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Nicotinic and PDGF-Receptor Function are Essential for Nicotine-Stimulated Mitogenesis in Human Vascular Smooth Muscle Cells

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Abstract Cigarette smoking is implicated in the formation of occlusive vascular diseases. Nicotine's role in this process is incompletely understood. Nicotine's effect on human aortic vascular smooth muscle cells (HaVSMC) and the role of the nicotinic receptor (nAChR), platelet-derived growth factor (PDGF), and the PDGF-receptor (PDGF-R) in this response were studied. Nicotine's mitogenic effect was characterized by three methods: thymidine incorporation, a viability/proliferation assay based on metabolic conversion of tetrazolium salt to formazan dye and cell counting. Nicotine administration (10^{-6} M) stimulated cell cycle entry marked by increased DNA synthesis, PCNA and cyclin D1 production, and increased cell division. Nicotinic receptor blockade with d-tubocurarine, a nicotinic AchR blocker, decreased nicotine-induced DNA synthesis, and cell division (0.33 ± 0.04, 0.77 ± 0.31-fold decrease, respectively). Nicotine increased cellular PDGF-BB transcript levels and protein release (ELISA: 1.6 ± 0.5-fold increase) but not PDGF-AA or PDGF–AB release. Nicotine increased PDGFβ-receptor protein content. PDGF inactivation with anti-PDGF antibody abolished nicotine-induced DNA synthesis (1.9 ± 0.08-fold decrease). PDGF-R blockade with the PDGF-R antagonist tyrphostin AG 1295 decreased nicotine-induced DNA synthesis and cell division (0.25 ± 0.01, 0.44 ± 0.2-fold decrease, respectively). PDGF-R blockade reversed nicotine-stimulated increases in PDGF release, PDGF-BB transcripts, and PDGF-receptor levels (0.68 ± 0.34; 0.46 ± 0.01; 0.28 ± 0.01-fold decrease, respectively). In conclusion, nicotine-mediated activation of nAChRs increases PDGF-BB transcription and protein production as well as PDGF β-receptor levels. PDGF-BB/PDGF-R interaction is vital in nicotine's mitogenic actions on human aortic smooth muscle cells. J. Cell. Biochem. 96: 986–995, 2005.

Key words: nicotine-stimulated mitogenesis; nicotinic acetylcholine receptor; PDGF-receptor; PDGF; smooth muscle cells

Occlusive vascular disease development and progression have been directly associated with cigarette smoking [Witteman et al., 1993]. The mechanisms by which cigarette smoke causes this phenomenon are not completely understood. Nicotine is an important component of cigarette smoke; however its role in the formation of these diseases remains unclear.

Effects of nicotine on the cardiovascular system are multifactorial since it acts upon nicotinic acetylcholine receptors (nAChRs) both in the central nervous system and on peripheral autonomic ganglia. Because most arteries lack direct cholinergic innervation, nicotine-induced alteration in vascular function has often been attributed to indirect effects of disturbed cholinergic transmission in autonomic ganglia. This may not be the case, as recent data demonstrate the presence of nAChRs on endothelial cells isolated from the arterial vascular wall [Macklin et al., 1998; Villablanca, 1998]. Similar receptors have also been found in rat vascular smooth muscle cells (VSMC) [Bruggmann et al., 2003]. nAChR outside of the neuromuscular junction consist of ligand-binding α-subunits (α2–α9) and additional β-subunits (β2–β4) arranged around a central ion channel. The endothelial nAChRs appear to include α3, α5, α7, β2, and β4

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subunits [Macklin et al., 1998]. The presence of neuronal nicotinic receptors at locations unrelated to synaptic activity such as blood vessels, bronchi and small and large intestine supports the hypothesis that acetylcholine may have cellular functions other than transmission of nerve signals [Conti-Fine et al., 2000].

Existing data implicate cigarette smoke in abnormalities of platelet function with resultant vascular complications [Dotevall et al., 1987; Rival et al., 1987]. Moreover, platelet-derived growth factor (PDGF), an important mitogen for mesenchyme-derived cells released by activated platelets, is known to be involved in the formation of vascular occlusive diseases [Ross, 1993]. PDGF is composed of dimeric molecules that may exist as homodimers or heterodimers of four peptide chains, PDGF-A, PDGF-B, PDGF-C, and PDGF-D [Raines, 2004]. The PDGFβ-receptor, the receptor most relevant to VSMC proliferation [Giese et al., 1999; Hart et al., 1999], binds both PDGF-BB and PDGF-AB but not PDGF-AA, which has only low mitogenic activity and no stimulatory effect on chemotaxis or actin filament reorganization [Claesson-Welsh et al., 1989]. Binding of PDGF leads to receptor dimerization, activation of its own intracellular tyrosine-kinase domain, and receptor autophosphorylation. In the case of the PDGFβ-receptor, a considerable number of autophosphorylation sites have been identified which allow for physical interaction with signal transduction molecules. These molecules appear to contribute to the generation of the proliferative response associated with the growth factor [Claesson-Welsh, 1994, 1996].

Despite recent advances in research examining nicotine-stimulated proliferative effects, no consistent association between nicotine and the PDGF receptor-ligand system has been established. The aim of our study was twofold: to examine nicotine's mitogenic effect on human arterial vascular smooth muscle cells (HaVSMCs) at concentrations similar to those found in smokers, and to investigate the role of nAChR, PDGF, and the PDGF-receptor in this response.

MATERIALS AND METHODS

Chemicals and Reagents

(−)-Nicotine acetate (N-5260), human recombinant PDGF-AB (P3326) and PDGF-BB (P4306), polyclonal anti-PDGF antibody (P6101), tyrphostin AG-1295 (T3932), and d-tubocurarine (T2379) were obtained from Sigma-Aldrich Co.; tritiated-thymidine from Perkin-Elmer Life Sciences, Inc.; and complete protease inhibitor cocktail tablets (1697498) from Roche Applied Sciences. All media and related products were purchased from Invitrogen Life Technologies.

Cell Culture

Explants of ascending thoracic aortas (from three non-smoking adult men, age = 40–65 years) were obtained from heart transplant donors or recipients. The study was approved by the Institutional Review Board of the University of Miami. VSMCs were isolated from these explants as previously described [Pauly et al., 1997]. Briefly, 3 cm length of thoracic aorta was digested with culture medium—Dulbecco's Minimum Essential Medium supplemented with F12 and fetal bovine serum (60:30:10)—containing 2 mg/ml of collagenase for 2 h. The tissue was then removed and cut into 1×1 mm pieces and cultured in 10 cm plates. When confluent areas of cell growth could be seen by inverted light microscopy, cells were trypsinated, expanded until passage three and then used for experiments or frozen into liquid nitrogen for further experiments. SMCs were identified by morphology and immunostaining using anti-smooth muscle alpha actin monoclonal antibody clone 4A1 (DAKO) (Fig. 1). Cell culture expansion was done in serum-rich medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin, glutamine 0.1 mM, sodium bicarbonate 0.75%, and 10% fetal bovine serum. Experimental culture and preparation of reagent aqueous solutions were completed in serum-poor medium consisting of DMEM supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin, glutamine 0.1 mM, sodium bicarbonate 0.75%, and 0.1% fetal bovine serum. The cultures were maintained at 37°C in an atmosphere of 5% CO_2 in air. Cells from passages 3–8 were used for experiments.

Assessment of HaVSMC Proliferation and Viability

Proliferation and viability were assessed by three distinct methods: tritiated thymidine incorporation, cell counting and XTT-based cell proliferation and viability assay [Mosmann, 1983; Gerlier et al., 1986]. For all experimental types, cells were seeded into 24-well plates at a concentration of 2×10^4 cells/well in serum-rich

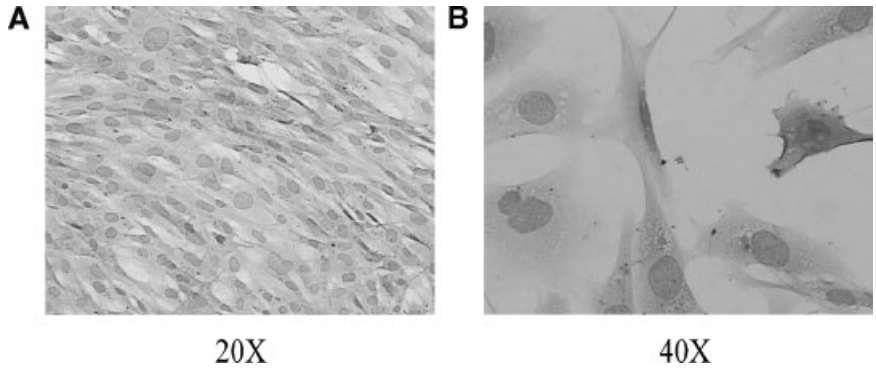

Fig. 1. Morphology of human aortic vascular smooth muscle cells (HaVSMC) in culture. HaVSMCs were isolated using the explant method and characterized by immunostaining with anti-smooth muscle alpha actin monoclonal antibody.

media for 24 h. Serum-poor conditions for 24 h followed to synchronize all cells at the G_0 phase of the cell cycle. Cultures subject to thymidine incorporation were incubated with reagents for 48 h. The cells were then pulsed with tritiated thymidine (1 U ci/well) for 6 h. Cellular DNA was precipitated with cold TCA 10% and solubilized with a mixture of NaOH 0.1 N and SDS 1% (v:v). Thymidine incorporation was determined with a scintillation counter. Thymidine uptake was normalized with respect to the total amount of protein and expressed as fold increase with respect to controls (untreated cells). Cell counts were performed after trypsinization with a single threshold Coulter counter (model ZF, Coulter Electronics, Miami, FL). Cell viability and mitochondrial activity were assessed by the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolically active cells (XTT-based colorimetric proliferation/viability assay no. 1465015, Roche Applied Sciences). The formazan dye is soluble in aqueous solution and is directly quantified by spectrophotometry ($\lambda =$ 490 nm, Bio-Rad Ultramark).

Nicotinic Acetylcholine and PDGF-Receptor Blockade

For receptor blockade d-tubocurarine, a nAChRs antagonist [Hakki et al., 2002] and tyrphostin AG 1295, a highly specific PDGF-receptor antagonist [Kovalenko et al., 1994, 1997; Fishbein et al., 2002; Karck et al., 2002] were utilized. Dose-response experiments (data not shown) demonstrated that a tyrphostin AG-1295 concentration of 50 μM and a d-tubocurarine concentration of 100 μM were sufficient to block PDGF and nicotinic receptors, respectively. These concentrations of AG-1295 and tubocurarine returned PDGF-BB (50 ng/ml) and nicotine (10^{-6} M)-stimulated DNA synthesis and XTT-based metabolic activity to baseline levels.

PDGF-AA, -AB, -BB Quantification in Conditioned Media

Cultured cells were stimulated in serum-poor medium with nicotine (10^{-6} M) alone or nicotine in combination with tyrphostin AG 1295 or d-tubocurarine. After 48 h of treatment, conditioned media was removed and stored at −80°C. Quantification of released PDGF in conditioned media was completed using human PDGF-AA (DAA00), PDGF-AB (DHD00B), PDGF-BB (DBB00) Quantikine ELISA kits (R&D Systems, Inc). Optical density readings were done at 490 nm with a spectrophotometer (Bio-Rad Ultramark). Final concentrations were expressed in pg/ml of PDGF determined by interpolation of reference curves.

Neutralization Assay of Released PDGF

The biologic effect of PDGF released secondary to nicotine treatment was assessed by inactivation of PDGF with polyclonal anti-PDGF antibody. Cells exposed to nicotine (10^{-6} M) alone and nicotine in combination with an excess of polyclonal anti-PDGF antibody (100 μg/ml) were subject to tritiated thymidine incorporation. After 48 h of incubation, cultures were pulsed with tritiated thymidine as described above.

Western Blot Analysis

Cell lysis was completed with lysis buffer composed of PBS supplemented with complete protease inhibitor cocktail (Roche), 0.1% Triton X-100 and 0.1% 2-mercaptoethanol. Lysates were subject to protein quantification using the micro-BCA method (No. 23232, Pierce). Protein concentration was confirmed by SDS–PAGE followed by Comassie Blue staining and protein densitometry to assure accurate protein concentration quantification. Total protein (1–3 μg) was resolved on SDS–PAGE and then electro-transferred to nitrocellulose membrane using a Mini Trans-Blot Transfer Cell (Bio-Rad). Specific antigen detection was performed with the following antibodies: PCNA (sc-25280), PDGFβ-receptor (sc-19995), cyclin D1 (sc-753) from Santa Cruz Biotechnology, Inc., and anti-smooth muscle α-actin clone 4A1 from DAKO (Denmark). Bound antibodies were detected with biotin-conjugated anti-mouse or goat antibody (Sigma Aldrich Co.) with subsequent incubation with streptavidin-HRP (DAKO). The membrane was then subject to chemiluminescence (Pierce) for signal recovery. Images were digitalized and signaling intensity quantified with ImageJ (NIH).

Real Time PCR for PDGF-B Transcript Quantification

Quantification of *PDGF B* and Glyceraldehayde-3-phospahte dehydrogenase (*GAPDH*) specific messengers was performed with RT^2 Real Time Gene Expression Assay Kits (SuperArray Bioscience Corporation, MD) QPH00488A and QPH00150A, respectively. Complementary DNAs were synthesized with SuperScripTM first-strand Synthesis system for RT-PCR (No. 11904-018, Invitrogen Life Technologies) starting with 2 μg of total RNA isolated by the Tri method. Thermocycling conditions using the LightCycler (Roche Diagnostics) were as follows: an initial denaturation at 95°C for 10 min followed by a 45-cycle amplification program consisting of heating to 95°C with a 30 s hold; annealing at 50°C with a 30 s hold; and extension at 72°C with a 30 s hold. The temperature transition rate for the amplification program was 20°C/s. The melting curve analysis was performed immediately after amplification and consisted of one cycle of heating at 20°C/s to 95°C with a 0 s hold, cooling at 20–45°C with a 10 s hold, and heating at 0.2°C/s to 95°C with a 0 s hold. Fluorescence signals of each capillary were acquired once per cycle for quantification analysis and continuously during the melting curve analysis. An increasing fluorescence signal for quantification analysis was confirmed by a corresponding Tm using melting curve analysis. The amount of GADPH transcripts was utilized as a normalizing factor. Final values were expressed as a normalized ratio of PDGF-B with respect to housekeeping GADPH.

Statistical Analysis

All cell culture experiments were repeated a minimum of three times in three independent primary HaVSMC cultures. Unless otherwise noted, quantitative data are expressed as mean ± standard deviation of nine values from three independent experiments. Comparisons were conducted using one-way ANOVA and Newman-Keuls post-hoc test. Differences were considered significant when P was less than 0.05.

RESULTS

Nicotine Induces Mitogenesis in Human Aortic Vascular Smooth Muscle Cells

HaVSMCs were cultured with increasing concentrations of nicotine and tritiated thymidine incorporation was determined. Compared to serum-poor medium, nicotine treatment stimulated DNA synthesis with peak levels observed at a concentration of 10^{-6} M (1.4 ± 0.05-fold increase vs. serum-poor control, $P = 0.02$). A bimodal pattern of nicotine-stimulated DNA synthesis was observed where synthesis was dose-dependently increased at low nicotine concentrations (10^{-8}–10^{-6} M) and inhibited at high nicotine concentrations (10^{-4} M, $P = 0.0067$ vs. DNA synthesis at peak concentration of 10^{-6} M) (Fig. 2A). Optimal duration of nicotine treatment was determined by time course experiments. Cells exposed to nicotine for time periods ranging from 24 to 72 h were subject to thymidine incorporation. DNA synthesis at 48 and 72 h of nicotine incubation was increased 0.5 ± 0.05 fold ($P = 0.048$) and 0.26 ± 0.05 fold ($P = 0.031$) when compared to 24 h, respectively (Fig. 2B). At 24 and 72 h, proliferation levels were not significantly different from control. To assure that the inhibition of DNA synthesis observed at higher and lower nicotine concentrations was not indicative of cell death, we used a XTT-based

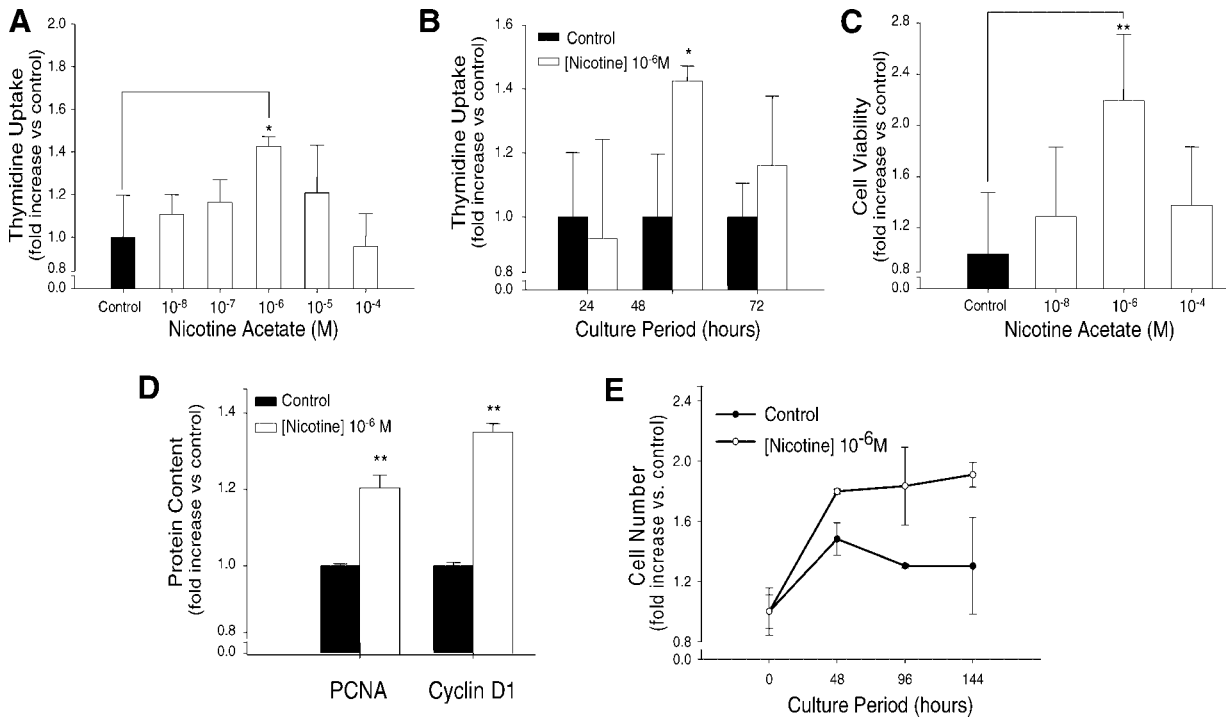

Fig. 2. Nicotine induces progression of HaVSMCs through the cell cycle. **A**, **B**: Nicotine-induced DNA synthesis is concentration (A) and time-dependent (B), as demonstrated by thymidine incorporation. **C**: Nicotine stimulates proliferation and cells remain viable as shown by XTT viability and proliferation assay. **D**: Nicotine induces PCNA and cyclin D1 production marking entry into the cell cycle as determined by Western blot. **E**: Growth curves show the nicotine stimulatory effect on HaVSMC division. Bar and points represent the average of nine values from three independent experiments. * $P < 0.05$ and ** $P < 0.01$.

colorimetric assay for the quantification of cell viability and proliferation. Cells cultured with a range of nicotine concentrations remained viable and proliferation was increased by 2.2 ± 0.5 ($P = 0.001$) fold at the same nicotine concentration that stimulated peak DNA synthesis (Fig. 2C). Therefore, similar to the DNA synthesis response, the proliferative response to nicotine was also concentration-dependent and bimodal.

We further confirmed nicotine's mitogenic effect by measuring entry of nicotine-exposed cells into the cell cycle using proliferating cell nuclear antigen (PCNA) and cyclin D1 [Pardee, 1989; Sherr, 1994; Baez et al., 1996; Sherr and Roberts, 1999] (Fig. 2D). Nicotine treatment (10^{-6} M) produced a slight but significant increase in PCNA (1.2 ± 0.02 fold, $n = 9$, $P = 0.008$) and cyclin D1 (1.34 ± 0.02 fold, $n = 9$, $P = 0.0004$) protein levels (Fig. 2D).

As changes in DNA synthesis and metabolic activity do not necessarily indicate changes in cellular mitotic index, we investigated whether the nicotine-stimulated changes presented above were reflective of cellular proliferation. Cells treated with nicotine (10^{-6} M) were subject to serial cell counting over an experimental period of 144 h and growth curves were compared to those of cells incubated in serum-poor medium alone. Growth curves showed that nicotine stimulated a 1.9 ± 0.08 ($P < 0.04$) fold increase in mitotic division with a lag time of 48 h and plateau reached at 96 h (Fig. 2E).

Effects of nAChR Blockade on Nicotine-Stimulated Mitogenesis

To demonstrate that nicotine was acting through a nicotinic receptor-dependent mechanism, we evaluated the effect of administration of d-tubocurarine (dTC), a nicotinic receptor antagonist. Blockade of nAChRs reduced nicotine-stimulated DNA synthesis by 0.33 ± 0.04 fold ($P = 0.008$) (Fig. 3A). XTT viability/proliferation assay was used to assess whether the inhibition of nicotine-stimulated DNA synthesis caused by receptor blockade was indicative of cell death produced by the receptor antagonist itself. Cells incubated with nicotine (10^{-6} M) in combination with d-tubocurarine for 48 h remained viable (Fig. 3B). More importantly, receptor antagonism reduced

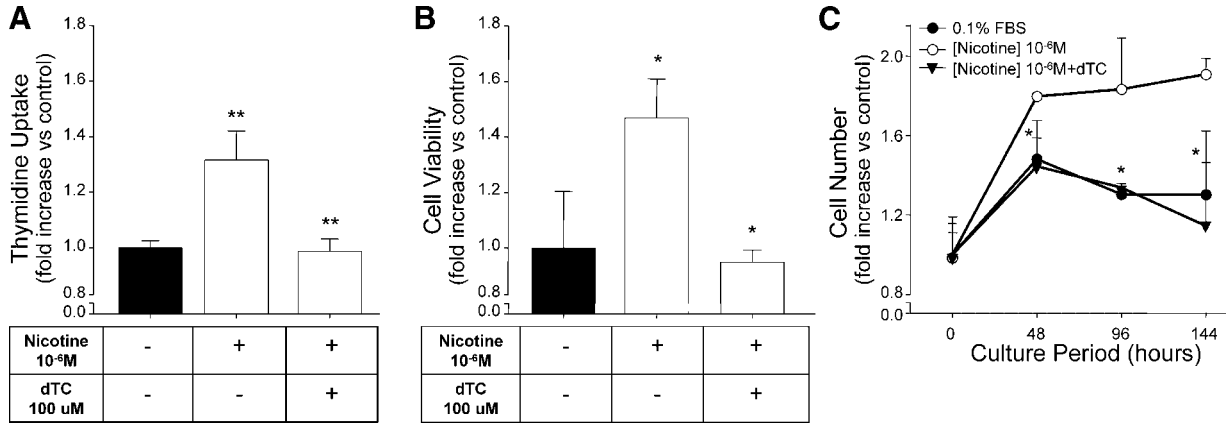

Fig. 3. Nicotinic acetylcholine receptor (nAChR) function is essential in nicotine's mitogenic effect. **A**, **B**: Blockade of nicotinic receptors ablates nicotine-stimulated increases in DNA synthesis and proliferation as demonstrated by thymidine incorporation (A). XTT-viability test showing that cells treated with nAChR antagonist remain viable (B). **C**: Growth curves showing that blockade of nAChRs significantly inhibit nicotine-stimulated cellular division. Bar and points represent the average of nine values from three independent experiments. * $P < 0.05$ and ** $P < 0.01$.

nicotine-stimulated proliferation by 0.5 ± 0.04 ($P = 0.01$) fold as measured by XTT colorimetric assay. Similarly, nAChR blockade mitigated nicotine-stimulated cell division by 0.77 ± 0.31 ($P < 0.04$) fold (Fig. 3C). Receptor blocked growth curves were not significantly different than those of untreated cells.

Effects of Nicotine on the PDGF Ligand-Receptor System

We next tested the hypothesis that nicotine induced the proliferation of HaVSMCs via the PDGF receptor-ligand pathway. Human PDGF-AA, -AB, and -BB ELISAs were performed to quantify PDGF release. Nicotine treatment (10^{-6} M for 48 h) increased the level of PDGF-BB by 1.6 ± 0.5 ($P < 0.05$) fold and had little effect on PDGF-AA or PDGF-AB release (Fig. 4A). As only PDGF-BB levels were increased in conditioned media, we sought to confirm these findings at the mRNA level. We observed that after 48 h of nicotine treatment, PDGF-B mRNA levels increased by 1.23 ± 0.12 ($P = 0.01$) fold compared to untreated cells (Fig. 4B).

The role of released PDGF in nicotine's mitogenic effect was determined by inactivation of PDGF activity with anti-PDGF antibodies (Fig. 4C). The addition of nicotine (10^{-6} M) to the culture medium resulted in an increase in tritiated thymidine uptake as compared to control. The addition of polyclonal anti-PDGF antibody to the medium of cell cultures exposed to nicotine decreased the tritiated thymidine uptake by 1.9 ± 0.08 ($P = 0.034$) fold, thus demonstrating the important autocrine effect PDGF has in nicotine-stimulated mitogenesis.

Because nicotine treatment induced upregulation of PDGF-BB both at transcript and protein levels, and PDGF activity was necessary for nicotine's mitogenic effect, Western blots for the PDGFβ-receptor, which is most relevant to VSMC proliferation, were performed (Fig. 4D). HaVSMCs exposed to nicotine for 48 h demonstrated a 1.4 ± 0.02 ($P = 0.0002$) fold increase in PDGFβ-receptor protein levels when compared to untreated cells.

Effect of PDGFβ-Receptor Blockade on Nicotine-Stimulated Mitogenesis

Nicotine treatment upregulated PDGF-BB and PDGFβ-receptor levels, therefore we investigated the importance of the PDGF-receptor in nicotine's mitogenic effect by blocking it with a specific antagonist, tyrphostin AG 1295. Cells were cultured with nicotine (10^{-6} M) alone or in combination with AG 1295 for 48 h and then were subject to tritiated thymidine incorporation and XTT-based cell viability and proliferation assay. PDGF-receptor blockade reduced nicotine-stimulated DNA synthesis and cellular proliferation by 0.25 ± 0.01 ($P = 0.027$) and 0.6 ± 0.01 ($P = 0.014$) fold, respectively (Fig. 5A,B). Growth curves of nicotine-treated cells also demonstrated that cell proliferation was diminished by 0.4 ± 0.2 fold ($P < 0.05$) with PDGF-receptor blockade (Fig. 5C). PDGF-receptor blockade reduced nicotine's effect on PDGF-BB levels in conditioned media by 0.68 ± 0.34

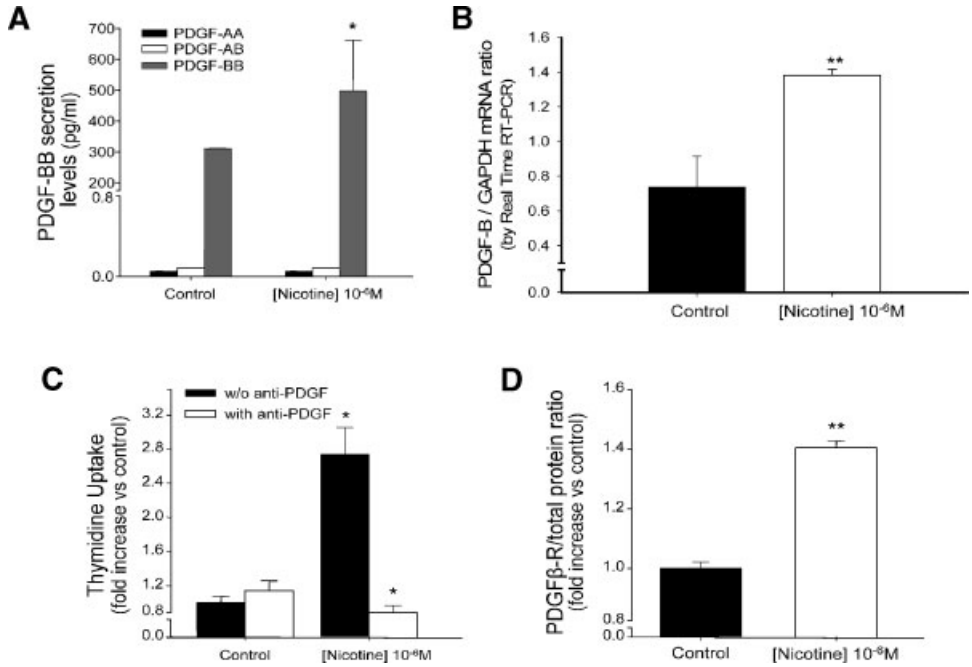

Fig. 4. Nicotine-induces alteration in PDGF receptor-ligand system. **A** (ELISA): Nicotine treatment induces increased release of PDGF-BB but not PDGF-AA or PDGF-AB. **B**: Nicotine treatment causes a significant increase in PDGF-BB transcript levels as determined by real time RT-PCR. **C**: Inactivation of PDGF with anti-PDGF antibody decreases nicotine-induced DNA synthesis. **D** (Western blot): Nicotine upregulates the PDGFβ-receptor. Bar represents the average of nine values from three independent experiments. * $P < 0.05$ and ** $P < 0.01$.

($P < 0.05$) fold, PDGF-B mRNA levels by 0.7 ± 0.04 ($P < 0.01$) fold, and returned PDGFβ-receptor protein content to control levels (0.28 ± 0.01-fold decrease, $P < 0.01$) (Fig. 6A–C).

DISCUSSION

A strong correlation has been established between cigarette smoking and the development and progression of vascular obliterative diseases such as atherosclerosis, coronary artery disease, myocardial infarction, cerebrovascular disease, and vascular graft failures [Hammond et al., 1954; Pittilo, 2000]. Although there is a large body of epidemiological data linking cigarette smoke and the development of vascular occlusive diseases, there is a paucity of

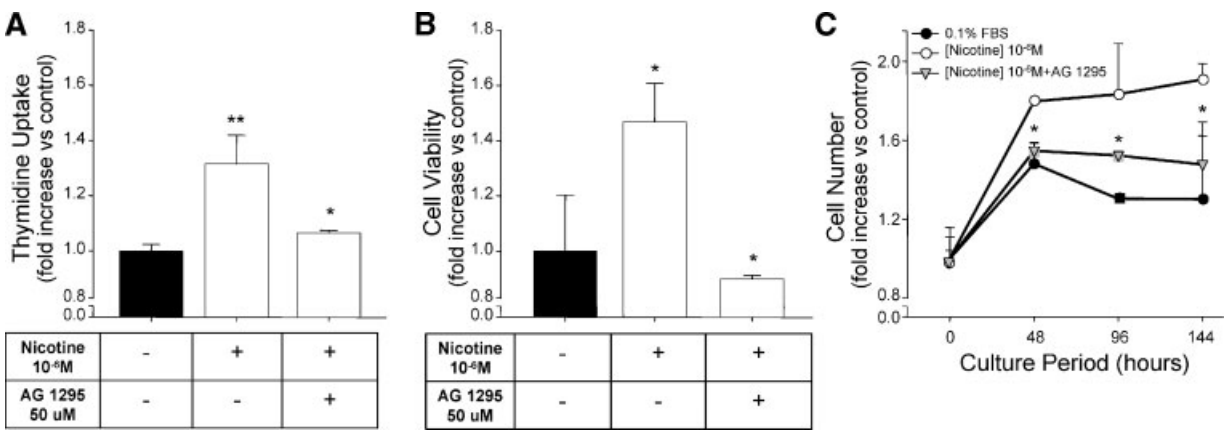

Fig. 5. PDGF-receptor function is crucial in nicotine's stimulatory effect. Quantitative evaluation of PDGF-receptor involvement in nicotine-stimulated mitogenesis by thymidine incorporation assay (**A**), XTT metabolic assay (**B**), cell counts (**C**). A and B, Blockade of PDGF-receptor activity ablates nicotine-stimulated increases in DNA synthesis and metabolic activity. Cells treated with PDGF-receptor antagonist remain viable. (C) Inhibition of PDGF-receptor activity significantly decreases nicotine-stimulated cellular division. Bar and points represent the average of nine values from three independent experiments. * $P < 0.05$ and ** $P < 0.01$.

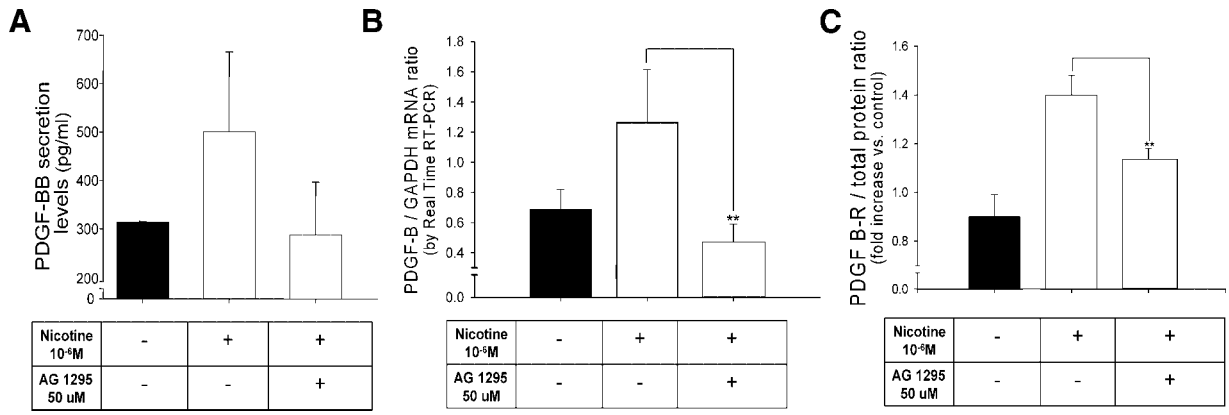

Fig. 6. Nicotine-induced increases in cellular PDGF release, PDGF-BB mRNA levels, and PDGF-receptor protein content are inhibited by blockade of the PDGF-receptor. Quantitative evaluation of PDGF-receptor blockade in nicotine-induced alteration of the PDGF receptor-ligand system by ELISA (**A**), real time RT-PCR (**B**), and western blot (**C**). Blockade of PDGF receptor activity with tyrphostin AG 1295 reverses nicotine's effect on cellular PDGF release (A), PDGF-BB transcripts (B), and PDGFβ-receptor upregulation (C) Bars represent the average of nine values from three independent experiments. * $P < 0.05$ and ** $P < 0.01$.

information concerning the cellular and molecular mechanisms whereby cigarette smoke influences this phenomenon. Furthermore, cigarette smoke contains over 4,000 constituents that may act alone or in concert to affect vascular wall structure and atherogenesis. Currently, it is still unclear which tobacco-derived products may play a role in the development of vascular occlusive diseases. Nicotine is an important constituent of whole cigarette smoke, but its role in vascular obliterative disease development and progression has not yet been clarified. Nicotine plasma concentration in active smokers, passive smokers, and smokeless nicotine users ranges between 10^{-5} and 10^{-8} M with peak levels occurring within 10 min after smoking initiation and declining with an average elimination half-life of about 2 h [Gritz et al., 1981; Darby et al., 1984].

Nicotine-associated vascular changes have often been attributed to disturbed cholinergic transmission in autonomic ganglia rather than to the action of nAChRs at the vascular cell level. Recent data demonstrating the presence of nAChRs on cells of the vascular wall dictate that this is not necessarily the case and prompted us to examine their involvement in nicotine-mediated proliferation.

In the present study, we provide further evidence for the presence of operational non-neuronal nAChRs on HaVSMC and demonstrate the role this receptor plays in nicotine-induced alterations in HaVSMC. Nicotine had the greatest mitogenic effect at a concentration of 10^{-6} M, a concentration similar to that observed in active and passive smokers. This mitogenic effect was time-dependent with maximal stimulation of DNA synthesis at 48 h treatment. Nicotine's mitogenic effect was reflected in the expansion of cell numbers as well as by increase in metabolic activity of cells exposed to nicotine. Moreover, cell cultures exposed to nicotine had an increase in cells entering the cell cycle as measured by PCNA and cyclin D1 protein levels. Inactivation of non-neuronal nAChRs expressed by HaVSMCs ablated the DNA synthetic, metabolic, and proliferative effects of nicotine treatment.

Available data indicate that nicotine causes other significant alterations in vascular cell function. Using cultured rodent arterial SMCs, Thyberg [1986] demonstrated that nicotine induces DNA synthesis and modulation of SMCs from contractile to synthetic phenotype. Smooth muscle cell recruitment to the intima and transition from a contractile to a synthetic phenotype are essential events in the development of vascular occlusive diseases [Thyberg et al., 1990]. It appears that this phenotypic alteration is driven by growth factors [Hedin et al., 1997]. SMCs that have undergone this phenotypic change are capable of proliferation, recruitment from vascular media to intima, and secretion of growth factors as well as extracellular matrix components [Thyberg, 1998]. Similarly, Carty and Soloway [1996] demonstrated in human SMCs that nicotine and its metabolite, cotinine, increased the production of basic fibroblast growth factor (bFGF), a major mitogen for SMCs, as well as several matrix

metalloproteinases. Although these authors did observe an increase in bFGF production, they did not find significant changes in PDGF-AB levels. In a recent study Cucina and associates [Cucina et al., 2000a,b] reported that nicotine-induced PDGF release, and this induction alters cytoskeletal organization in bovine endothelial and aortic smooth muscle cells. However, the effect of nicotine on PDGF release from human VSMCs remains unknown.

Our study demonstrated that nicotine administration significantly increased cellular release of PDGF-BB from human VSMCs. Moreover, nicotine increased PDGF-BB gene transcripts within 48 h of exposure. Neutralization of PDGF activity with anti-PDGF antibodies abolished nicotine-stimulated DNA synthesis, suggesting the important autocrine or paracrine effect of PDGF release secondary to nicotine treatment. In addition, exposure to nicotine caused an upregulation of PDGFβ-receptor expression. Nicotine's effect on PDGF-BB transcripts and PDGF receptor upregulation was inhibited by blockade of PDGF-receptors. Taken together, our data suggest that nicotine causes an upregulation of the PDGFβ-receptor and increase in PDGF release which, in an autocrine or paracrine fashion, acts upon local SMCs to stimulate proliferation.

In conclusion, we demonstrated that nicotinic acetylcholine and PDGF-receptor function are essential for nicotine-induced mitogenesis in human VSMCs. We provide further evidence for the existence of functional nAChRs on cells of the human vascular wall. Neutralization of nicotinic receptor and PDGF receptor activity reverses nicotine-stimulated DNA synthesis, cell proliferation, and metabolic activity. We demonstrate that nicotine does indeed alter PDGF release by increasing PDGF-BB levels. Once nicotine binds to nAChRs, PDGF-BB transcripts are increased, the PDGF β-receptor is upregulated, and SMCs are activated to increase PDGF-BB release. PDGF-BB acts in an autocrine or paracrine fashion to stimulate cellular proliferation and is vital in nicotine's mitogenic actions on HaVSMCs.

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