Regulation of angiotensin-converting enzyme production by nicotine in human endothelial cells

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Epidemiological studies have shown a strong correlation between cigarette smoking and the development and progression of cardiovascular disease such as atherosclerosis (3). The mechanism for the increased cardiovascular risk of cigarette smoke is not well understood, but it is presumed to be related to endothelial dysfunction (19).

Cigarette smoke contains >4000 chemical constituents. It is not clear which components of cigarette smoke contribute to the pathogenesis of cardiovascular disease.

Nicotine is the most widely studied compound of cigarette smoke. Nicotine has a variety of effects on vascular biology that may contribute to atherosclerosis (13). In vivo, nicotine has been reported to impair endothelium-dependent dilatation in human veins (4). In vitro, nicotine has been shown to be mitogenic for endothelial cells, smooth muscle cells, and fibroblasts (17, 24). It modulates the expression of various proteins such as basic fibroblast growth factor (8), transforming growth factor-β (8), tumor necrosis factor-α (1), plasminogen activator inhibitor-1 (28), nitric oxide synthase (23), and vascular endothelial growth factor (VEGF; Ref. 7) in endothelial cells.

Angiotensin-converting enzyme (ACE), a widely distributed enzyme mainly located on the luminal surface of vascular endothelium, catalyzes the proteolytic cleavage of angiotensin (ANG) I to ANG II and has bradykinin-degrading activity (22). Thus ACE participates in the control of vascular resistance by generating ANG II and degrading bradykinin. ANG II also acts as a vascular growth factor participating in angiogenesis, vascular remodeling, response to vascular wall injury, and atherogenesis (27). As a regulator of ANG II production, ACE may have an important role in atherosclerosis and hypertension.

It has been reported that the renin-angiotensin-aldosterone system is activated in smokers (16) and that ACE inhibitors improve endothelial function in smokers (5). In the present study, we investigated the role of nicotine in ACE regulation in human endothelial cells.

MATERIALS AND METHODS

Endothelial Cell Culture

With the approval of the Committee of Ethics of the Department of Obstetrics and Gynecology, University of Helsinki, endothelial cells were prepared from human umbilical cord veins (HUVECs) according to the method of Jaffe et al. (15). Veins were cannulated, washed with phosphate-buffered saline (PBS), and treated with 0.5% collagenase (Sigma; St. Louis, MO) in PBS for 15 min at room temperature, and cells were then collected by centrifugation. Cells were grown to confluence in 0.2% gelatin (Sigma)-coated cell culture flasks (Costar; Cambridge, MA) in medium 199 (GIBCO; Belmont, CA) supplemented with 20% fetal calf serum (GIBCO), 20 μg/ml endothelial cell growth supplement (Sigma), 12 U/ml heparin (Sigma), 100 U/ml G-penicillin, 100 μg/ml streptomycin (GIBCO), and 2 mM L-glutamine (GIBCO) at 37°C in humidified 5% carbon dioxide in air. The cells were detached in a solution of 0.125% trypsin and 0.02% Na2-EDTA (GIBCO) and subcultured on 48-well cell culture plates (Costar) coated with 0.2% gelatin solution. The cells were identified as endothelial cells by their typical cobblestone appearance and the presence of von Willebrand factor as demonstrated by immunofluorescence method using rabbit immunoglobulin to human von Willebrand factor (Dakopatts; Glostrup, Denmark); >90% of the cells stained positively.

Experimental Design

Confluent endothelial cell subcultures at passages 1–2 were incubated in medium 199 supplemented with 5% fetal calf serum. To study the effects of nicotine on basal ACE production, cell cultures were incubated with nicotine (0.1–1 μM; Sigma) for 4–24 h. Effects of nicotine on stimulated ACE production were studied by coincubating endothelial cells with nicotine and VEGF. The concentration of nicotine, 0.1–1 μM; Sigma), was chosen on the basis of a dose–response curve established in preliminary studies. Values are given as means ± SE. Statistical differences between groups were assessed by analysis of variance followed by Bonferroni’s multiple comparisons test. P < 0.05 was considered statistically significant.
VEGF used here (0.5 nM; Sigma) was earlier shown to be effective in stimulating ACE production (21). To study whether PKC was involved in the nicotine-signaling pathway, endothelial cells were preincubated with the PKC inhibitor 3-[N-(dimethylamino)propyl]-3-indolyl]-4-(3-indolyl)maleimide [GF-109203X (GFX); 2.5 μM; Calbiochem; San Diego, CA] for 30 min and then with nicotine (0.1 μM) and VEGF (0.5 nM) for an additional 24 h. To study whether the effects of nicotine were mediated via nicotinic acetylcholine receptors (nAChRs), endothelial cells were preincubated for 30 min with the specific nAChR blocker hexamethonium (1–200 μM; Sigma) and then with nicotine (0.1–1 μM) and VEGF (0.5 nM) for an additional 24 h. After the incubation time had passed, ACE assay was performed as described below.

The effects of test substances on cellular viability and growth were tested by a CellTiter 96 Cell Proliferation/Cytotoxicity Assay Kit (Promega; Madison, WI) and by protein assay using a BCA (bicinchoninic acid) Protein Assay Kit (Pierce; Rockford, IL).

ACE Inhibitor-Binding Assay

The amount of ACE in intact endothelial cells was measured using an inhibitor-binding assay and characterized in our laboratory (11, 20). Briefly, the lisinopril analog 351A [a p-hydroxybenzamidine derivative of N-(1-carboxy-3-phenylpropyl)-l-lysyl-L-proline; MerckSharp&Dohme; Rahway, NJ] was labeled with 125I (IMS 125I-labeled 351A) that was bound (in cpm) per 105 cells, which is proportional to the amount of ACE on the cell membrane (11). The amount of ACE is given as the quantity of inhibitor (in cpm) per 106 cells, which is proportional to the amount of ACE on the cell membrane (11). The method was previously shown by us (11) and others (26) to closely match the enzyme-activity method.

ACE mRNA Measurement

Endothelial cells grown on gelatin-coated tissue-culture plates were incubated with nicotine (0.1 μM) and VEGF (0.5 nM) for 24 h.

Total RNA and cDNA preparation. Total RNA from HUVECs was isolated using a GenElute Mammalian Total RNA Kit (Sigma) according to the manufacturer’s instructions. After DNase I treatment (Sigma), RNA concentrations were measured using the RiboGreen fluorescent nucleic acid stain (Molecular Probes; Eugene, OR) and were checked by agarose gel electrophoresis. Total RNA (0.5 μg) was transcribed into cDNA using murine Moloney leukemia virus reverse transcriptase (Life Technologies; Paisley, UK) and oligo(dT)12–18 (500 μg/μl) primer.

Quantification of ACE mRNA and 28S rRNA. Quantification of the mRNAs was performed by real-time PCR using LightCycler technology (Roche Diagnostics; Mannheim, Germany). The cDNA (2 μl, diluted 1:10) was brought to a final volume of 20 μl that contained 4 mM MgCl2, 2 μl of LightCycler-FastStart DNA SYBR Green I Mix (Roche Diagnostics), and 0.5 μM of primers in H2O. After initial activation of the DNA polymerase at 95°C for 10 min, the amplification conditions were as follows: 40 cycles of denaturation at 95°C for 15 s, annealing for 5 s at 54°C (28S rRNA), 3 s at 56°C (ACE), and extension at 72°C. The extension times (in s) were calculated from the amplicon size [base pairs (bp)/25]. Fluorescence data were acquired at the end of each extension phase. After amplification, a melting-curve analysis was made from 65 to 95°C with a heating rate of 0.1°C/s and continuous fluorescence acquisition.

The primers used for 28S rRNA (GenBank accession no. X00525) were as follows: forward primer, 5'-TTG AAA ATC CGG GGG AGA G; reverse primer, 5'-ACA TTG TTC CAA CAT GGC AG; and amplicon size, 100 bp. For ACE (GenBank accession no. AF118569), the forward primer was 5'-ACC AAT GAC GAG CAA AGA; reverse primer was 5'-GGT GTG TTT GAA CTG A, and amplicon size was 207 bp. Standard curves were created from specific PCR products. The signals of the samples of interest were then quantified from the standard curve, and ACE expression was determined relative to 28S rRNA.

Immunoblotting of Phosphorylated PKC

Before experiments were started, confluent HUVECs were incubated in medium 199 with 0.2% BSA for 4 h. Nicotine (0.1 μM) and VEGF (0.5 nM) were then added, and incubation continued for 15 min. GF-109203X (2.5 μM) was added 30 min before nicotine and VEGF additions. Phosphorylated and nonphosphorylated PKC levels were measured using phospho-PKC (pan) antibody (Cell Signaling Technology; Beverly, MA) and PKC (MC5) antibody (Santa Cruz Biotechnology). Western blots were performed according to the manufacturer’s instructions (Cell Signaling Technology). Briefly, cells were rinsed with ice-cold PBS and lysed with SDS sample buffer that
contained 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromophenol blue and were immediately scraped off the plate and sonicated. An aliquot of sample was boiled for 5 min, centrifuged, and applied to SDS-PAGE gel for Western blotting. Equal amounts of protein in the supernatants were subjected to SDS-PAGE. A 10% SDS-PAGE bis-acrylamide gel was run at 100 V for 1.5 h. After transfer of samples to nitrocellulose membranes, the membranes were blotted overnight with primary antibodies (phospho-PKC or PKC antibody, 1:1,000 dilution) and the membranes were then incubated with horseradish peroxidase-conjugated secondary antibody at a 1:2,000 dilution (Cell Signaling Technology). The bands were visualized with Western Blot Chemiluminescence Reagent Plus (NEL Life Science Products; Boston, MA) after autoradiography according to manufacturer’s instructions. Bands were quantified by Science Lab 99 Image Gauge software (Fuji Photo Film).

Statistical Evaluation

Results are expressed as means ± SD of three to eight replicate determinations from three or four separate experiments. ANOVA and subsequent Bonferroni’s multiple-comparison tests were applied.

RESULTS

Effects of Nicotine on Basal and VEGF-Stimulated ACE Production

Nicotine alone did not modulate basal ACE production measured after 4–24 h of treatment (Fig. 1). When endothelial cell cultures were cotreated with VEGF and nicotine, the stimulatory effect of VEGF on ACE was significantly potentiated after 18 and 24 h, whereas no stimulatory effect was measured after 4 h (Fig. 1A).

Hexamethonium (1–200 μM), a nAChR blocker, had no effect on nicotine and VEGF cotreatment-induced ACE production, which suggests that the effect of nicotine was not mediated via nAChRs (data not shown).

Effects of Nicotine on ACE mRNA Levels

To study whether the potentiating effect of nicotine on VEGF-induced ACE production was also found at the mRNA level, real-time PCR was used to quantify the levels of ACE

![Fig. 2](http://ajpheart.physiology.org/)

Fig. 2. Effect of nicotine (0.1 μM) on VEGF (0.5 nM)-induced ACE mRNA levels after 24 h of incubation as detected by LightCycler technology. Relative ACE mRNA levels normalized to 28S rRNA are shown. Values are means ± SD; n = 9; ***P < 0.001 vs. control; +++P < 0.001 vs. VEGF.

![Fig. 4](http://ajpheart.physiology.org/)

Fig. 4. Effect of GFX on VEGF- and nicotine-induced ACE upregulation in HUVECs after 24 h of incubation. GFX (2.5 μM) was added 30 min before VEGF (0.5 nM) and nicotine (0.1 μM). Values are means ± SD; n = 16; ***P < 0.001 vs. control.

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mRNA and 28S rRNA. Levels of the 28S rRNA did not vary significantly between treatments. Nicotine treatment caused potentiation of VEGF-induced ACE mRNA levels, whereas nicotine alone did not modulate ACE mRNA levels (Fig. 2).

Effects of Nicotine on PKC Phosphorylation

Incubation of endothelial cells with nicotine (0.1 μM) alone did not induce PKC phosphorylation. VEGF (0.5 nM) treatment induced PKC phosphorylation measured after 15 min, and this effect was potentiated by cotreatment with nicotine (0.1 μM; Fig. 3, A and B). Nonphosphorylated PKC levels were not significantly modulated by treatments (Fig. 3A). Preincubation of the cell cultures for 30 min with the selective PKC inhibitor GFX (2.5 μM) totally blocked VEGF (0.5 nM)- and nicotine (0.1 μM)-induced PKC phosphorylation (Fig. 3C).

Effects of GFX on ACE Upregulation

The selective PKC inhibitor GFX (2.5 μM) did not modulate basal ACE production after 24 h of incubation. When the cell cultures were preincubated with GFX (2.5 μM) for 15 min and then incubated together with nicotine (0.1 μM) and VEGF (0.5 nM) for 24 h, ACE production was downregulated to control levels (Fig. 4).

DISCUSSION

We show here that nicotine in combination with VEGF potentiated VEGF-induced ACE upregulation in human endothelial cells, whereas nicotine treatment alone did not modify ACE levels even at a high concentration. This kind of effect was not restricted to nicotine, as cotinine, which is the major product of nicotine metabolism, had a similar VEGF-potentiating effect (unpublished results from our laboratory). An effective concentration of nicotine (100 nM) shown here to potentiate VEGF-induced ACE production was within the range measured in plasma of cigarette smokers (2).

We have earlier shown that VEGF is a potent ACE inducer in endothelial cell culture (21). The renin-angiotensin system plays a role in many cardiovascular disorders in which angiogenesis is induced. This includes myointimal proliferation after vascular injury, atherosclerosis, and diabetic angiopathy (10). Association of enhanced vascular ACE expression with the development of coronary atherosclerosis in humans has been demonstrated (10, 18). Furthermore, ACE inhibitors have vasculoprotective effects that may contribute to prevention of coronary atherosclerosis (6) and reduction of left ventricular hypertrophy of hypertensive patients (9).

Nicotine has been reported to accelerate the growth of atherosclerotic plaques and tumors in experimental animals, and it has been suggested (13) that VEGF has a role in these processes. Thus synergistic interaction of nicotine and VEGF in ACE induction may enforce detrimental growth processes in the vascular wall by increasing ANG II generation and bradykinin degradation.

Nicotine reportedly increased VEGF expression in an intact porcine carotid artery perfusion system (7). Increase of VEGF expression probably does not explain the ACE induction in the present study, as nicotine alone did not modulate ACE expression.

To study the intracellular mechanism by which nicotine potentiated VEGF-induced ACE production, we focused on the intracellular signaling molecule PKC, which has been implicated in the regulation of ACE production in endothelial cells (21, 25). We have previously reported that PKC is a mediator involved in VEGF-induced ACE production (21).

Here we show that inhibition of PKC by the selective PKC inhibitor GFX totally blocked ACE upregulation induced by VEGF and nicotine cotreatment. Furthermore, we studied whether PKC phosphorylation was modulated by nicotine. Nicotine enhanced VEGF-stimulated PKC phosphorylation, whereas nicotine alone did not modify PKC phosphorylation. These data suggest that nicotine potentiated ACE production by potentiotizing VEGF-induced PKC phosphorylation. The mechanism causing this potentiation remains to be studied. The results of the present study suggest that nicotine potentiated VEGF-induced ACE production by means other than via nAChRs. Indeed, it has been reported that nicotine could bypass nAChR activation and directly modulate intracellular signaling (12, 23).

Nicotine has been associated with impaired endothelial function and increased neovascularization, which contribute to the development of atherosclerosis (4, 13). Because cigarette smoke consists of numerous chemical compounds, the mechanism by which smoking induces endothelial dysfunction may be multifactorial. The present study suggests that interaction of nicotine and VEGF in ACE induction may contribute to the pathogenesis of smoking-related cardiovascular disease.

GRANTS

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