Nicotine stimulates DNA synthesis and proliferation in vascular endothelial cells in vitro

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Villablancas, Amparo C. Nicotine stimulates DNA synthesis and proliferation in vascular endothelial cells in vitro. J. Appl. Physiol. 84(6): 2089–2098, 1998.—Nicotine is a major component of cigarette smoke and has been postulated to play an important role in atherosclerosis and malignancy. Endothelial cell growth may be regulated by nicotine, yet operative mechanisms at the endothelial level are poorly understood. We studied the effects of nicotine (10^{-14}, 10^{-8} M) on endothelial DNA synthesis, DNA repair, proliferation, and cytotoxicity by using cultures of bovine pulmonary artery endothelial cells. Assays were performed on cells incubated with nicotine in the presence and absence of hydroxyurea (an inhibitor of scheduled DNA synthesis), serum, human platelet-poor plasma, and platelet-derived growth factor and endothelial cell growth factor (PDGF and PDECGF, respectively). Nicotine significantly stimulated endothelial cell DNA synthesis and proliferation at concentrations lower than those obtained in blood after smoking (<10^{-8} M). The stimulatory effects of nicotine were enhanced by serum (0.5%) and PDECGF and were blocked by the nicotinic-receptor antagonist hexamethonium. The response to nicotine was bimodal because cytotoxicity was observed at higher concentrations (>10^{-6} M). This study has implications for understanding cellular mechanisms of nicotine action. The results may be important in tumor angiogenesis, atherogenesis, and vascular dysfunction in smokers.

There is overwhelming evidence that an association exists between cigarette smoking and a number of pathological conditions, including cardiopulmonary diseases and malignancies (3, 9, 38). Cigarette smoke is a complex mixture of over 4,000 chemical constituents that are distributed in particulate and gaseous phases. Because of the complexity of smoke, identifying the components responsible for pathophysiological relationships and discerning mechanisms by which their effects are mediated are very difficult.

Interactions of components of cigarette smoke with the endothelium, the vascular lining, have attracted considerable interest because of earlier observations that suggested smoke morphologically altered the vasculature (4). Indirect evidence from endothelial cells (EC) in culture and from whole-organ experiments demonstrated morphological changes with exposure to cigarette smoke (6, 29). These include endothelial denudation and endothelial loss without denudation. Nonde-nuding changes are characterized by an increase in the number of circulating EC consistent with a desquamating effect (40). In addition, ultrastructural phenotypic changes are also manifest in the endothelium after exposure to cigarette smoke (23, 41). These changes have been characterized by extensive vacuolation, giant cell formation, and mitochondrial swelling. For these reasons, the primary response of the vascular endothelium to smoke constituents has been presumed to be cytotoxicity and injury. It is important to point out that previous studies have not directly demonstrated this effect, however.

Nicotine may not be as important as other components of cigarette smoke in mediating morphological changes of endothelial injury. Instead, several lines of experimental evidence suggest that nicotine’s primary role is in regulating endothelial function and that functional changes may occur in the absence of accompanying morphological changes. Indeed, significant functional perturbations in endothelial function have been identified in response to nicotine (23, 33). Additionally, increased production of prostacyclin (5) and enhanced macromolecular transport (1, 12) have been reported to occur in the endothelium in response to nicotine.

Functional alterations in the endothelium have the potential for significant pathological sequelae because the endothelium regulates vascular permeability, antithrombogenesis, and vasoreactivity. The endothelium is the innermost lining of the vasculature and consists of a contact-inhibited quiescent cell monolayer with a low turnover rate (28). Therefore, the endothelium is also important in maintaining normal vascular architecture and can participate in vascular repair in response to injury or wounding. An additional role of the endothelium is in angiogenesis (2), which is necessary to maintain the growth, viability, and metastatic potential of many malignant tumors (36). Despite the physiological importance of nicotine, few studies have directly addressed its effects at the cellular level in the endothelium.

Studies have suggested that nicotine may regulate DNA synthesis in EC, but results have been inconclusive and contradictory. Whereas nicotine has been demonstrated to cause initiation of DNA synthesis in serum-free cultures of arterial smooth muscle cells (31), it has not been convincingly shown to stimulate endothelial proliferation. Although previous reports have indicated that nicotine can stimulate DNA synthesis (5, 41), it has not been determined whether DNA synthesis reflected cellular proliferation or injury, and the study methods did not allow determination of a direct mitogenic effect. Other investigators have reported that nicotine does not stimulate DNA synthesis in EC (4). Therefore, the effects of nicotine on endothelial growth control are uncertain.

This study was undertaken to investigate the possible regulatory effects of nicotine on EC growth and DNA synthesis. An additional goal of our study was to examine interactions of nicotine with growth cofactors.
present in the endothelial environment. Our approach enabled us to differentiate nicotine's effects on endothelial DNA synthesis, DNA injury, proliferation, and cytotoxicity. The findings of our study may have implications for understanding pathological conditions associated with smoking that result in alterations in vascular function, including growth control, response to injury, and neovascularization.

MATERIALS AND METHODS

Materials

Chemicals. Nicotine (free base), bovine insulin, hydroxyurea, and hexamethonium were purchased from Sigma Chemical (St. Louis, MO), and [methyl-3H]thymidine (40–60 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Human platelet-derived growth factor (PDGF-AB chain) was purchased from J R Scientific (Woodland, CA). Platelet-derived endothelial cell growth factor (PDECGF) was prepared from the plasma fraction of human platelets (18) and partially purified (~50%) by carboxymethyl and quaternary aminoethyl Sephadex chromatography and ammonium sulfate precipitation as described previously (10). PDECGF stimulated EC DNA synthesis by three- to fourfold over control [serum-free medium 199 (M199) alone] at concentrations >1:125 (vol/vol).

Tissue culture supplies. M199 was purchased from Fisher (Pittsburgh, PA); fetal bovine (FBS) and iron-supplemented calf serum were from Hyclone (Logan, UT); penicillin/streptomycin/fungizone, trypsin, and EDTA were obtained from Sigma Chemical; tissue culture plates were from Falcon (Lincoln Park, NJ); tissue culture flasks were from Corning Glass Works (Corning, NY); and cell scrapers were from Costar (Van Nuys, CA).

Cell Culture

Calf pulmonary artery EC (passage 5) were obtained from the American Type Culture Collection (CRL 1733; ATCC, Rockville, MD) and as a generous gift from Una Ryan (passage 10). Cells were cultured as monolayers in M199 supplemented with 5% FBS, 5% iron-supplemented calf serum, and 10−5 M thymidine, hereafter referred to as growth medium, plus antibiotics (penicillin, 100 U/ml; streptomycin, 0.1 mg/ml; and amphotericin B, 0.25 µg/ml) in plastic culture flasks in a humidified atmosphere of 95% air-5% CO2 at 37°C. Cells were passaged weekly enzymatically at a split ratio of 1:2 to 1:4 by using a cell scraper and were subcultured an average of 3–4 times (range 1–10 times) after they were received before being used for studies. No differences were seen for the range of subcultures used in the cellular responses to the study factors tested.

Step-Down Procedure and Conditions Used in DNA Synthesis and Proliferation Studies

The conditions used for these studies were chosen because EC in vivo are in growth quiescence and experience a very low turnover rate and because we wanted to study the ability of nicotine and other study factors to stimulate cells to enter the G0/G1 phase, with a much smaller percentage of cells in S phase, flow cytometric analysis was performed. For this analysis, cells were plated and deprived of thymidine as described above. Cells were then trypsinized (0.25%), centrifuged, washed, and resuspended in PBS. The cultured cells were fixed with 95% cold ETOH and stored at 5°C. Flow cytometric analysis was performed within 1 wk of cell harvest at Los Alamos National Laboratories by using the following protocol: the ETOH-fixed cells were centrifuged, washed in PBS, resuspended for 30 min in a solution containing propidium iodide (50 µg/ml) and RNAse (100 U/ml), filtered through a nylon mesh (35 µm), and then analyzed by flow cytometry. As demonstrated in Fig. 1, flow cytometric analysis confirmed that the proportion of cells in S phase was low and demonstrated that the majority of cells were in G1 phase.

DNA Synthesis Assay

For these studies, EC were cultured and plated as described above. Immediately before each experiment, the
conditioned medium was removed from the wells and the desired growth factors or nicotine, freshly prepared, were added to the cells in serum-free M199. [3H]thymidine was incubated into each well (0.1 μCi/well) immediately after the desired growth stimuli were added. I dentically prepared, but non-growth stimulus-treated cells, were used for control cells, which were incubated with serum-free M199 alone.

Time course experiments were conducted by incubating cells (3–6 wells/experimental point) with nicotine for time periods ranging from 8 h to 6 days. For the longer incubations, the conditioned medium was discarded and [3H]thymidine and nicotine were readded after the third day. Time-course response curves demonstrated that 36 h were sufficient to detect the maximal response. Therefore, all subsequent assays were performed for this time period.

After incubation with nicotine for the desired time interval, cells were washed with 0.15 M NaCl, DNA was precipitated with 0.3 M HCl for 20 min at 5°C, and cells were washed again with 0.3 M HCl, rinsed with 95% EtOH, allowed to air dry, and then solubilized with 0.5 M NaOH. An aliquot from each well was taken for liquid scintillation counting, and data were expressed as counts per minute (cpm) of [3H]thymidine incorporated into DNA. Incubating cells with HCl during the assay procedure precipitated the DNA and permeabilized the cells so that any low levels of thymidine associated with intracellular pools were released and removed during the subsequent wash step. In this manner, measurements of [3H]thymidine incorporated into DNA corresponded to de novo DNA synthesis and not to shifts in levels of the cold-thymidine precursor pool.

Proliferation Studies

To determine whether an increase in [3H]thymidine incorporated into DNA was reflective of cellular proliferation, EC were cultured and plated in a manner identical to that described above for the DNA synthesis studies. Cells to be counted were then incubated in the presence and absence of nicotine (5–10 wells/experimental point) for 36 h without added [3H]thymidine. Before cells were counted, the conditioned medium was emptied from the wells and the cells were washed with PBS, pH 7.4. Cells were detached from the wells by incubating them with trypsin in EDTA for 2 min at 37°C, as described above. Growth medium was added to stop the reaction, and the contents of each well were then mixed by using a P-200 micropipette. This procedure was repeated once more to ensure that all cells were removed from the wells. The cells for each experimental point were pooled, centrifuged, and resuspended in M199 containing 50% vol/vol trypsin blue. Cell counts were performed by using a Neubauer counting chamber (C, A. Hauser and Son, Philadelphia, PA). Nonviable cells (~1–3 high-powered field) were identified by trypsin blue staining and excluded from counting.

Preparation of Human Platelet-Poor Plasma (PPP)

PPP was prepared as previously described (24). Fresh (24–48 h after collection) human plasma units (~300 ml/unit) were obtained from the Sacramento Blood Bank. To avoid platelet rupture and associated growth factor release, the plasma was collected and handled so as to minimize agitation and avoid freeze-thawing. In addition, before processing, proteolytic enzyme inhibitors (4 ml/unit) were added to each unit of plasma (1 mM phenylmethylsulfonyl fluoride, 100 mM 6-aminocaproic acid, and 1% vol/vol aprotinin). The plasma was then centrifuged at 13,800 g for 1 h at 5°C. A small red blood cell pellet was formed and discarded. Next, the plasma was recentrifuged and the supernatant was heated for 30 min at 56°C to precipitate fibrinogen. A small precipitate was removed, and the plasma was centrifuged for a third time and then extensively dialyzed (cutoff: 3,500 mol wt) against PBS overnight at 5°C. The dialyzed plasma was centrifuged once more and then passed through a 0.45-μm filter. Before use, the plasma was bioassayed and shown to not stimulate the growth of sparse populations of BALB/c 3T3 fibroblasts (kindly provided by W. J. Pledger) and to not differ significantly from control (M199 alone) when incubated with EC, with 20% FBS serving as a positive control.

Data Analysis

DNA synthesis experiments were performed in 3–6 wells/point, and cell proliferation assay experiments were performed in 5–10 wells/point. Results were expressed as means ± SE of data (cpm or cell number) from experiments repeated at least three times (n = 3). Data involving comparisons of experimental treatment groups against a control group were first analyzed by one-way ANOVA followed by Dunnett’s post hoc test. Comparisons within treatment groups were first analyzed by two-way ANOVA followed by the Bonferroni correction using modified t-tests. Significance was determined at the P < 0.05 level.

RESULTS

Dose-Dependent Effects of Nicotine on Endothelial DNA Synthesis, Proliferation, and Cytotoxicity

To determine the effect of nicotine on EC growth, we first investigated the effect of nicotine on EC DNA synthesis. For these studies, EC were incubated with nicotine and incorporation of tritiated thymidine into DNA was determined. Nicotine stimulated initiation of DNA synthesis in EC cultures (Fig. 2A). Compared with blank (serum-free M199 alone), a significant increase in DNA synthesis was observed when EC were incubated with nicotine at 10–14–10–10 M. The stimulatory response was more than 100% greater than control at the lowest nicotine concentration tested (10–14 M). The peak response was observed at 10–10 M nicotine and corresponded to a 152% increase in DNA synthesis over blank. We also observed that, compared with blank (2,900 ± 480 cpm), there was inhibition of DNA synthesis (to 800 ± 70 and 344 ± 31 cpm) at 10–6 and 10–4 M nicotine, respectively. Therefore, the pattern for DNA synthesis in response to nicotine was bimodal, with stimulation at low nicotine concentrations (<10–8 M) and inhibition at high nicotine concentrations (>10–6 M).

Because changes in DNA synthesis are not necessarily indicative of changes in cellular mitotic index, we also investigated whether the nicotine-stimulated increase in DNA synthesis was reflective of cellular proliferation, and, conversely, whether the nicotine-stimulated decrease in DNA synthesis was reflective of cell death. For these studies, EC were incubated with nicotine and subjected to cell counts. As shown in Fig. 2B, nicotine stimulated EC proliferation, as determined by an increase in the number of cells present in the wells, at nicotine concentrations of 10–14–10–8 M. The proliferative response was significantly greater than blank over this entire nicotine concentration range. The peak increase in EC number (180% greater than blank) was observed at the same nicotine concentration (10–10 M) that stimulated peak DNA synthesis. In contrast, compared with blank, a marked decrease in
cell number (from 4,995 ± 660 to 29 ± 3 cpm) was observed when EC were incubated with the highest concentration of nicotine tested, 10^{-4} M. Therefore, like the DNA synthesis response, the proliferative response to nicotine was also concentration dependent and bimodal. However, compared with the DNA synthesis response, the proliferative response displayed a slight concentration shift to the right (see also Fig. 5B).

The pronounced reduction in cell number observed at high nicotine concentrations could be consistent with cell death. Trypan blue exclusion confirmed cytotoxic effects at the highest concentrations of nicotine. Furthermore, at high nicotine concentrations morphological changes consistent with apoptosis, including loss of cytoplasmic integrity, cell shrinkage, membrane blebbing, or apoptotic bodies, were not observed by light microscopy. Thus the reduction in cell number appeared to be related to cell death.

**Effect of Nicotine on Reparative vs. Scheduled DNA Synthesis**

To evaluate whether any of the observed changes in DNA synthesis in response to nicotine were due to reparative DNA synthesis (as a result of nicotine-induced DNA injury) or whether they were entirely representative of de novo DNA synthesis (as a result of nicotine-induced EC mitogenesis), cells were incubated with nicotine in the presence of hydroxyurea. By interfering with dATP production, hydroxyurea blocks the transition between the G1 and S phases of the cell cycle and thereby inhibits scheduled DNA synthesis (13). For these studies, the effect of nicotine on both EC DNA synthesis and proliferation was evaluated by incubating the cells with nicotine in the presence and absence of hydroxyurea (1 mM).

As seen in Fig. 3A, when EC were incubated with nicotine (10^{-12} - 10^{-9} M) in the presence of hydroxyurea (1 mM), the increase in DNA synthesis that was previously seen in response to nicotine alone was abolished, and DNA synthesis returned to control levels. Similarly, as demonstrated in Fig. 3B, treatment of EC with relevant concentrations of nicotine (10^{-12} and 10^{-10} M) in the presence of hydroxyurea abolished the increase in cell number that resulted when cells were exposed to nicotine alone. Hydroxyurea alone had no significant effect on EC DNA synthesis or proliferation compared with blank. Therefore, because nicotine alone stimulated DNA synthesis and proliferation in the absence of hydroxyurea, and all of the nicotine-stimulated endothelial proliferation and incorporation of thymidine was sensitive to hydroxyurea, our results indicated that DNA synthesis in response to nicotine was not consistent with nicotine-induced DNA repair as a consequence of DNA injury. Rather, our results indicated that nicotine-stimulated DNA synthesis in EC was consistent with scheduled de novo DNA synthesis, which in turn was associated with EC proliferation.

**Time-Dependent Effects of Nicotine on DNA Synthesis**

We were next interested in evaluating the time dependence, if any, of the effects of nicotine on DNA synthesis. For these studies, changes in DNA synthesis

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**Fig. 2.** A: effect of nicotine on endothelial DNA synthesis. Counts per minute (cpm; mean ± SE; n = 3) of [3H]thymidine incorporated into DNA after incubation of endothelial cells with nicotine (solid bars) are shown. Blank (B; open bar) indicates control and represents values obtained for serum-free medium 199 alone. Comparisons were made between nicotine and blank. *Significant differences. B: effect of nicotine on endothelial proliferation and cytotoxicity. Effect of increasing concentrations of nicotine on endothelial cell number (mean ± SE; n = 3) is demonstrated. B is defined as in A. Comparisons were made between nicotine and blank. *Significant differences.
after exposure of EC to nicotine (10^{-14}-10^{-4} M) were examined over various time intervals ranging from 16 to 48 h. There were no appreciable changes in basal DNA synthesis levels for control cells that were not exposed to nicotine (0% change; solid horizontal line in Fig. 4) over this same time interval. As demonstrated in Fig. 4, after treatment of EC with nicotine, the earliest increase in DNA synthesis occurred after 18 h. Thereafter, DNA synthesis increased gradually and reached a peak that was, on average, 200% greater than control levels after 32 h of incubation. By 40 h of incubation, DNA levels returned to baseline. The concentrations of nicotine that displayed a peak increase in DNA synthesis at 32 h corresponded to those nicotine concentrations that stimulated EC proliferation (10^{-14}-10^{-8} M).

The inhibitory effects of high nicotine concentrations (10^{-6}-10^{-4} M) on DNA synthesis were, on average, 70% lower than control. The inhibitory response occurred early (within 16 h of incubation) and persisted throughout the length of the incubation time period. These data indicate that inhibition of DNA synthesis in response to high concentrations of nicotine was not preceded by initiation of DNA synthesis and suggest that high nicotine concentrations were indeed associated with early endothelial cytotoxicity.

**Effect of Growth Cofactors on Nicotine-Stimulated Endothelial Mitogenesis and Cytotoxicity**

To further characterize the mitogenic response of EC to nicotine and evaluate whether modifying the growth environment mediated the proliferative or cytotoxic responses of nicotine, the effects of endothelial growth cofactors were also investigated. For these studies, we incubated EC with nicotine in the presence and absence of serum, PPP, and PDGFs, as described below.

Serum was chosen because of its previously described ability to induce DNA synthesis (32). Also, given that exposure of EC to serum would be expected to occur near wounds or sites of vascular injury, we were interested in studying the effects of nicotine on EC

![Fig. 3. A: effect of hydroxyurea (HU) on nicotine-stimulated endothelial DNA synthesis. Values are means ± SE; n = 3. Shown are cpm of [\textsuperscript{3}H]thymidine incorporated into DNA for cells incubated with nicotine in the presence (hatched bars) or absence (solid bars) of HU (1 mM). Crosshatched bar, response to HU alone. B is defined as in Fig. 2. B: effect of HU on nicotine-stimulated endothelial proliferation. Values are means ± SE; n = 3. Bars are defined as in A. *Significant differences between nicotine and nicotine + HU. **Significant differences between nicotine and blank.](image)

![Fig. 4. Time-dependent effects of nicotine on endothelial DNA synthesis. Endothelial cells were incubated with nicotine for various time periods (16-48 h). Values are means ± SE; n = 3. Bars are defined as in A. **Significant differences between nicotine and control at 32 h, P < 0.05.](image)
growth under these conditions. However, because serum itself is a potent EC mitogen, our approach was to use serum in low concentrations that would not independently result in stimulation of EC growth. As shown in Fig. 5A, incubation of EC with nicotine in the presence of low concentrations of FBS (0.5%) resulted in highly significant potentiation of the DNA synthesis response to nicotine over a wide nicotine concentration range (10^{-12}-10^{-7} M). The magnitude of the effect was proportionately greater at the higher nicotine concentrations. Serum had a synergistic effect with nicotine (10^{-8} and 10^{-7} M) because the responses were 182% and 190% greater, respectively, than the responses to nicotine alone. The effect of FBS alone (0.5%) on DNA synthesis was not significantly different from control.

In contrast, as demonstrated in Fig. 5B, when EC were incubated with nicotine and FBS (0.5%), significant potentiation of nicotine-stimulated proliferation was seen over a narrower nicotine concentration range (10^{-8}-10^{-6} M). At these nicotine concentrations (10^{-8} and 10^{-7} M), cell counts for nicotine in the presence of FBS were 44% and 157% greater, respectively, than cell counts with nicotine alone. FBS had no effect on the cytotoxic response of EC to the highest nicotine concentration studied (10^{-4} M). These findings suggest that serum factors may be important in facilitating the endothelial proliferative response at near-physiological nicotine concentrations and in attenuating nicotine-mediated cytotoxicity at high (>10^{-6} M) nicotine concentrations.

However, in the in vivo environment, EC are primarily exposed to plasma and plasma components. Therefore, to compare the response of EC exposed to nicotine and serum with that of cells exposed to nicotine and plasma, we incubated EC with nicotine in the presence of human PPP. In contrast to the enhanced stimulation of DNA synthesis seen with nicotine in the presence of FBS, PPP had no effect in modulating the proliferative response of EC to nicotine or in modulating the cytotoxic effects of nicotine (data not shown).

We subsequently wanted to investigate the possibility that growth factors, particularly PDGFs, could interact with nicotine in the growth response of EC. For these studies, cells were incubated with nicotine and two human PDGFs of demonstrated importance in EC mitogenesis: PDECGF and PDGF. When EC were incubated with nicotine in the presence of partially purified PDECGF (as described in MATERIALS AND METHODS) at dilutions of 1:125, nicotine-stimulated DNA synthesis was significantly enhanced, on average, by 93% over a wide nicotine concentration range (10^{-14}-10^{-6} M) (Fig. 6). As with FBS, the potentiating effect of PDECGF was of greatest significance at higher concentrations of nicotine (10^{-8}-10^{-6} M). When cells were coincubated with PDECGF and high nicotine concentrations (10^{-8} and 10^{-6} M), DNA synthesis was enhanced over nicotine-stimulated DNA synthesis alone by 56 and 266%, respectively. Therefore, PDECGF not only prevented inhibition of DNA synthesis at 10^{-6} M nicotine but also led to stimulation of endothelial DNA synthesis at this nicotine concentration. However, like serum, PDECGF offered no protection against the
cytotoxic effect of nicotine at the highest nicotine concentration studied (10^{-4} M). The effect of PDECGF alone on DNA synthesis was not significantly different from blank.

In contrast to PDECGF, our studies with nicotine and PDGF (AB chain, 25 ng/ml) indicated that PDGF did not significantly modulate EC DNA synthesis compared with nicotine alone (data not shown). These findings suggest that only certain PDGFs (i.e., PDECGF but not PDGF) may play a significant role in enhancing the growth response of EC to nicotine.

Effect of Nicotinic Receptor Inhibition

Our DNA synthesis and cell proliferation studies demonstrated an endothelial proliferative response to nicotine at physiological concentrations or lower. The results suggested that the response might involve interactions with cellular nicotinic receptors. To study this possibility as a mechanism for the nicotine-mediated effects on EC growth, we incubated EC with nicotine and the ganglionic nicotinic-receptor blocker hexamethonium. As shown in Table 1, when EC were incubated with nicotine in the presence of hexamethonium (10^{-4} M) for 32 h, hexamethonium completely and significantly blocked EC DNA synthesis at nicotine concentrations that previously resulted in stimulation of DNA synthesis (10^{-14}-10^{-8} M). The effect of hexamethonium alone on EC DNA synthesis was not significantly different from blank (9,940 ± 479 vs. 8,841 ± 431 cpm, respectively). In separate experiments, it was demonstrated that the concentration of hexamethonium used for these studies had no effect on EC growth in response to another endothelial mitogen (basic-fibroblast growth factor, 1-10 ng/ml) over a 2- to 3-day incubation time period. These data suggest that a mechanism for the mitogenic effects of nicotine on EC may involve interactions with nicotinic receptors. Interestingly, however, hexamethonium did not alter the inhibitory effects of high concentrations (10^{-6}-10^{-4} M) of nicotine on DNA synthesis. The lack of response to hexamethonium at high nicotine concentrations was likely due to early nicotine-mediated cytotoxicity and direct cell kill. This suggests that at high nicotine concentrations the mechanism for cytotoxicity may be independent of nicotinic receptors.

**DISCUSSION**

The examination of cell growth is an important aspect in the understanding of nicotine's effects on vascular function. This study provides the first evidence in pulmonary artery EC indicating that EC growth is regulated by nicotine, an important constituent of cigarette smoke. In this investigation we showed that nicotine had a bimodal effect on EC growth function that could result in either cellular proliferation or cytotoxicity. The increase in DNA synthesis associated with cellular proliferation occurred in a time-dependent manner. Nicotine had no effect on inducing DNA injury. Regulation of proliferative and cytotoxic responses was entirely dependent on nicotine concentration and was modulated by serum factors and PDECGF. Furthermore, EC growth regulation by nicotine was mediated through interactions with nicotinic receptors because the stimulatory effect was sensitive to treatment with hexamethonium, a nicotinic-receptor antagonist. The proliferative effects of nicotine occurred at physiologically relevant concentrations.

Nicotine's effects on the vasculature involve complex and concurrent processes. Most previous studies on the cellular effects of the products of cigarette smoke, including nicotine, have shown them to be deleterious, and investigators have therefore assumed constituents of cigarette smoke to be associated with cellular injury. For example, investigations of the effect of nicotine on EC have indicated that nicotine can be associated with

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<th>Nicotine Concentration, M</th>
<th>cpm of [H]Thymidine Incorporated into DNA</th>
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<tr>
<td></td>
<td>Nicotine alone</td>
</tr>
<tr>
<td>10^{-14}</td>
<td>12,780 ± 240</td>
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<td>10^{-12}</td>
<td>16,650 ± 573</td>
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<td>1,005 ± 46</td>
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Values are means ± SE for 32-h incubation; n = 3. cpm, counts per minute. *P < 0.05 compared with nicotine alone.
cell loss and desquamation (40). These features suggest cellular toxicity in response to nicotine.

Our studies are consistent with cytotoxic effects of nicotine on EC. However, cytotoxicity was only seen at high nicotine concentrations (>10^{-6} M) and appeared to be due to direct cell kill, rather than vascular cell injury. In contrast to the assumptions of previous studies, data from this study demonstrated that cellular cytotoxicity was associated with inhibition of DNA synthesis, not stimulation of DNA synthesis. In addition, our results indicated that nicotine treatment was not associated with injury to DNA.

Previous studies have demonstrated that nicotine can stimulate DNA synthesis. For example, nicotine stimulates DNA synthesis in cervical cells (35), smooth muscle cells (31), and human cancer cell lines (27). Previous models also have demonstrated that nicotine stimulates DNA synthesis in EC. However, these studies have inferred that stimulation of DNA synthesis was indicative of cellular injury because [3H]thymidine incorporation into DNA can be a marker of injury. However, [3H]thymidine incorporation into DNA may also be indicative of cellular proliferation. This possibility had not been investigated previously in EC.

The model used in the present study is a more physiological model compared with previous ones in that EC were studied with the majority of cells in growth arrest and with cells in serum-free conditions. Using this model, we performed direct determinations of the significance of nicotine-stimulated DNA synthesis in EC. Our studies showed that, over a broad range of concentrations, including those found in smokers (8, 26), nicotine did not impair proliferation of EC and did not appear to result in EC injury. To the contrary, the number of cells, and scheduled [3H]thymidine incorporation into DNA, were significantly increased. Therefore, nicotine was mitogenic because nicotine-stimulated DNA synthesis resulted in cellular proliferation. Our findings are in agreement with previously described mitogenic effects of nicotine in other cell types, including human cervical cells (35) and pulmonary neuroendocrine cells (19).

Although mechanisms accounting for nicotine-stimulated cellular proliferation in EC are not known, they could involve interactions of nicotine with cellular nicotinic cholinergic receptors. Our studies demonstrated that the proliferative effects of nicotine in EC were completely inhibited by the nicotinic-receptor antagonist hexamethonium. Previous evidence exists for the presence of functional nicotinic receptors on vascular cells. For example, in deendothelialized saphenous arterial strips, nicotine-induced vascular relaxation was abolished by hexamethonium (20). Similarly, in dog retinal central arteries that responded to nicotine with relaxation, vasodilatation was abolished by treatment with hexamethonium (32). Although there are no previous data demonstrating nicotinic growth control in EC, nicotinic control of cell growth has been reported in newborn hamster lung cells (19) and human lung cancer cell lines (16). Taken together, these data support the concept that nicotinic receptors may be important not only in vascular function but also in growth control of vascular cells.

Although the pathophysiological effect of nicotine stimulation of endothelial proliferation is not clear, the following scheme is postulated to explain the potential significance of our results: nicotine is believed to function as a tumor promoter (17, 37). Angiogenesis, known to take place in lung tumors (39), correlates not only with tumor growth but also with a tumor's metastatic potential (36). Furthermore, it has been recently demonstrated that angiostatin, a circulating EC inhibitor, suppresses angiogenesis and mediates suppression of tumor growth and tumor metastases (21, 22) in lung carcinoma. Angiogenesis is a complex multistep process that includes the proliferation of EC (2). If nicotine stimulates tumor growth and EC proliferation, then nicotine could be of significance in vascular support of malignant tumors, and specifically, in nicotine-mediated tumor angiogenesis. No studies to date have investigated a role for nicotine in the direct stimulation of angiogenesis or neovascularization. This area remains an important and underinvestigated area of study.

Nicotine-dependent carcinogenesis has been demonstrated to proceed by growth factor-mediated mechanisms (25). Our experiments demonstrated that the regulation of DNA synthesis and cell growth by nicotine was enhanced in EC by PDGFs, specifically PDECGF. This growth factor has a previously well-demonstrated role in stimulating EC proliferation (10). It is also of interest to the theoretic scheme proposed above, that PDECGF has recently been shown to be an important angiogenic stimulus in several in vivo assays (7). PDECGF is also important in tumor systems and is differentially expressed in human lung carcinoma cell lines (11). Previous studies have demonstrated that growth factors can regulate cellular effects of nicotine by modulation of nicotinic-receptor expression and number (14), and enhanced [3H]nicotine binding to nicotinic receptors, as has been shown for nicotinic growth factor on adrenal PC-12 cells (15). Nicotine can, in turn, regulate the expression of growth factor receptors (25, 30), enhance the intracellular stability of growth factors, and protect against their degradation (25). Although we are unable to state by which of these mechanisms PDECGF and nicotine are able to interact to enhance nicotine-stimulated EC growth, it is clear that growth factors can enhance the effects of nicotine. These interactions may be of particular importance in growth factor-mediated mechanisms of nicotine-dependent carcinogenesis and angiogenesis, as has previously been suggested (25). In our system, PDGF did not enhance the endothelial growth response to nicotine. The lack of response to PDGF may be due to the source of EC. Microvessel EC are believed to have PDGF receptors, whereas those of large vessels do not.

The proliferative effects of nicotine could also be significant in terms of cardiovascular disease. Although atherosclerosis is primarily characterized by neointimal smooth muscle cell proliferation, endothelial proliferation occurs during reendothelialization in response
to vascular injury and denudation. Nicotine could be of importance in this process. In addition, chronic ischemic conditions may be accompanied by a local angiogenic response, and nicotine might be postulated to have a stimulatory effect in this regard. The stimulatory interaction between nicotine and platelet growth factors could be relevant to cellular proliferative responses within the atherosclerotic plaque.

Finally, the time-response studies indicated that thymidine counts peaked at 32 h but then decreased with longer incubation periods. The reasons for the reduction in counts after 32 h were not clear. Potential explanations include 1) loss of cell attachment to the underlying plastic with longer incubation periods; 2) complete utilization of the added thymidine label with longer incubation times; or 3) apparent dilution of the thymidine label caused by incomplete depletion of unlabeled intracellular thymidine pools during cell permeabilization and washes. However, it is important to point out that the present study was done at peak thymidine incorporation (32 h) and not at later time periods.

In conclusion, the results of this study indicate that nicotine can regulate EC growth and suggest nicotinic control of growth. The response exhibits complexity in that it is bimodal, with both proliferation and cytotoxicity observed. That nicotine can stimulate EC proliferation is of interest and contrary to results from earlier experiments that suggested an inhibitory role for nicotine in cell growth. Differentiation between the bimodal effects of nicotine on EC growth appear to be linked to nicotine concentration and the cellular environment, and the proliferative response appears to be mediated via nicotinic receptors. Taken together, our findings may be of significance in conditions affected by nicotine that are also dependent on endothelial proliferation and angiogenesis, including tumors, vascular wound, and cardiovascular disease.

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