Nicotine Increases Plasminogen Activator Inhibitor-1 Production by Human Brain Endothelial Cells via Protein Kinase C–Associated Pathway

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Background and Purpose—Smoking both increases stroke risk and reduces the risk of thrombolysis-associated intracerebral hemorrhage. Plasminogen activator inhibitor-1 (PAI-1) is a major regulator of fibrinolysis; elevation of PAI-1 is associated with an increased risk of thrombotic disorders. We studied the effect of nicotine, an important constituent of cigarette smoke, on PAI-1 production by human brain endothelial cells.

Methods—Adult human central nervous system endothelial cells (CNS-EC) were used for tissue culture experiments. We analyzed culture supernatant for PAI-1 protein and measured PAI-1 mRNA (by Northern blot analysis) and protein kinase C (PK-C) activity.

Results—Nicotine at 100 nmol/L increased PAI-1 protein production and mRNA expression by CNS-EC. After 72 hours of exposure to nicotine, the concentration of secreted PAI-1 in the cell supernatant was increased 1.90 ± 0.2 fold compared with untreated cells. PAI-1 mRNA also increased approximately twofold. Inhibition of PK-C completely abolished this effect. Nicotine had no effect on the concentration of tissue plasminogen activator.

Conclusions—Nicotine increases brain endothelial cell PAI-1 mRNA expression and protein production via PK-C–dependent pathway. These findings provide new insights into why smoking may be associated with predisposition to thrombosis and inversely associated with intracerebral hemorrhage after therapeutic tissue plasminogen activator therapy. (Stroke. 1999;30:651-655.)

Key Words: cerebral ischemia ■ endothelium ■ nicotine ■ plasminogen activator inhibitor 1 ■ protein kinase C
would be relevant to understanding the regulation of PAI-1 production.

Materials and Methods

Cell Culture

CNS-ECs were derived from human brain as previously described in detail. Cells were cultured in RPMI-1640 medium (GIBCO Labs) supplemented with 100 ng/mL endothelial cell growth factor Endogro (VECTEC), 2 mmol/L L-glutamine, 10 mmol/L Hepes, 24 mmol/L sodium bicarbonate, 300 USP U/mL heparin, 1% penicillin/streptomycin, and 10% fetal calf serum. We used subconfluent preparations of cells up until passage 4 to 5 only; and Endogro-free medium was used beginning 24 hours before the experiment. The purity of CNS-EC (>95%) was confirmed by immunocytochemical staining for factor VIII, glial fibrillary acidic protein, and the macrophage maker CD11b, as previously described. The PK-C inhibitor GF-109203-X (GF) (Calbiochem) was added to the cells 30 minutes before nicotine treatment. GF at 1 µmol/L was used in inhibition experiments; this dose completely inhibited PK-C in CNS-EC without affecting cell viability (authors’ unpublished observations). Nicotine sulfate (Sigma) was used at 100 nmol/L, on the basis of titration experiments and physiological range.

PK-C Assay

ECs were treated as described in the previous sections. The PK-C assays were performed using PK-C assay kits obtained from GIBCO. Briefly, the experimental treatments were terminated by replacing the medium with the cell extraction buffer (20 mmol/L TRIS, 0.5 mmol/L EDTA, 0.5 mmol/L EGTA, 25 µg/mL each aprotinin and leupeptin, pH 7.5) at room temperature. The cells were then homogenized with a precooled Dounce homogenizer, and the cytosol and membrane fractions were separated by centrifugation (10 000 g, 30 minutes), at 4°C. The supernatants were collected as the cytosol fraction. The pellets were resuspended in 0.5 mL extraction buffer with the detergent (1% NP-40). Membrane fractions were obtained by centrifugation (10 000 g, 10 minutes, 4°C) and the supernatant was collected. The determinations of PK-C activity in the cytosol and membrane fractions were performed according to the manufacturer’s instructions. The specific PK-C substrate was a synthetic peptide from myelin basic protein (amino acids 4 to 14) with an acetylated N-terminal glutamine, and the specific inhibitor was a peptide (amino acids 19 to 36) derived from the same protein that binds to the “pseudosubstrate” region of the regulatory domain. The specific PK-C activity was determined as the difference between phosphorylation of the PK-C-specific substrate in the absence or presence of the specific PK-C inhibitor. The data are presented as ratios of the membrane and the total (cytosol+membrane) PK-C activities.

PAI-1 Production

PAI-1 production was evaluated using the commercially available ELISA kit (American Diagnostics Inc). Briefly, cells were grown in culture to 70% to 80% confluency in 10% fetal calf serum. Culture supernatants (100 µL) were removed after 24, 48, 72, and 96 hours and evaluated for PAI-1 content. The ELISA determined the amounts of bound and free PAI-1 present. Each data point was generated by samples run in triplicate; and each experiment was repeated at least 3 times.

RNA Analysis

RNA from 2 to 3×10^6 cultured cells was isolated 24 hours after treatment and prepared according to a modification of the acid phenol method using the Trisol reagent (Life Technologies) as specified by the manufacturer. Twelve micrograms of total RNA was denatured and fractionated on a 1.2% agarose gel containing formaldehyde, then transferred to Hybond-N nylon membrane (Amer sham), and hybridized with [32P]UTP Klenow-labeled random-primed probes (Boehringer Mannheim) according to manufacturer’s instructions. Prehybridization was performed in Quikhyb hybridization solution (Stratagene) for 15 minutes at 68°C. Subsequently the labeled probe plus 1 mg salmon sperm DNA was added to the hybridization solution mixture and incubated for 1 hour at 68°C. The hybridized membrane was washed twice with 2× standard saline citrate, 0.1% sodium dodecyl sulfate at 37°C, then washed with 0.1× standard saline citrate, 0.1% sodium dodecyl sulfate buffer for 15 minutes at 60°C. Membranes were exposed to Hyperfilm-MP (Amersham) for at least 24 hours at −70°C. The human PAI-1 probe used in hybridization, obtained from plasmid designated as pPAI-1 1.3, is 1253 bp in length and binds to the full-length PAI-1 mRNA (3.0 kb). Densities of hybridized bands were determined by scanning and analyzed using the NIH Image software program. Blots were rehybridized with labeled polymerase chain reaction products of glyceraldehyde-3 phosphate dehydrogenase (GAPDH; Stratagene), the housekeeping gene, to control for total amount of RNA present in the cells and precision of RNA loading.

Statistics

All experiments were performed 3 times unless otherwise stated. The error bars on all figures correspond to SEM. Statistical comparisons between groups were performed using unpaired Student’s t tests or 2-way ANOVA. All data presented as significant in this study have the value of P<0.02, unless otherwise indicated.

Results

We investigated the effects of physiological concentrations of nicotine on PAI-1 production by human brain–derived endothelial cells. CNS-ECs were cultured in the absence or presence of nicotine (100 nmol/L) for 96 hours. Aliquots of supernatant were removed after 24, 48, 72 and 96 hours and analyzed for secreted PAI-1 protein using the ELISA technique. The results of 4 experiments (each in triplicate) on different CNS-EC primary cell cultures demonstrated a significant increase in PAI-1 production following nicotine treatment after 72 hours with a continued increase through 96 hours of culture. Primary cultures typically exhibit great variability in baseline levels of PAI-1 production, ranging from 9.6 to 33.4 µg/mL per 10^6 cells (27.1±2.9; SEM), and after 72 hours of nicotine treatment, ranging from 31.8 to 75.2 µg/mL per 10^6 cells (53.8±5.8; SEM). The results of nicotine treatment were therefore assessed in terms of relative PAI-1 production for each treated culture as compared with its respective control. Nicotine treatment increased relative PAI-1 production by 1.9±0.2 fold (P<0.02). To determine whether nicotine affects PAI-1 mRNA expression, CNS-ECs were exposed to nicotine for 24 hours, and RNA was isolated and analyzed for PAI-1 mRNA. The results (Figure 1A, 1B) show that PAI-1 mRNA was increased approximately twofold in the presence of nicotine. The data presented are representative of 3 experiments performed. Incubation with nicotine for 1, 4, or 6 hours showed no significant increase in PAI-1 mRNA above control values (data not shown). Thus, nicotine increases PAI-1 production on mRNA and protein levels. In contrast, incubation of the CNS-ECs with nicotine (100 nmol/L) for 72 hours did not significantly affect tPA production (14.4±1.7 ng/mL · 10^6 cells for the control versus 15.5±1.6 ng/mL · 10^6 cells for nicotine-treated cells).

We next determined whether the PK-C–associated intracellular signal transduction pathway is involved in a nicotine-induced increase in PAI-1 production. The effect of nicotine on PK-C activity was measured as a function of time. Figure 2 shows that nicotine (100 nmol/L) indeed caused an increase
in PK-C activity in ECs. The increase in PK-C activity was significant within 30 seconds, attained maximum levels at 2 minutes, and sharply declined thereafter, reaching control levels at 30 minutes (Figure 2). To determine the significance of the nicotine-induced PK-C activation in the observed increase in PAI-1 production, we measured the effect of nicotine in the presence of the PK-C inhibitor GF. Figure 1 demonstrates that in the presence of GF, nicotine has no effect on PAI-1 mRNA levels. Similar results were obtained with another PK-C inhibitor calphostin C (data not shown). Furthermore, Figure 3 shows that GF completely inhibited nicotine-induced PAI-1 protein production, demonstrating that in CNS-ECs PAI-1 mRNA expression and protein production are dependent on the activation of PK-C.

Discussion

We have shown that nicotine increases PAI-1 production by human CNS-ECs. After 72 hours of incubation, PAI-1 protein production increased twofold; this paralleled the increase in PAI-1 mRNA expression. These effects were completely abolished by inhibition of PK-C, indicating that nicotine induces increased PAI-1 production in CNS-ECs via a PK-C–dependent pathway.

We observed the effects of nicotine at 100 nmol/L, within the concentration range of nicotine achieved in the serum of smokers.21,22 Previous studies of the biological effects of nicotine in various vascular systems have shown that mice chronically exposed to nicotine exhibit subendothelial edema, endothelial cell swelling, cytoplasmic vacuolation, and mitochondrial swelling.23 Exposure of bovine EC to nicotine caused giant cell formation, ruffled membrane, and extensive cellular vacuolation.24,25 Other studies found no significant effects of nicotine on EC at the submicromolar concentrations. No change of functions such as growth properties, morphology, total protein and collagen synthesis and turnover, lactate production, DNA synthesis, and platelet-subendothelial interaction/adhesion was observed in bovine aortic ECs with up to 10^{-4} mol/L nicotine in 1 to 4 days.26 Thus, there is no consensus in the literature regarding the effects of nicotine on ECs.

This uncertainty may be related to the use of different nicotine concentrations and duration of the treatment and may depend on the target tissue. Specifically, there are significant physiological differences between micro- and macrovascular ECs. Several growth factors are specific for one or the other type.27–29 Coagulation factors are expressed differently between large vessels and capillaries. Thrombin stimulates ECs from large vessels but not from the capillaries.30 Elevation of glucose resulted in decreased PAI-1 mRNA in brain ECs but not in human umbilical vein ECs (HUVECs) or bovine aortic ECs.31 The PK-C activator PMA suppresses the basic fibroblast growth factor–induced proliferation of capillary ECs but has no effect on aortic ECs.32 Also, vanadate treatment leads to an inhibition of protein tyrosine kinase activity in the aortic ECs but not in the capillary ECs.33 Thus, these ECs differ not only in the array of growth factor receptors present on their
surfaces but also in their intracellular regulatory mechanisms. For these reasons we have used human adult CNS-derived ECs, emphasizing the relevance to stroke. Our results, which associated increased PAI-1 production with activation of PK-C, agree with other studies in which PK-C activation was shown to increase PAI-1 production in various ECs, including HUVECs, HUVECs, bovine CNS-ECs, and bovine aortic ECs. Furthermore, our data are consistent with clinical findings showing increased plasma levels of PAI-1 in smokers.38,39

There is a complex relationship between smoking and hemorrhagic transformation after tPA treatment for stroke. Hemorrhagic transformation is related to disruption of the microvascular basal lamina after cerebral ischemia, and it is unclear how that process is modified by therapeutic thrombolysis and smoking. However, it is known that smokers have increased plasma levels of PAI-1, and there is substantial evidence emphasizing the importance of PAI-1 in thrombolysis after acute myocardial infarction. Measurements of PAI-1 predict outcome in these patients: high baseline PAI-1 levels are associated with occluded coronary arteries 90 minutes after treatment. Moreover, neutralizing antibodies to PAI-1 produce enhanced efficacy of therapeutic thrombolysis in an experimental model of coronary artery thrombosis. In the setting of acute arterial thrombosis, endothelial- and platelet-derived PAI-1 bind to fibrin strands and inactivate tPA.

It should be emphasized that nicotine is only one component of cigarette smoke. It is thought that much of the toxic effects of smoking are due to a variety of gas-phase constituents and to tar rather than nicotine. It is unclear to what extent nicotine contributes to hemostatic alterations in humans. Subjects receiving transdermal or oral nicotine have plasma levels of platelet-derived PAI-1 levels associated with occluded coronary arteries 90 minutes after treatment. Moreover, neutralizing antibodies to PAI-1 produce enhanced efficacy of therapeutic thrombolysis in an experimental model of coronary artery thrombosis. In the setting of acute arterial thrombosis, endothelial- and platelet-derived PAI-1 bind to fibrin strands and inactivate tPA.

In summary, our results demonstrate that nicotine induces PAI-1 production in CNS-ECs and that this process is mediated through the PK-C-dependent pathway. Enhanced production of PAI-1 may serve to increase stroke risk by increasing predisposition to thrombosis and to reduce hemorrhagic risk by inactivating therapeutic tPA. Thus, clarification of the mechanism of PAI-1 regulation by nicotine may allow therapeutic modification of both harmful and potentially useful effects.

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References
Nicotine is known to have several effects on blood vessels, including vasoconstriction, increased production of superoxide anion, and impairment of endothelium-dependent relaxation.1,2 The preceding article summarizes a study that examined effects of nicotine on production of plasminogen activator inhibitor-1 (PAI-1) by cerebral endothelium. PAI-1 is a major regulator of fibrinolysis. Increased levels of PAI-1 are present in atherosclerotic lesions,3 and elevated plasma levels of PAI-1 are associated with increased risk for thrombotic disorders.

These are several findings in this study. First, physiologically relevant concentrations of nicotine increased endothelial production of PAI-1 by approximately 2-fold. Second, a molecular analysis indicated that increased expression of PAI-1 in response to nicotine occurred at the mRNA and protein levels. Third, biochemical and pharmacological evidence suggests that the mechanism responsible for increased production of PAI-1 in response to nicotine involves activation of protein kinase C. These new findings, along with other known effects of nicotine on blood vessels, support the concept that this important component of cigarette smoke may contribute to vascular dysfunction and a predisposition to thrombosis.

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References
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