consisted of deriving primary cultures from medial explants instead of enzymatic dispersion, passage number (pass 2–6), level of confluency or serum presence (~70% confluence versus 2 or 11 days after reaching confluence in the presence of 10% FBS or after 2–3 additional days in serum-free defined medium), plating surface (glass, plastic, laminin, soluble or fibrillar type I collagen, elastin, fibronectin, or RGD peptide). The reduction in mRNA was not prevented by exposure to the following agents and conditions for up to 48 h in serum-free defined medium: 1 \(\mu\)M norepinephrine, 10 \(nM\) angiotensin II, 80 \(mM\) KCl, 10 \(nM\) dexamethasone, 10 \(nM\) diethylstilbestrol, 25 \(nM\) cyclic guanosine monophosphate, 25 \(nM\) forskolin, 10 \(nM\) cis-9-retinoic acid, 20 \(ng/ml\) fibroblast growth factor 2, 20 \(ng/ml\) platelet-derived growth factor-BB, normal tissue oxygen levels (21 mm Hg ambient PO\(_2\)), and 60-Hz phasic stretch at 20% elongation on Flexercell plates (Flexcell, Inc., McKeesport, PA). Similar relative levels of \(a_{1A}\) and \(a_{1D}\)-AR mRNAs to those in aorta SMCs (Table 1) were found by RPA of SMC cultures derived from adult rat superior mesenteric artery, pulmonary artery, vena cava (data not shown), and adventitia (media cannot be separated from adventitia in this vessel). These validation studies were required because of the mixed SMC and fibroblasts in this vessel.

**\(a_{2B}\)** and **\(a_{2C}\)**-AR Are Not Expressed by Medial or Adventitial Cells. Whether using the more sensitive single-tube method (Fig. 3, left) or the standard method with separate RT and PCR reactions (data not shown), 40 cycles of PCR with up to 2 \(\mu\)g of RNA did not detect \(a_{2B}\) or \(a_{2C}\)-AR mRNAs in media, adventitia, cultured SMCs, or AFBs from adult rat aorta (Fig. 3, left). \(a_{2B}\)-AR mRNA was also not detected in aorta tissues and cells by RPAs (Fig. 3, right), but was detected in intact vena cava and pass 4 SMCs cultured from it (not shown), in agreement with our previous report using qualitative RT-PCR that used different primer pairs than herein (Ping and Faber, 1993). \(a_{2C}\)-AR expression by RT-PCR was not confirmed by RPAs because we (Ping and Faber, 1993) and others have not detected \(a_{2C}\)-AR mRNA in rat aorta using different primer pairs than herein.

To address potential contamination by medial SMCs, RPAs (Fig. 4) and immunohistochemistry (Fig. 5C) did not detect \(\alpha\)-SM-actin in aorta adventitia after separation from media or when examined intact, respectively. This is in agreement with the absence of detection of \(\alpha\)-SM-actin mRNA in adventitia (Chen et al., 1995). Adventitial collagen and AFB density are indicated in Fig. 5, A and B.

**\(\alpha\)-AR Densities: Validation of Assays Using Cloned Fibroblasts and Kidney.** Saturation and competition binding assays were first optimized using cloned fibroblast cultures and rat kidney. These validation studies were required because of 1) limited availability of thoracic aorta media and adventitia (media cannot be separated from adventitia in abdominal aorta), 2) low density of the total \(\alpha\)-AR populatio-
tion on aorta, and 3) the need to differentiate all three α1-AR subtypes (based on the above mRNA studies). First, three Rat1 fibroblast cell lines each stably expressing one of the full-length cloned rat α1-AR subtypes were assayed. B\text{max} for α1D-, α1B-, and α1A-ARs in fibroblasts were 1234 ± 76, 3733 ± 97, and 520 ± 35 fmol/mg of protein, respectively (n = 3 for each cell line; nonspecific binding averaged 6, 3, and 9%, respectively). Membranes from each cell line were then combined in a 7:2:1 α1D-, α1B-, and α1A-AR receptor proportion to mimic a possible aorta media distribution suggested by the above (Table 1) abundance of media mRNA as α1D-AR > α1B-AR > α1A-AR, labeled with 0.3 nM [3H]prazosin (the average K\text{d} for [3H]prazosin obtained from the preceding saturation binding studies of the three fibroblast lines), and competed with the α1D-AR antagonist BMY and the α1A-AR antagonist KMD. Reported pK\text{a} values for BMY at cloned rat receptors range at α1D-AR from 8.2 to 9.1 (average = 8.6), for α1B-AR from 6.2 to 7.0 (average = 6.5), and for α1A-AR from 6.1 to 7.3 (average = 6.5) (Goetz et al., 1995; Piascik et al., 1995; Suzuki et al., 1997), demonstrating α1D-AR selectivity of 126-fold. KMD exhibits pK\text{a} values for the cloned rat α1A-AR and submandibular gland α1A-AR of 9.3 and 9.8 (average = 9.6), and showed 56- and 583-fold selectivity versus α1D- and α1B-ARs, respectively (Shibata et al., 1995). BMY and KMD competition assays each fit the two-site model best, and yielded 60 ± 4 and 15 ± 3% as high-affinity sites, respectively (70 and 10% expected) (Fig. 6).

The robustness of these antagonists was then tested in adult rat kidney membranes (Fig. 7, left), B\text{max} and K\text{d} values for saturation binding assays agreed with published values (Bylund, 1987). KMD competition against 0.25 nM [3H]prazosin gave 45 ± 5% high affinity (taken as α1A-AR) and 55 ± 6% low affinity (taken as α1B- and/or α1D-AR) (Fig. 7, right); since BMY gave 0% high-affinity sites (taken as α1A-AR), the low-affinity KMD sites are presumed to be the α1B-AR. These data, and the indirect determination of α1B-AR density that they yield, agree with binding and functional blood flow studies of rat kidneys showing that α1A- and α1B-AR predominate, while α1D-AR is essentially undetectable (cf. Blue et al., 1995; Canessa et al., 1995; Yang et al., 1997; Salomonsen et al., 2001). These studies validate our assays and confirm the selectivity of BMY and KMD. The indirect estimate of α1B-AR density is required because α1B-AR competitive antagonists have limited selectivity suitable for competition binding assays when multiple α1-AR subtypes are suspected of being present (Dockerty, 1998).

It was not feasible to obtain full saturation and competition assays for aorta media and adventitia, due to limited tissue availability and the requirement of highly purified membrane protein for measuring vascular α1-ARs (e.g., 72 rats were required for Fig. 9 data). Therefore, a “two-point” ligand competition analysis (45, 55, and 0%, respectively) shown in Fig. 7. These validation studies predict that these two-point binding assay method, both media and adventitia have similar densities of total α1-ARs (B\text{max} = 101 and 96 fmol/mg, respectively; Fig. 9). Media expressed predominantly α1D-
AR, whereas adventitia expressed predominantly $\alpha_{1A}$-AR (Fig. 9). The relative abundance of receptor subtypes and their mRNAs agree for media, but not for adventitia where mRNA amounts are $\alpha_{1D}$-AR > $\alpha_{1A}$-AR >> $\alpha_{1B}$-AR (Table 1).

Complete saturation binding assays were used in cultured SMCs and AFBs because membrane protein was not limited. Total $\alpha_1$-AR density was approximately 2-fold higher for SMCs than AFBs (Fig. 10 and Table 1). BMY identified 34% high-affinity ($\alpha_{1D}$-AR) sites and 66% low-affinity sites in SMCs, and 18% high affinity ($\alpha_{1D}$-AR) and 82% low affinity in AFBs. Since culture had little or no effect on $\alpha_{1B}$-AR (or $\alpha_{1D}$-AR) mRNA levels, but reduced $\alpha_{1A}$-AR mRNA in both cell types to almost undetectable levels (Table 1), the low-affinity KMD sites are likely to be $\alpha_{1B}$-AR. Consistent with this, KMD only exhibited low-affinity, single-site competition in both cell types (presumably competing at $\alpha_{1D}$- and $\alpha_{1B}$-AR). These data are interpreted as an absence of $\alpha_{1A}$-AR expression. Moreover, in good agreement with BMY's published selectivity (see above), BMY displayed $pK_i$ values of 8.4 and 8.2 for SMCs and AFBs at the high-affinity receptor ($\alpha_{1D}$-AR) and 6.5 for both cell types at their low-affinity site (presumed $\alpha_{1B}$-AR). Notably, the ratio of $\alpha_{1D}$-AR:$\alpha_{1B}$-AR receptor density in SMCs (1:2) and AFBs (1:4) is opposite the ratio of mRNAs (23:1 and 22:1, respectively, Table 1). Total $\alpha_1$-AR densities ($B_{\text{max}}$) in these purified membrane preparations of SMCs and AFBs are 5- and 3.8-fold higher, respectively, than in standard crude membrane preparations obtained from SMCs (Eckhart et al., 1996) and AFBs (data not shown) when assayed at the same passage number and at 2 days postconfluence plus 2 more days in serum-free defined media. We also found the yield of purified membrane protein to be 5 times lower than that obtained for crude membrane fractions from identical numbers of cultured SMCs. $\alpha_2$-AR density was not determined in the present study because only $\alpha_{2D}$-AR mRNA was expressed in media, adventitia, SMCs, and AFBs, and because radioligand binding data for total $\alpha_2$-AR density have already been reported for rat aorta (Regan, 1988; Daniel et al., 1991).

**Stimulation of Adventitial Fibroblasts with Norepinephrine Induces Proliferation and Protein Synthesis.** Previously, we found that 24-h norepinephrine treatment of Rat1 fibroblasts stably expressing cloned $\alpha_{1A}$-, $\alpha_{1B}$-, or $\alpha_{1D}$-AR caused similar dose-dependent increases in protein synthesis. The same effect was obtained for aorta SMCs and
was blocked by prazosin but was unaffected by propranolol or rauwolscine, thus identifying $\alpha_1$-ARs as being responsible for this effect (Xin et al., 1997). To determine whether native $\alpha$-ARs detected herein on AFBs are functionally coupled to cellular growth pathways, cell proliferation and protein synthesis ($^{35}$S)methionine incorporation were measured in postconfluent AFBs that had been growth-arrested for 24 h in serum-free defined medium. Norepinephrine caused increases in protein synthesis and cell number (Fig. 11).

**Discussion**

A major finding of this study was that aorta media and, unexpectedly, adventitia expressed all three $\alpha_1$-AR subtypes, wherein total $\alpha_1$-AR density was as high in the adventitia, which is composed mostly of AFBs, as in the media composed of SMCs. However, the percentage distribution of $\alpha_{1A}$-, $\alpha_{1B}$-, and $\alpha_{1D}$-AR in media (19, 26, and 55%, respectively) differed from that in adventitia (44, 37, and 19%, respectively).

mRNA distributions qualitatively followed these receptor distributions (i.e., $\alpha_{1A}$-AR < $\alpha_{1B}$-AR < $\alpha_{1D}$-AR for media; $\alpha_{1A}$-AR > $\alpha_{1B}$-AR > $\alpha_{1D}$-AR for adventitia), but absolute transcript abundances differed greatly between the two layers. Among $\alpha_2$-ARs, only $\alpha_{2D}$-AR transcripts were detected, and levels were 21-fold higher in adventitia than in media. Similar to quiescent SMCs, where norepinephrine induced $\alpha_1$-AR-mediated protein synthesis and hypertrophy (Xin et al., 1997), norepinephrine induced protein synthesis and proliferation of quiescent AFBs. This is the first quantitation of $\alpha_1$-AR subtypes in media and adventitia.

**Distribution of mRNA**

Scofield et al. (1995) used competitive RT-PCR and found that $\alpha_{1A}$-, $\alpha_{1B}$-, and $\alpha_{1D}$-AR mRNA distribution in intact aorta of adult rat was 67, 15, and 18%, respectively. A similar distribution was found in intact rat aorta using RPAs (Xu and Han, 1996). The major source of disagreement between these and our results likely reflects absence of removal of intima and separation of media from adventitia in those studies, as well as methodological differences. Using RPAs (Yang et al., 1999), we also found all three $\alpha_1$-AR transcripts and the $\alpha_2D$-AR in media and adventitia of adult rat aorta in qualitatively similar proportions to those reported herein. Using competitive RT-PCR, we have

![Fig. 8. Assay optimization study III: two-point ligand binding assay tested for ability to estimate $B_{max}$ and proportion of $\alpha_1$-AR subtypes in membrane protein samples of limited abundance. Membranes from Rat1 fibroblasts, each expressing one of the three $\alpha_1$-ARs, were mixed in a 1:1:1 proportion; membranes from rat kidney were also analyzed (not shown). $B_{max}$ (fmol/mg) estimated from $K_i$ and saturating concentrations of $[^3H]$prazosin were 865 ± 54 for Rat1 membrane mixture and 114 ± 6 for rat kidney; these values agreed with $B_{max}$ determined from full saturation analysis of Figs. 6 and 7 data (1141 ± 43 and 106 ± 4, respectively). Estimates of subtype percentages for Rat1 fibroblasts are given in the figure (and for kidney membranes they were $\alpha_{1A}$-AR = 42 ± 9%, $\alpha_{1B}$-AR = 52 ± 13%, $\alpha_{1D}$-AR = 5 ± 5%; n = 2), and are based on displacement of 5 nM $[^3H]$prazosin binding by 200 nM BMY and 30 nM KMD (see Results for calculation of receptor densities). These data also agree closely with values obtained from full competition assays of Rat1 fibroblasts and rat kidney shown in Figs. 6 and 7. n = number of different membrane preparations assayed in duplicate. *p < 0.05 versus control.](image1)

![Fig. 9. Two-point ligand binding analysis shows that rat thoracic aorta media (SMCs) and adventitia (AFBs) express the same density of total of $\alpha_1$-ARs ($B_{max}$), despite approximately 10-fold lower levels of mRNAs for each $\alpha_1$-AR subtype in adventitia (Table 1). Media expresses predominately $\alpha_{1A}$-AR, whereas adventitia expresses predominantly $\alpha_{1B}$- and $\alpha_{1D}$-AR. The relative abundance of receptors and their mRNAs agree for media, but not for adventitia, where mRNAs are $\alpha_{1A}$-AR > $\alpha_{1B}$-AR > $\alpha_{1D}$-AR (Table 1) (see Results for calculation of receptor densities). Unlike previous cloned fibroblasts and kidney membranes (Figs. 6–8), vascular tissue/cell assays in Figs. 9 and 10 were done on highly purified microsomal fractions. n = number of independent membrane preparations each from 12 rats (72 adult rats total) assayed in duplicate.](image2)
In the present study, we used the primers described by Richman and Regan (1998), but were again unable to detect \( \alpha_{2B} \)- or \( \alpha_{2C} \)-AR transcripts in aorta media, adventitia, or cells cultured from these layers, regardless of dispersion, explant, or other conditions. Also, RPA failed to detect \( \alpha_{2D} \)-AR in media, SMCs, adventitia, or AFBs (\( \alpha_{2C} \)-AR RPA was not tested). In situ hybridization of rabbit aorta (conducted with RPAs) detected \( \alpha_{2D} \)-AR on medial SMCs and endothelial cells, but did not detect \( \alpha_{2B} \) - or \( \alpha_{2C} \)-AR; adventitia was not examined (Handy et al., 1998). In rat tail artery, in situ hybridization of \( \alpha_{2D} \) - and \( \alpha_{2C} \)-AR, but not \( \alpha_{2B} \)-AR, was detected (McNeill et al., 1999), whereas all three \( \alpha_{2} \)-ARs were detected by RT-PCR (Phillips et al., 1997). As in aorta, we have also confirmed the absence of expression of mRNA for \( \alpha_{2B} \)- and \( \alpha_{2C} \)-AR by RT-PCR in separated media and adventitia of the adult rat common carotid artery (J. E. Faber and N. Yang, unpublished data).

**\( \alpha_{1} \)-AR Density.** Given that fibroblasts are normally non-contractile and considered “passive” matrix-maintaining cells, it is surprising that AFBs express \( \alpha_{1} \)-ARs in the same total abundance as in the medial SMC layer. Moreover, \( \alpha_{2D} \)-AR mRNA levels were 21-fold greater in adventitia than in media. We are unaware of other reports of \( \alpha_{1} \)-ARs on tissue fibroblasts. We confirmed herein that Rat1, Cos7, and 3T3 fibroblast cell lines do not express \( \alpha_{1} \)- or \( \alpha_{2} \)-AR transcripts (not shown). In addition, freshly dispersed rat cardiac fibroblasts do not express \( \alpha_{1} \)-AR mRNAs or receptors, although they do express \( \beta_{2} \)-ARs and angiotensin receptors (Stewart et al., 1994). Our finding that substantial amounts of mRNAs and receptors for \( \alpha_{1} \)-AR subtypes are expressed by adventitial cells could explain some of the disagreement in transcript and receptor subtype levels reported previously for intact arteries.

Media SMCs expressed the same total \( \alpha_{1} \)-AR density as fresh media (111 versus 101 fmol/mg), and \( \alpha_{1} \)-AR density decreased modestly for cultured AFBs when compared with fresh adventitia (48 versus 96 fmol/mg). Thus, our culture conditions largely preserved total \( \alpha_{1} \)-AR density. In contrast, expression of detectable \( \alpha_{1A} \)- and \( \alpha_{2D} \)-AR binding sites was lost in cultures of both cell types (and mRNA levels were reduced 200- to 7000-fold), even when cells were assayed as early as passage 2, and despite 30 different culture conditions tested (see Results). We are unaware of other studies that have compared expression of adrenergic mRNAs or receptor densities in different vascular wall cell types in situ versus in culture.

**Function of Multiple \( \alpha \)-ARs on Vascular SMCs and Adventitial Fibroblasts.** The predominance of the \( \alpha_{1D} \)-AR population in rat aorta media is consistent with studies showing it mediates contraction of aorta (Goetz et al., 1995; Piascik et al., 1995; Deng et al., 1996), as well as the carotid artery and a number of conduit and resistance blood vessels in the rat (Leech and Faber, 1996; Docherty, 1998). The \( \alpha_{1D} \)-AR appears to be the main determinant of resting sympathetic tone and arterial pressure in rats (Scott et al., 1999), although \( \alpha_{1A} \)-AR signals constriction in certain other blood vessels (e.g., mesenteric resistance vessels) and regional circulations such as the kidney (Piascik et al., 1996; Docherty, 1998; Salomonsson et al., 2001). A contractile role for the \( \alpha_{1B} \)-AR in rat appears more restricted, having been impli-
ated for tail and mesenteric arteries (Plasick et al., 1996; Docherty, 1998) and rat venous vessels (cf. Leech and Faber, 1996). The functions of the α1A- and α1B-ARs in aorta medial SMCs remain unknown.

The present results identified α2AR mRNA in rat aorta. This subtype signals constriction of arterioles and venules in rat skeletal muscle (Leech and Faber, 1996). Absence of α2AR mRNA in rat aorta vascular cells is consistent with the lack of an α2AR contraction in some blood vessels. Similarly, we also failed to detect α2AR mRNA in aorta layers and cultured SMCs from other vessels, except rat vena cava and pulmonary artery. Thus the α2AR- and α2AR may not contribute to the α2AR-dependent contraction of rat aorta (Lues and Schumann, 1984).

As in our previous studies of SMCs, norepinephrine caused proliferation and protein synthesis in AFBs. In the present study α1A- and α1DAR mRNA declined to almost undetectable levels when AFBs were placed into culture, and no α1A/AR binding sites could be detected. Since β-ARs, at least on SMCs, cause inhibition of proliferation, norepinephrine stimulation of proliferation of AFBs may be mediated by the α1D- and/or α1D-ARs present on these cells. However, studies with α- and β-AR antagonists are required to test this hypothesis. One or more of the α-AR subtypes on AFBs may signal a trophic action of catecholamines on vascular wall growth and remodeling. There is growing evidence of a role for adventitial fibroblasts and their myofibroblast phenotype in a number of vascular wall diseases, including fibrosis and intimal hyperplasia in atherosclerosis, graft rejection, and restenosis (Gutterman, 1999). Migration to intima, matrix regulation, and contracture by AFBs and myofibroblasts may be modulated by catecholamines through one or more of the α-ARs we detected.

It has been proposed that an AFB population in adult vessels may represent an embryonic mesenchymal precursor cell that gives rise to SMCs during vasculogenesis (Faggin et al., 1999). Such a developmental link between SMCs and AFBs may, together with the presence of dense adrenergic innervation in the adventitia of most arteries, underlie the presence of α-ARs on these fibroblasts. Additional studies capable of single cell resolution will be required to determine whether a given SMC and AFB expresses all four α-AR subtypes in the intact media and adventitia, or if instead variation exists among SMCs and AFB types as a function of different phenotypes or location of the cells within their layers. There is evidence that medial SMCs in rats, larger species, and humans appear to be composed of more than one phenotype (Bochaton-Piallat et al., 1996; Frid et al., 1999).

In conclusion, all three α1-AR subtype mRNAs and receptors are expressed by both medial SMCs and adventitial fibroblasts in rat aorta, and with the same overall α1-AR density in each layer, but with opposite relative proportions. Like SMCs, stimulation of AFBs with norepinephrine induces proliferation and protein synthesis. These findings demonstrate that vascular fibroblasts, like SMCs, are also capable of expressing abundant α-ARs, and underscore the importance of differentiating between these cell types when quantitating mRNAs or receptors and drawing inferences from these data about the role of different α-AR subtypes in mediating vascular functions. Moreover, they raise the possibility that adrenergic nerves, which are abundant in the adventitia of most vessels, or elevated plasma catecholamines may regulate fibroblast involvement in vascular wall growth, remodeling, and disease.

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