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Platelet-Derived Growth Factor Inhibits α1D-Adrenergic Receptor Expression in Vascular Smooth Muscle Cells In Vitro and Ex Vivo

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ABSTRACT

Indirect evidence suggests that stimulation of α1-adrenergic receptors (ARs) increases smooth muscle cell (SMC) growth in the growing and adult artery and worsens atherosclerosis and restenosis after balloon injury. In support of a direct adrenergic effect, we have previously shown that α1D-AR stimulation induces SMC hypertrophy in cell and vessel organ culture. Because interactions between α1-ARs and peptide growth factors may be important in normal and pathological SMC growth, herein we examined regulation of α1D-AR expression by growth factors. Platelet-derived growth factor (PDGF)-BB dose- and time-dependently lowered α1D mRNA in cultured quiescent SMCs (e.g., 58% inhibition at 20 ng/ml, 24 h, $p < .05$), whereas other α1-AR transcripts were unaffected. This same selective effect was seen in the medial layer of aorta in ex vivo organ culture. However, PDGF-AA, insulin-like growth factor-1, insulin, epidermal growth factor, endothelin, histamine, and serotonin had no effect, whereas thrombin induced a modest (1.8-fold) increase. PDGF-BB inhibition of α1D-AR mRNA was accompanied by a 42% reduction in total α1-AR density ($p < .05$) and a functional decrease in norepinephrine-mediated protein synthesis. α1D mRNA half-life was not significantly affected by PDGF-BB (3.8 versus 3.2 h). However, transcriptional activity of the α1D promoter was inhibited. Reduction in α1D-AR mRNA depended partly on new protein synthesis, and was abolished by protein kinase C inhibition, whereas phosphatidylinositol 3 kinase and mitogen-activated protein kinase kinase inhibition had no effect. These data demonstrate that PDGF-β receptor stimulation (because PDGF-AA had no effect) induces a selective inhibition of α1D-AR expression and hence norepinephrine-mediated SMC growth. This down-regulation may lessen additive or synergistic growth effects of catecholamines with other growth factors in vascular hypertrophic diseases.

Augmented smooth muscle cell (SMC) growth is central to the development of atherosclerosis, restenosis after angioplasty, neointima hyperplasia after vascular grafting, and hypertensive wall hypertrophy. Indirect evidence has suggested that sympathetic nervous system stimulation of α1-adrenergic receptors (ARs) may exert a trophic influence over SMCs during normal development and also contribute to the pathogenesis of vascular hypertrophic diseases. For example, sympathectomy attenuates vessel growth and wall hypertrophy in normal and hypertensive animals, respectively (Head, 1991). Hyperinnervation of blood vessels by noradrenergic fibers in the genetic spontaneously hypertensive rat correlates with wall hypertrophy and SMC hyperplasia therein (Head, 1991). Chronic systemic administration of α1-AR antagonists, beginning at the time of balloon angioplasty injury, reduces media DNA synthesis by 50% when measured at day 2 in rat aorta (Jackson et al., 1988), and reduces neointimal thickening by 50% when measured at 2 weeks in rat carotid artery (Fingerle el al., 1991) and by 50 and 85% when measured at 2 and 12 weeks, respectively, in rabbit aorta (O'Malley et al., 1989, 1991) after injury. Recently, deBlois et al. (1996) found that infusion of nonepinephrine (NE) or phenylephrine for 2 weeks increased DNA synthesis 6- to 7-fold in rat carotid artery.

In vitro studies argue that the above-mentioned in vivo growth effects may arise from a direct trophic action of α1-ARs, in addition to potential indirect hemodynamic and humoral actions. Thus, activation of α1-ARs, but not α2- or β-adrenoceptors, increases proliferation of subconfluent cultured SMCs (Hu et al., 1996; Yu et al., 1996) and induces hypertrophy in postconfluent cells and intact aorta media (Chen et al., 1995; Xin et al., 1997). We recently demonstrated that, among three α1-ARs expressed by rat thoracic aorta SMCs, the α1D mediates hypertrophy (Xin et al., 1997).

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ABBREVIATIONS: SMC, smooth muscle cell; AR, adrenergic receptor; NE, norepinephrine; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; IGF-1, insulin-like growth factor 1; PKC, protein kinase C; RT-PCR, reverse transcriptase-polymerase chain reaction; PI3, phosphatidylinositol 3; MAPKK, mitogen-activated protein kinase kinase; ANG, angiotensin.

α1D-AR mediation of SMC growth may provide a mechanism to link increases in wall mass to prolonged increases in sympathetic activity and attendant hypertension. This would oppose both an increase in wall stress and reduced catecholamine sensitivity due to αAR desensitization.

Despite this evidence for direct trophic actions of SMC α1-AR stimulation, little is known about whether peptide growth factors modulate α1-AR expression. Peptide growth factors are strongly induced and regulate SMC migration, proliferation, matrix formation, and intimal lesion growth in vascular hypertrophic diseases (Schwartz et al., 1995). For example, platelet-derived growth factor (PDGF) ligands and receptors evidence prolonged activation after balloon injury and are required for full neointimal formation (Panek et al., 1997). A similar up-regulation of PDGF-B is evident in SMCs from growing arteries of postnatal rats (Rafty and Khachigian, 1998) and in adult rat carotid neointimal SMCs (Majesky et al., 1992). Interestingly, expression of the α1D-AR appears to be suppressed in the aorta and peripheral resistance vasculature of young immature rats (Ibara et al., 1997). In addition, binding of the α1 antagonist [^{3}H]prazosin to the intact rat carotid artery evidences an early (day 3) reduction that is sustained after-balloon injury (Bruijns et al., 1998). However, whether SMC α1-AR expression is altered by PDGF or other growth factors that are up-regulated during normal or hypertrophic vessel wall growth is unknown.

Thus, the aim of the present study was to determine if growth factors known to be induced by vascular injury and in the growing vascular wall of young animals alter α1-AR expression. We found that PDGF-BB reduces expression of α1D-, but not α1A- or α1B-ARs through activation of PDGF-β receptors in cultured rat aorta SMCs and in media of ex vivo rat aorta in organ culture. This action was not shared by PDGF-AA, epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), insulin, and several other growth agonists. α1D-AR down-regulation is blocked by protein kinase C (PKC) inhibition, is accompanied by the predicted functional outcome, i.e., reduced NE-mediated SMC growth, and appears to depend on reduced α1D-AR transcription rather than on decreased α1D-AR mRNA stability.

Materials and Methods

Cell Culture. Methods for culture of rat thoracic aorta SMCs, derived from 200-g male Sprague-Dawley rats, have been described in detail (Chen et al., 1995). Confluent SMCs were growth-arrested for 24 h in serum-free, defined media consisting of 50% Dulbecco's modified Eagle's medium, 50% F-12 media supplemented with 5 mg/l transferrin, 35.2 mg/l ascorbic acid, 6 μg/ml selenium, 100 U/ml penicillin, and 100 μg/ml streptomycin. After lifting with 0.10% trypsin-EDTA, SMCs were seeded at 3000 to 5000 cells/cm^2 and used in passages 4 to 6.

RNA Extraction and Reverse-Transcription Polymerase Chain Reaction (RT-PCR). Total cellular RNA was extracted with the acid guanidine thiocyanate-phenol-chloroform method (Eckhart et al., 1996). RNA concentration was determined spectrophotometrically at 260 nm. Purity, assessed according to an A_{260}/A_{280} ratio, was 1.8 to 2.0, and quality was gauged by electrophoresis on a 1.2% agarose gel after denaturation with formaldehyde. Possible genomic DNA contamination was eliminated by treatment of RNA samples with DNase and verified by no-RT controls. Oligonucleotide primers for amplifying rat α1A-, α1B-, and α1D-AR mRNAs were synthesized commercially by BRL (Life Technologies, Inc., Grand Island, NY). Primers were designed to amplify ~200-base pair, third-loop segments of similar location for the three α1-AR subtypes. The sequences for each primer were as follows: α1A-AR sense, 5′-CGAGTCTACGTAGTAGCC-3′; α1A-AR antisense, 5′-GTCTTGGCAGCTTTCTTC-3′; α1B-AR sense, 5′-ATCGTGGCCAAGAGGACC-3′; α1B-AR antisense, 5′-TTTGGCTGCTTTCTTTTC-3′; α1D-AR sense, 5′-GCGTGTACGTGGTCGCAC-3′; α1D-AR antisense, 5′-CTTGGCAGCCTTTTTC-3′. RT-PCR was performed with the single-tube method with *Tth*11 DNA polymerase (Promega Biotec, Madison, WI) for both RT and PCR amplification as described by the manufacturer with modification. Briefly, *Tth*11 DNA polymerase (5 U) was used to synthesize single-stranded cDNA at 65°C, 20 min. Total sample RNA for α1B- and α1D-AR assay was 100 ng/reaction. Because of especially low expression of α1A-AR mRNA, 400 ng of sample RNA was used for RT-PCR of α1A-AR mRNA in each reaction. RT was performed in a 20-μl volume consisting of sample RNA and 1 mM $MnCl_2$, 10 mM Tris-HCl, pH 8.3, 90 mM KCl, 200 μM dNTP for A,T,C,G, and 0.15 μM antisense primer. The reaction was stopped by placing the tubes on ice. Tubes then received chelating buffer [4% (v/v) glycerol, 8 mM Tris-HCl, pH 8.3, 80 mM KCl, 0.04% (w/v) Tween 20, 0.6 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid], 2 mM $MgCl_2$, and 0.15 μM sense primer for one of the three α1-AR subtypes and 1 μCi [^{32}P]dCTP in a total volume of 100 μl for 35 cycles at 94°C and 62°C for 15 and 30 s, respectively. Twenty microliters of the PCR products was subjected to electrophoresis in 1.2% agarose gel. The amplified ethidium bromide-stained products were then photographed, excised, and counted with a Wallac 1450 Micro-β-counter. RT-PCR of cyclophilin (30 cycles against 10-ng RNA) was included for each RNA sample as an internal control to which all α1-AR mRNA scintillometry values were normalized; shorter film exposures for cyclophilin are given in figures (Clements and Faber, 1997; Xin et al., 1997). With cloned fibroblast cell lines each stably expressing one of the three α1-AR subtypes, together with positive and negative control tissues, we have verified with quantitative, competitive RT-PCR that the above-mentioned relative RT-PCR α1-AR assays (using the above-mentioned sample RNA amounts and cycle numbers) have comparable efficiencies of amplification and yield products that are on the midpoint of the linear portion of the curve relating cycle number to amount of product (Yang et al., 1999). We also have verified that our RT-PCR assay for cyclophilin yields a product that is on the midpoint of the linear portion of its curve in SMCs. All RT-PCR products were verified by sequencing.

Measurement of α1-AR Density. Radioligand binding assays were performed as described (Eckhart et al., 1996). SMC crude membrane protein was collected after 24 h of PDGF-BB or vehicle treatment, and protein concentration was measured by the BCA method (Pierce Chemical Co., Rockford, IL). Saturation binding was determined with [^{3}H]prazosin (New England Nuclear, Boston, MA) (0.01–3.0 nmol/l). Each sample was assayed at 4°C in duplicate with 180 μg of crude membrane protein. Phentolamine at a final concentration of 10 μmol/l was used to determine nonspecific binding (<15% at the K_d). Reactions were incubated for 45 min at 25°C, immediately filtered through Whatman GF/C filters with a Millipore filter manifold, and washed three times. Filters were dried, placed in Ecoscint H (National Diagnostics, Inc., Manville, NJ), and counted in a liquid scintillation counter.

Intact Aorta Organ Culture. Rat thoracic aortae were isolated as described previously (Chen et al., 1995; Eckhart et al., 1996; Clements and Faber, 1997) and allowed to equilibrate for 3 h in 10% serum-containing medium at 37°C in a 5% CO_2 incubator. After a change to serum-free defined medium, vessels were treated with 20 ng/ml PDGF-BB for 24 h. Vessels were then rinsed with fresh media and incubated for 23 min at 37°C in an enzyme solution, followed by microdissection of the media layer from adventitial layer at 4°C. Endothelial cells were removed by gentle rubbing with a cotton-tipped applicator, with virtually none remaining as revealed by histochemistry (Eckhart et al., 1996). Media was frozen in liquid N_2,

powered, and RNA extracted. More than 99% of the cells present in the dissected medial layer are SMCs, based on size, morphology, and α-smooth muscle actin staining after enzymatic dispersion; the other <1% of freshly dispersed cells are one-third the diameter of the SMCs and are probably adherent adventitial fibroblasts.

Protein Synthesis. Protein synthesis was measured by [^{35}S]methionine incorporation (Xin et al., 1997). Confluent SMCs were growth arrested for 24 h in serum-free defined medium and then pretreated with PDGF-BB (20 ng/ml), EGF (20 ng/ml), or vehicle for 24 h, washed, and exposed for 24 h to 1 μM NE in media containing low methionine (2 mg/l), and 100 μM ascorbate. [^{35}S]methionine (1 μCi/ml, 1000 Ci/mmol; Amersham Corp., Arlington Heights, IL) was added 6 h before cell harvest, and incorporation was measured. Vehicle-exposed control cells were treated identically.

***α*1D-AR mRNA Stability.** Stability of α1D-AR mRNA was measured as described (Eckhart et al., 1996). Confluent SMCs were serum-deprived for 24 h and then exposed to PDGF-BB (20 ng/ml) or vehicle for 12 h. Transcription was then arrested by addition of actinomycin D or 5,6-dichloro-1-D-ribofuranosyl benzimidazole at final concentrations of 5 μg/ml or 75 μmol/l, respectively. These concentrations have been shown to inhibit mRNA transcription by >95% without compromising cellular integrity (Eckhart et al., 1996). The cells were incubated for up to 8 h after addition of either agent, and total RNA was extracted and subjected to RT-PCR.

Transcription Analysis. A 1.6-kb 5′-flanking region of the rat α1D-AR gene was cloned from a rat liver genomic library, sequenced (Genbank accession no. AF071014) and fused into the promoter/enhancer-less pGL3 Basic vector (Promega Biotec). This DNA construct was transiently transfected (5 μg/well) by calcium phosphate precipitation into confluent aorta SMCs cultured in 6-well plates, along with β-galactosidase luciferase vector (1.5 μg/well) to control for transfection efficiency (Eckhart et al., 1997). After 8 h, the cells were washed twice and maintained in serum-free defined media with or without addition of PDGF-BB (2, 20, 50 ng/ml), IGF-1 (80 ng/ml), EGF (20 ng/ml), or thrombin (10 nM) for 24 h. Luciferase activities from cellular extracts were determined with the luciferase assay system (Promega Biotec) as described by the manufacturer and were normalized to β-galactosidase activity. pGL3 control vector containing the simian virus 40 promoter and enhancer was transfected in each experiment in additional plates to monitor transcription efficiency.

Statistical Analysis. InPlot (GraphPad Software, San Diego, CA) was used to analyze the radioligand-binding data. mRNA half-life determination was based on first-order kinetics and linear regression analysis with Instat (GraphPad Software). Data are given as means ± S.E. Differences were analyzed by *t* test and/or ANOVA, followed by the Bonferroni test for multiple comparisons. A value of $p < .05$ was considered significant.

Results

PDGF-BB Specifically Inhibits α1D mRNA Expression in Cultured SMCs. We first examined the effect of growth factors on α1D-AR mRNA in 1-day postconfluent, quiescent SMCs. PDGF-BB (20 ng/ml) rapidly decreased α1D mRNA by 58% after 24 h. In contrast, the same concentration of EGF and IGF-1 and 100 nM insulin did not affect α1D mRNA expression (Fig. 1, top). Higher concentrations of IGF-1 (50–100 ng/ml) induced a small increase in α1D mRNA (maximum 124% of control, $p < .05$, $n = 3$–5; data not shown). mRNA for cyclophilin was unaffected by these growth factors or other stimuli tested in this or the other experiments described in *Results*, and was therefore used as an internal control. PDGF-BB-induced α1D down-regulation was dose- and time-dependent (Fig. 1, bottom). Inhibition was evident at 10 ng/ml when measured at 24 h, was detected within 4 h of exposure to 20 ng/ml, and reached a maximal response at 8 to 12 h for 20 ng/ml.

PDGF-BB and the other agents tested in Fig. 1 are known mitogens for subconfluent SMCs, and for confluent SMCs particularly if their competence is promoted by the presence of serum or insulin. Although only PDGF-BB reduced α1D mRNA, we nevertheless examined whether this PDGF-BB action was dependent on a growth effect possibly not shared by the other growth factors tested. Confluent quiescent SMCs in serum-free media (without insulin) were treated with 20 ng/ml PDGF-BB, IGF-1, and EGF for 24 h [the approximate time point for maximal PDGF-BB-induced decrease in α1D-AR mRNA (and receptor number; see below)]. Cell number, protein, and total RNA were then measured. All three growth factors elicited a similar modest stimulatory effect on cell number (112–130% of control), protein (116–138% of control), and total RNA (110–130% of control) per plate ($n = 2$–3 for each growth factor; data not shown). Thus, receptors for the growth factors were present and concentrations were effective for modest growth effects of these agents, yet only PDGF-BB inhibited α1D-AR mRNA. We also tested several G protein-coupled receptor agonists that reportedly

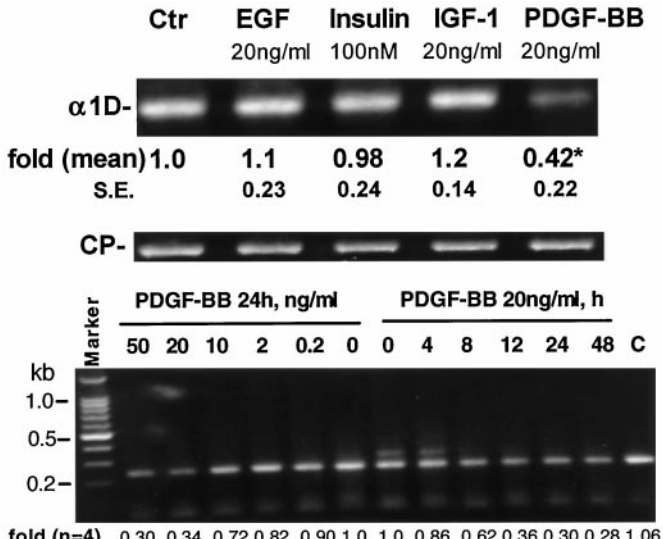

Fig. 1. Top, PDGF-BB selectively inhibits rat α1D-R mRNA by SMCs. Confluent rat aorta SMCs in serum-free media (without insulin) were exposed to EGF (20 ng/ml), isulin (100 nM), IGF-1 (20 ng/ml),or PDGF-BB (20 ng/ml) for 24 h. Control groups (Ctr) received vehicle treatment. Total RNA was isolated and subjected to RT-PCR. RT-PCR for cyclophilin (CP) mRNA was included as internal control. PCR product bands were excised from gels for radioisotope measurement. α1DAR mRNA values were normalized to cyclophilin and expressed as fold changes from time-matched controls. Data are a representative autoradiogram and means ± S.E. from four independent experiments. *$p < .05$ versus control (two-tailed *t* test). High concentrations of IGF-1 (50–100 ng/ml) also had no significant effect on α1D-AR mRNA. Bottom, PDGF-BB dose- and time-dependently inhibits α1D-AR mRNA expression. Confluent serum-deprived SMCs were exposed to PDGF-BB at the doses (left, 0–50 ng/ml) and times (right, 0–48 h) indicated, and examined with RT-PCR as in (top). Fold change (average from four independent experiments) represents the relative cpm between PDGF-BB-treated and vehicle-treated "0 h" groups after normalization to cyclophilin (CP). C in the representative autoradiogram is the 48-h time-matched and vehicle-treated group. DNA marker was included to indicate the 218-base α1D PCR product. *$p < .05$ versus control (ANOVA plus Bonferroni two-tailed *t* test).

stimulate proliferation of competent rat aorta SMCs at the selected concentrations. Endothelin-1 (20 nM, 8 h, $n = 2$), histamine (1 μM, 8 h, $n = 2$), and serotonin (1 μM, 24 h, $n = 2$) had no effect on α1D mRNA expression. Angiotensin II (ANG II) (100 nM, 24 h, $n = 2$) and PGF2α (1 μM, 8 h, $n = 2$) reduced α1D mRNA by 25 and 35%, respectively. The ANG II effects confirm our previous findings (Clements and Faber, 1997). In contrast, thrombin (10 nM, 8 h, $n = 2$) caused a 1.8-fold increase in α1D mRNA (data not shown). Collectively, these results demonstrate that PDGF-BB specifically mediates down-regulation of α1D-AR mRNA.

PDGF-BB Does Not Inhibit α1A- or α1B-AR mRNA in Cultured SMCs. Cultured quiescent SMCs express all three α1-AR mRNAs at different levels (α1D, α1B, and α1A levels by quantitative RT-PCR are 115:6:1, respectively) (Yang et al. 1999). We therefore examined whether PDGF-BB inhibition is selective for α1D-AR mRNA expression. SMCs were treated with PDGF-BB (20 ng/ml; 24 h) and relative α1-AR mRNA levels were detected by RT-PCR. PDGF-BB caused significant inhibition of α1D-AR but had no effect on α1B, or α1A-AR mRNAs (Fig. 2, top).

PDGF-BB Down-Regulates α1D mRNA in Ex Vivo Rat Aorta Organ Culture. To determine if the above-mentioned results in cell culture mimic the response of SMCs in the intact vascular wall, ex vivo rat thoracic aorta were maintained in organ culture and exposed to 20 ng/ml PDGF-BB for 24 h. The medial SMC layer was then separated from adventitia and intima layers (see *Materials and Methods*). Media RNA was extracted and analyzed by RT-PCR. As shown in Fig. 2 (bottom), PDGF-BB decreased α1D mRNA by 40% at 24 h in the media layer. However, α1B-AR mRNA was unaffected. Thus, rat aorta SMCs in primary culture behave similarly to SMCs in the intact vascular wall for PDGF-BB repression of SMC α1D mRNA. In our previous studies with RNase protection assays, α1A was not detectable in media from fresh rat aorta or in cultured SMCs from the same vessel with up to 100 μg of RNA (Chen et al., 1995; Eckhart et al., 1996, 1997; Clements and Faber, 1997). However, we are able to detect α1A transcripts with RT-PCR (Fig. 2, top), although 4-fold more RNA is required than for α1B or α1D measurement. For this reason and because α1A was not affected by PDGF-BB (Fig. 2, top), we did not assay for α1A in media of vessels maintained in organ culture.

PDGF-BB Decreases α1-AR Density. To test whether PDGF-BB inhibition of α1D-AR mRNA reduces receptor density, [^{3}H]prazosin-binding assays were performed to detect total α1AR density. All saturation-binding assays best fit a one-site model. Nonspecific binding increased linearily, was $<$15% of total binding at the K_d, and equaled 35% at saturation (1 nM). As shown by Fig. 3, PDGF-BB (20 ng/ml; 24 h) decreased maximum specific binding (B_{max}) by 42% compared with vehicle-treated, time-matched control cells (from 23.6 to 13.8 fmol/mg). α1-AR affinity (K_d) for [^{3}H]prazosin was unaffected by PDGF-BB treatment (204 versus 184 pmol/l). Because PDGF-BB did not affect α1B- and α1A-AR mRNAs (Fig. 2, top) and α1D appears to be the dominant α1-AR (80%) expressed in cultured SMCs (Eckhart et al., 1996), these results suggest that PDGF-BB inhibition of α1D-AR mRNA is associated with a down-regulation of α1D-AR density. However, the low basal expression of total α1ARs, effect of PDGF-BB to decrease expression, and limited availability of cell membrane and selective antagonists for all three subtypes, precluded determination of individual subtype densities.

Down-Regulation of α1D-AR Expression Results in Functional Inhibition of NE-Induced SMC Protein Synthesis. We previously demonstrated that the α1D-AR mediates SMC protein synthesis and hypertrophy induced by NE (Xin et al., 1997). Thus, the functional significance of α1D down-regulation by PDGF-BB was assessed for NE-induced protein synthesis in cultured SMCs. Pretreatment for 24 h with 20 ng/ml PDGF-BB, followed by 24-h exposure to NE alone, attenuated dose-dependent NE-induced protein synthesis (Fig. 4, left). In contrast, pretreatment with 20 ng/ml EGF for 24 h, which had no effect on α1D-AR mRNA (Fig. 1, top), did not attenuate but instead augmented the growth response induced by NE (Fig. 4, right). These results demonstrate that PDGF-BB inhibition of α1D-AR expression leads to a reduction in NE-mediated SMC growth.

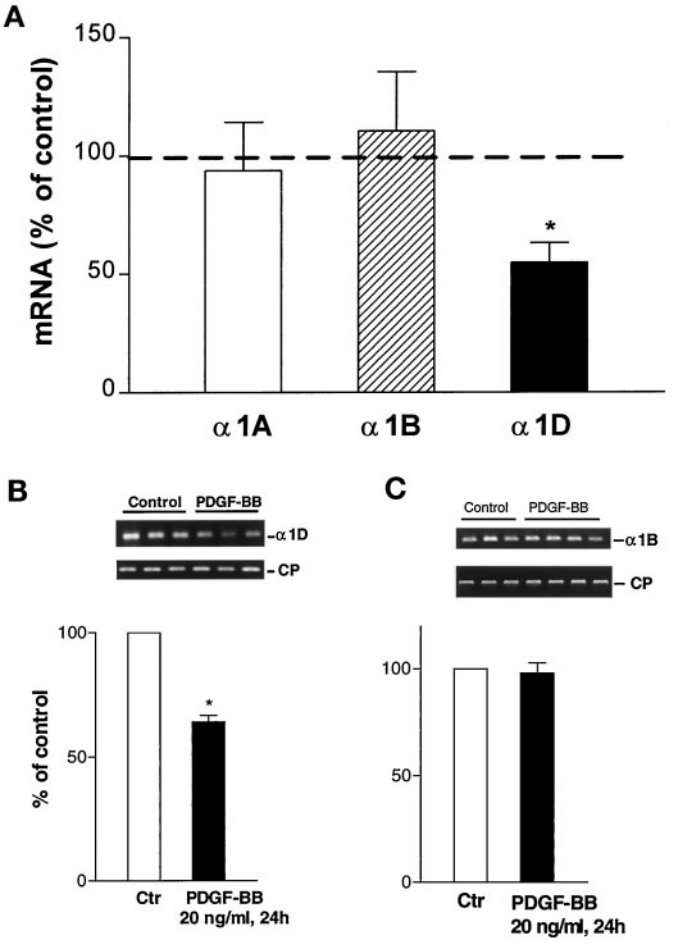

Fig. 2. Top, PDGF-BB selectively inhibits α1D-AR mRNA in cultured SMCs. Confluent serum-deprived SMCs were exposed to 20 ng/ml PDGF-BB or vehicle for 24 h and subjected to RT-PCR. Each bar represents the mean ± S.E. percentage of change of PDGF-BB-treated versus vehicle-treated group after normalization to cyclophilin (CP) from four independent experiments. *$p < .05$ versus control (one-tailed t test). Bottom, PDGF-BB reduces α1D-AR mRNA expression ex vivo. Rat thoracic aorta were maintained in organ culture in serum-free media (without insulin), and received 20 ng/ml PDGF-BB or vehicle (Ctr) for 24 h. Medial layer was carefully separated from adventitia and intima and assayed by RT-PCR. Changes in α1D- (A) and α1B-AR (B) mRNAs are given as mean ± S.E. percentage of control groups after normalization to cyclophilin (CP). *$p < .05$ versus control group (one-tailed t test).

PDGF-β Receptor Mediates α1D-AR Down-Regulation. PDGF is a dimer of two proteins, A and B, capable of forming three ligands (AA, AB, and BB) that bind and dimerize two PDGF receptors, α and β, in either homo- or heterodymeric pairs. The cytosolic domains of the two receptors are different, and differences in signal transduction pathways, as well as gene transcription, also have been observed (Hughes et al., 1996). To elucidate the mechanism of PDGF-BB-induced α1D-AR down-regulation, we first studied which PDGF receptor(s) mediates this inhibition. PDGF-A only binds α receptors, whereas the PDGF-B can bind either β- or α-receptors. We therefore compared the effect of PDGF-AA on expression of α1D mRNA. PDGF-AA (10–500 ng/ml; 24 h) had no effect on α1D mRNA expression. Exposure for 24 h to PDGF-AA at 10, 20, 100, 300, and 500 ng/ml caused 1.12-, 0.98-, 1.14-, 1.02-, and 1.06-fold changes, respectively, over control ($p > .05$ for all; $n = 4$). Cyclophilin expression also was not affected by PDGF-AA and was used to normalize α1D-AR mRNA signal. These data suggest that PDGF-β receptor activation, in either the αβ- and/or ββ-dimeric forms, signals inhibition of α1D-AR expression.

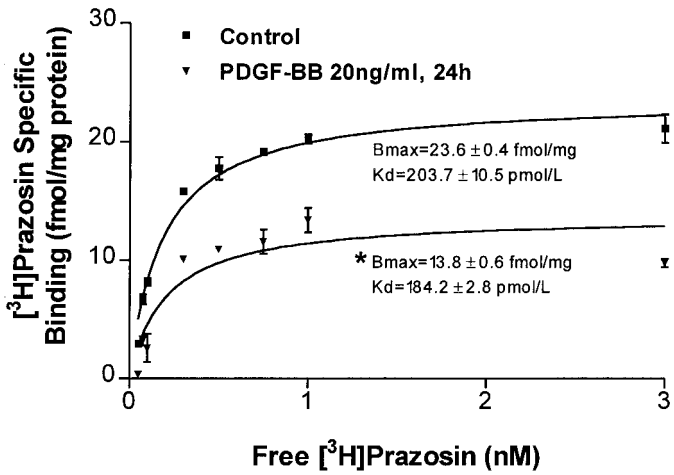

Fig. 3. PDGF-BB down-regulates α1-AR density in cultured SMCs. Radioligand-binding assays were performed with [^{3}H]prazosin and 180 μg of crude membrane protein from cultured aorta SMCs. Confluent serum-deprived SMCs were exposed to 20 ng/ml PDGF-BB or vehicle for 24 h. Receptor density (B_{max}) and dissociation constant (K_d) were analyzed with InPlot (GraphPad Software). Data are represented as means ± S.E. from three independent experiments. *$p < .05$ versus control (two-tailed t test).

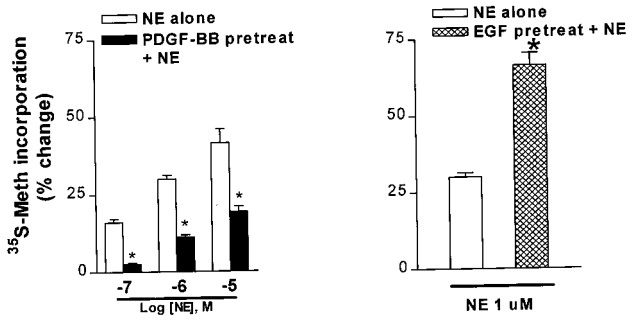

Fig. 4. PDGF-BB-mediated α1D-AR down-regulation attenuates NE-induced SMC growth. Confluent serum-deprived SMCs were pretreated with PDGF-BB (20 ng/ml; left) or EGF (20 ng/ml; right) for 24 h, followed by a 24-h exposure to concentrations of NE as indicated. [^{35}S]methionine incorporation was measured as described in *Materials and Methods*. Results normalized to vehicle-treated, time-matched groups. Data K_d expressed as means ± S.E. from three to four independent experiments. *$p < .05$ versus NE alone (left, ANOVA plus Bonferroni test; right, two-tailed t test).

Postreceptor Signaling of PDGF-BB Down-Regulation of α1D Expression. Intracellular signals arising from PDGF receptor activation are not fully elucidated. Based on studies using a variety of cell types, at least three major pathways, Ras, phosphatidylinositol 3 (PI3) kinase, and PKC, couple to activation of the PDGF-β receptor (Hughes et al., 1996). To begin to investigate the signal transduction pathway activated by the PDGF-β receptor that reduces α1D-AR expression, SMCs were pretreated for 1 h with the selective antagonists tyrphostin 9A (inhibitor of PDGF receptor tyrosine kinase phosphorylation), PD 98059 [mitogen-activated protein kinase kinase (MAPKK) inhibitor], wortmannin (PI3 kinase inhibitor), and calphostin C (PKC inhibitor), followed by addition of 20 ng/ml PDGF-BB or vehicle for 24 h (Fig. 5). Concentrations of these inhibitors were chosen from previous articles of their specificity from this (Xin et al., 1997) and other (Jarvis et al., 1994; Burger et al., 1995; Dudley et al., 1995; Ui et al., 1995; Servant et al., 1996) laboratories (additional references given in Calbiochem catalog): enzyme inhibitory IC_{50} values for tyrphostin 9A = 500 nM, PD 98059 = 2 uM, wortmannin = 5 to 10 nM, and calphostin C = 50 nM. The reduction of α1D mRNA by PDGF-BB (antagonist vehicle group) was blocked by pretreatment with tyrphostin 9A (Fig. 5), confirming the requirement for PDGF receptor tyrosine phosphorylation for this down-regulation. Calphostin C also blocked the PDGF-BB inhibition, suggesting that activation of a PKC isoform(s) is required. Calphostin C is significantly less potent for blockade of other kinases (IC_{50} values >5–50 μm for PKA, PKG, etc.; references given in Calbiochem catalog). In contrast, PD 98059 and wortmannin did not attenuate PDGF-BB-induced down-regulation even at concentrations 10- to 500-fold greater than their IC_{50} values, suggesting that the MAPKK/MAPK and PI3 kinase pathways are not involved.

PDGF-BB Does Not Significantly Decrease α1D-AR mRNA Half-Life. To determine whether PDGF-BB inhibition of α1D-AR expression is through increased degradation of mRNA, we measured transcript half-life ($T_{1/2}$) for the α1D-AR. SMCs were pretreated with 20 ng/ml PDGF-BB or vehicle for 12 h. Then α1D mRNA was measured at 0, 1, 2, 4, and 8 h in the presence of transcription inhibitors actinomycin D (5 μg/ml) or 5,6-dichloro-1-D-ribofuranosyl benzimidine (75 μM). Half-life of α1D mRNA for vehicle-treated SMCs was 3.8 h, which confirms our previous findings (Eckhart et al., 1996). PDGF-BB did not significantly alter $T_{1/2}$ (3.2 h)

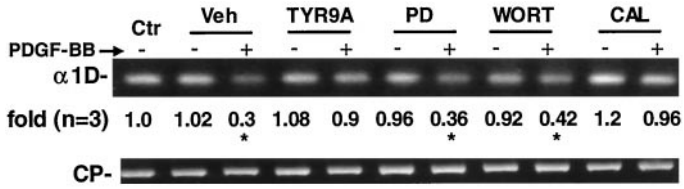

Fig. 5. Calphostin C attenuates PDGF-BB-mediated reduction of α1D-AR mRNA. Confluent serum-deprived SMCs were pretreated with tyrphostin 9A (TYR9A; 10 uM), PD 98059 (PD; 20 uM), wortmannin (WORT; 1 uM), or calphostin C (CAL; 125 nM) for 1 h followed by addition of 20 ng/ml PDGF-BB or vehicle (Veh) for 24 h. Total RNA was subjected to RT-PCR as in Fig. 1. Data are from three to four independent experiments and represented as average fold change after normalization to cyclophilin (CP). *$p < .05$ versus vehicle-treated groups (one-tailed t test).

(Fig. 6, left). Time-matched controls showed that α1D-AR mRNA levels did not change over 48 h in culture.

PDGF-BB Repression of α1D-AR mRNA Partly Requires New Protein Synthesis. Transcriptional regulation requires interaction of existing and/or nascent nuclear transcription factor proteins. To explore whether new protein synthesis is required for PDGF-BB down-regulation of α1D mRNA, SMCs were pretreated with the protein synthesis inhibitor cycloheximide (25 μg/ml) for 45 min, and then treated with PDGF-BB or vehicle for 12 h. Cycloheximide partially attenuated PDGF-BB reduction of α1D mRNA (Fig. 6, right), and alone had a small inhibitory effect on SMC protein synthesis. These data suggest a partial requirement of new protein synthesis for α1D down-regulation by PDGF-BB.

PDGF-BB Lowers Transcriptional Activity of α1D Gene Promoter-Reporter Construct. We then examined whether transcription of the α1D gene might be reduced by PDGF-BB. A 1.6-kb fragment of the 5′-flanking region of α1D gene was cloned, sequenced, and fused into a luciferase reporter gene (pGL3 Basic vector) (Xin et al., 1999). SMCs were then transiently transfected with this α1D 5′-flanking region construct, along with β-galactosidase plasmid to normalize for transfection efficiency. The 5′-flanking region exhibited strong promoter activity (10-fold over pGL3 basic). Treatment of SMCs with 20 ng/ml PDGF-BB for 24 h dose-dependently decreased transcriptional activity of the reporter construct (Fig. 7). Similar to their absence of effect on α1D mRNA levels (Fig. 1, left), however, EGF and IGF-1 had no effect on transcriptional activity (Fig. 7). These results suggest that PDGF-BB represses α1D transcription and further confirm absence of correlation of α1D repression with the modest SMC growth induced by these growth factors (see above).

Discussion

The major findings in this study are that PDGF-BB, acting through the PDGF-β receptor and a presumed PKC (i.e., calphostin C-dependent) pathway, inhibits α1D-AR mRNA and receptor expression by ~50% after a 24-h exposure in cultured rat SMCs and ex vivo aorta organ culture. PDGF-AA, EGF, IGF-1, and insulin, as well as thrombin, endothelin, histamine, and serotonin had no effect. Moreover, PDGF-BB had no effect on α1A-AR, α1B-AR, or cyclophilin mRNA levels, indicating the selectivity of the response for the α1D-AR. Reduction in α1D-AR expression by PDGF-BB appears to extend from diminished α1D-AR gene transcrip-

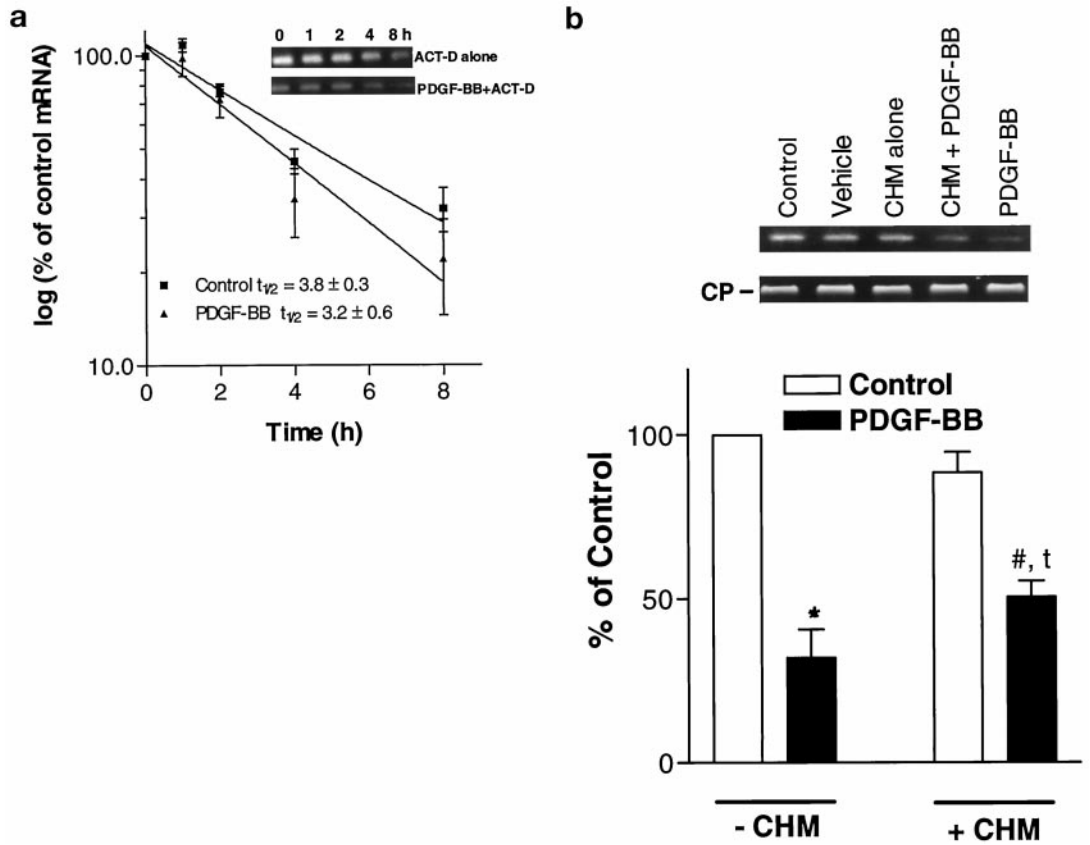

Fig. 6. Left, PDGF-BB does not alter stability of α1D-AR mRNA. Confluent serum-deprived SMCs were treated with PDGF-BB (20 ng/ml; △) or vehicle (control; ■) for 12 h before addition of actinomycin D (ACT-D; 5 μg/ml) or 5,6-dichloro-1-D-ribofuranosyl benzimide (75 μmol/l). Total RNA was extracted at the indicated times after their addition. RT-PCR was performed and the cyclophilin-corrected values were plotted as a percentage of control to determine mRNA half-life ($T_{1/2}$) in hours. Values are means ± S.E. from five independent experiments (representative autoradiogram shown). Right, PDGF-BB repression of α1D-AR mRNA partly requires new protein synthesis. Confluent serum-deprived SMCs were pretreated with cycloheximide (CHM; 25 μg/ml) or its vehicle (control; first white bar) for 45 min and then PDGF-BB (20 ng/ml) or its vehicle (vehicle; second white bar) for 12 h. Total RNA was extracted and assayed by RT-PCR. Cyclophilin expression (CP) served as an internal control for normalization. Results are means ± S.E. from four independent experiments (representative autoradiogram shown). Two-way ANOVA: *$p < .01$ versus control without CHM pretreatment; #$p < .05$ versus control with CHM pretreatment; †$p < .05$ versus PDGF-BB without CHM pretreatment.

tion that is partly dependent on new protein synthesis. This down-regulation results in a functional outcome, i.e., reduced catecholamine-induced SMC growth, consistent with our previous results that α1D-AR stimulation directly mediates SMC growth (Xin et al., 1997). α1D-AR-mediated SMC hypertrophy does not appear to depend on release of a soluble growth factor because we found no effect of conditioned medium, collected from cells treated with 1 μM NE for 8 or 24 h, on protein synthesis by naive SMCs ($n \geq 6$ for all groups) (Xin, 1998).

PDGF-BB-mediated inhibition of α1D-AR expression is not simply a consequence of growth factor-induced SMC proliferation. PDGF, EGF, IGF-1, and insulin are all mitotic for SMCs and other mesenchymal cells in vitro (Jawien et al., 1992; Giannella-Neto et al., 1992; Ko et al., 1993; Nickenig and Murphy, 1994; Nickenig et al., 1996; Dixon et al., 1996), depending on the competence of the cells for growth as determined by cell confluence, phenotype, and presence of other factors in the media such as serum or insulin. We compared the growth effects of these growth factors with their effect on α1D-AR gene expression in the same postconfluent serum/insulin-free conditions, and found that they all caused a similar modest growth stimulation (10–38% increase) of SMC number and/or total protein and/or RNA per plate. However, only PDGF-BB lowered α1D expression (Fig. 1), demonstrating the specificity of the response. This finding is different from the reduction in AT_{1A} ANG II receptor mRNA and protein in serum-deprived rat aorta SMCs that was produced by 50 ng/ml PDGF-BB and also 20 ng/ml EGF and bFGF (Nickenig and Murphy, 1994). Also different from our study, recent evidence has shown that this AT_{1A} receptor decline induced by these heterologous growth factors, is correlated with their induction of SMC proliferation and involves a decline in both AT_{1A} mRNA transcription and stability (Nickenig et al., 1996). Although both AT_{1A} and α1D-ARs have been implicated in SMC growth, the proliferation-associated decline in AT_{1A} versus proliferation-independent decline in α1D-ARs induced by PDGF-BB suggest a different influence on AT_{1A} receptor versus α1D-AR expression in conditions in vivo when local PDGF ligands are augmented but SMC proliferation arrested (e.g., after the initial wave of SMC proliferation during balloon or in-stent restenosis). The selectivity of PDGF-BB inhibition of α1D-AR expression, as suggested by lack of effect on α1A-AR, α1B-AR, and cyclophilin transcripts, is further supported by an opposite effect on the G protein-coupled bradykinin B2 receptor. PDGF-BB (but not -AA) and EGF (all at 20 ng/ml) increased bradykinin receptor expression in rat arterial SMCs by a PKC-dependent mechanism that was completely blocked by transcriptional inhibitors but unaffected by cycloheximide (Dixon et al., 1996).

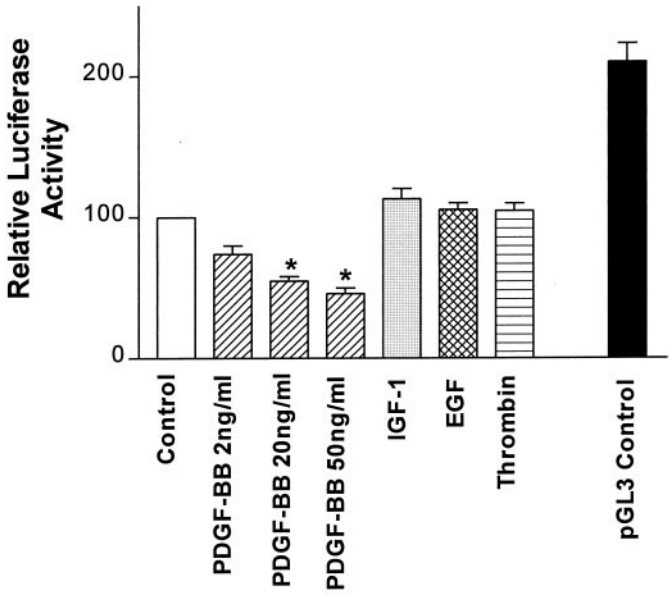

Fig. 7. PDGF-BB decreases α1-AR gene promoter activity. A 1.6-kb 5′-flanking region of the rat α1D-AR gene was cloned and fused into the promoter/enhancer-less pGL3 Basic vector. Confluent SMCs were then transfected with the construct (5 μg/well) plus β-galactosidase plasmid to normalize for transfection efficiency. After 8 h of transfection, cells were exposed for 24 h to PDGF-BB (2, 20, 50 ng/ml), IGF-1 (100 ng/ml), EGF (20 ng/ml), or thrombin (10 nM; 8 h). Luciferase activities in treated groups are expressed as percentage of time-matched control (white bar) that received vehicle. Basal activity of α1D 5′-flanking region (control bar) was ~10-fold higher than activity of pGL3 Basic vector alone. pGL3 control (black bar) represents luciferase activity of cells transfected with plasmid containing the simian virus 40 promoter and enhancer. Data (means ± S.E.) are from four to six independent experiments. *$p < .05$ versus control (ANOVA plus Bonferroni test).

Our finding that α1D-AR mRNA expression was minimally increased (24%) by IGF-1 is less than the reported 3-fold up-regulation of α1D-AR mRNA (as assessed by RNase protection) in rat aorta SMCs by similar levels of IGF-1 as used in the present study (Hu et al., 1996). Besides differences in assay methods, the variance in the two studies may relate to differences in culture conditions (postconfluent serum-free in our study versus subconfluent in Hu et al., 1996). Consistent with this possibility, Giannella-Neto et al. (1992) showed that as rat aorta SMCs reach confluence, expression of insulin/IGF-1 receptor strongly declines, whereas expression of IGF-binding protein 4, which inhibits IGF-1 actions on SMCs, is induced.

We used enzymatic blockade to obtain a preliminary indication of the intracellular signal transduction pathways activated by PDGF-BB that mediate suppression of α1D expression. PDGF receptor dimerization, and its subsequent autophosphorylation by the intrinsic tyrosine kinase portion of the receptor, triggers several intracellular signaling pathways, chiefly those led by MAPKK, PI3 kinase, and PKC (Hughes et al., 1996). The specific inhibitor of PDGF receptor tyrosine kinase activity, tyrphostin 9A, blocked PDGF-BB-induced α1D down-regulation, demonstrating the requirement of PDGF receptor phosphorylation for this response. This is contrary to the proliferation-associated EGF-induced down-regulation of AT_{1A} receptors in SMCs that that does not require EGF receptor autophosphorylation (Ullian et al., 1997). The well known mitogenic action of PDGF is dependent on PDGF receptor dimerization and phosphorylation, subsequent activation of membrane-bound Ras, and in turn, activation of Raf/MAPKK/mitogen-activated protein K and PI3 kinase pathways leading to altered gene expression (Hughes et al., 1996). However, in contrast to PDGF-induced cell proliferation, both PD 98059 and wortmmanin (at concentrations 10- to 500-fold higher than their IC_{50} values) did not affect PDGF-BB-induced α1D down-regulation, indicating MAPKK and PI3 kinase pathways do not mediate this response. The complete blockade by calphostin C of PDGF-BB-mediated α1D down-regulation suggests a requirement of PKC activation, although this needs to be confirmed by

experiments to up- and down-regulate PKC and to identify the specific isoform(s) involved. In other studies, we have evidence that PDGF-BB induces activator protein-2 binding to a *cis* element within the proximal α1D gene promoter that leads to repression of transcription (Xin et al., 1999). Consistent with this, PKC activation has been reported to increase activator protein-2 binding to *cis* elements in several other genes in other cell types (Imagawa et al., 1987; Hyman et al., 1989).

PDGF-BB down-regulation of α1D-AR expression may be involved in the differences in α1-AR expression in the growing arteries of immature versus adult animals. Rat aorta SMCs cultured from 2-week-old rat pups express high levels of PDGF-B ligand and mRNA, whereas in SMCs from 12-week-old adults both are low (Rafty and Khachigian, 1998). Unlike adults, pup SMCs also secrete PDGF-like activity and do not require serum (enriched in PDGF) for growth (Majesky et al., 1990a). α1-AR expression changes with maturation although the responsible mechanisms are unknown. For example, contraction of aorta from the 1-month-old immature rat exhibits α1B-like pharmacology (Gurdal et al., 1995), whereas contraction in the adult is mediated by the α1D-AR (Goetz et al., 1995). Similar pharmacological evidence for reduced α1D-AR expression in immature rats also has been reported for resistance vessels (Ibara et al., 1997). It is possible that the PDGF-BB down-regulation of α1D-AR expression, identified in the present study, serves as an ontogenic switch in α1D-AR expression in the maturing vasculature.

Reduced α1D-AR expression by PDGF-BB also may be important in modifying effects of catecholamines in vascular wall hypertrophic diseases, such as in intimal hyperplasia and restenosis after angioplasty. For example, several studies have shown that systemic pharmacological α1-AR blockade reduces SMC growth and neointimal lesion formation after balloon angioplasty injury (Jackson et al., 1988; O'Malley et al., 1989, 1991; Fingerle et al., 1991), suggesting that α1-AR stimulation augments restenosis. In normal rat carotid, mRNA and protein for PDGF-A and PDGF-B were reportedly very low (Majesky et al., 1990a); protein for PDGF-α receptor was present, whereas β-receptor was undetectable (Panek et al., 1997). PDGF-A and -B mRNA content did not change during 4 weeks after injury (Majesky et al., 1990b); protein and phosphorylation state changed little for PDGF-α receptor, whereas PDGF-β receptor activation, i.e., protein phosphorylation state and association with P85-PI3 kinase, increased dramatically over day 2 to day 5 and remained high through day 20 studied (Panek et al., 1997). Injury increased PDGF-B and receptor β-mRNA 4- to 7-fold by day 4 and remained similarly elevated on day 21, whereas PDGF-A and receptor-α were little affected in rabbit femoral artery (Uchida et al., 1996). Collectively, these studies show in different species that PDGF-BB and receptor-β are strongly induced and follow a time course of maintained induction for several weeks after balloon injury. This is consistent with evidence for a prominent role of PDGF in wall repair and neointimal growth after angioplasty (Jawien et al., 1992).

Thus, according to our current findings, PDGF-β receptor stimulation after injury would promote down-regulation of α1D-AR expression. Consistent with this, 2-week infusion of NE, which increases arterial pressure by 20 mm Hg, between 3 and 5 weeks after balloon injury induced less DNA synthesis in the injured than noninjured rat carotid media (deBlois et al., 1996). And binding of 0.3 nM [^{3}H]prazosin to the intact rat carotid artery evidences an early (3 day) ~50% reduction that is sustained for at least 20 weeks after balloon injury (Bruijns et al., 1998). Moreover, we have found with quantitative RT-PCR that α1D-AR mRNA and α1AR receptor density in rat carotid media and neointima is significantly decreased by 50–60% at 4, 21, and 42 days after balloon injury (Faber et al., 1999). However, despite this down-regulation the prevailing α1-ARs may still contribute to restenosis (Jackson et al., 1988; O'Malley et al., 1989, 1991; Fingerle et al., 1991).

In summary, our results suggest that PDGF-BB potently reduces α1D-AR expression at the message, protein, and functional growth-promoting level in association with a reduction in transcription. Additional studies are required to test the hypothesis that this down-regulation may prevent excessive sympathetic influence on vascular wall growth when local autocrine/paracrine PDGF-β receptor stimulation is high, such as in the growing or injured vessels. Moreover, potential additive or synergistic growth effects between α1D-AR and peptide growth factor stimulation (e.g., NE plus EGF, Fig. 6; Bobik et al., 1990; Majesky et al., 1990a) could still permit sympathetic catecholamines to contribute importantly to growth of the immature artery and worsen excessive growth after injury, especially if this potential negative feedback mechanism is compromized.

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