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Effect of Nicotine and Nicotine Metabolites on Angiotensin Converting Enzyme (ACE) in Human Endothelial Cells

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Short title: Nicotine Induces ACE in HUVEC
ABSTRACT

Nicotine has been shown to induce endothelial dysfunction, which is an early marker of atherosclerosis. Nicotine undergoes extensive metabolism in the liver, forming a number of major and minor metabolites. There are very limited data on the effect of nicotine metabolites on the cardiovascular system. This study investigates the effects of nicotine and the nicotine metabolites; cotinine, cotinine-N-oxide, nicotine-1’-N-oxide, norcotinine, trans-3’-hydroxycotinine, on angiotensin converting enzyme (ACE) in human endothelial cells. Cultured endothelial cells obtained from human umbilical cord vein (HUVEC) were stimulated with nicotine or nicotine metabolites in concentrations similar to those observed in plasma during smoking. ACE activity and expression were analysed using commercial kits. The results showed that nicotine and nicotine metabolites can increase both activity and expression of ACE. However, a marked individual variation in the response to the drugs was observed. This variation was not associated with the ACE insertion/deletion polymorphism.

Tobacco contains numerous chemical compounds, and the underlying cause for development of atherosclerosis in smokers is probably multifactorial. The results from this study could explain one cellular mechanism by which smoking exerts negative effect on the vascular system.

KEYWORDS

Angiotensin Converting Enzyme, Atherosclerosis, Endothelial cells, Nicotine, Nicotine metabolites, Tobacco
INTRODUCTION

Cardiovascular diseases are considered to be the major cause of death in Western countries. There is a strong association between cigarette smoking and increased risk of atherosclerosis and other cardiovascular diseases (Ambrose and Barua 2004; Burns 2003; Michael Pittilo 2000). The mechanism behind the development of atherosclerosis in smokers is not fully understood, but a number of studies have suggested that nicotine most likely is an important factor, for review see (Benowitz 1997). Use of oral snuff, where nicotine is absorbed through the oral mucosa, and nicotine infusion have been shown to cause endothelial dysfunction (Neunteufl et al. 2002; Rohani and Agewall 2004). In addition, a large-scale study has shown that snuff use is associated with an increased risk of fatal myocardial infarction (Hergens et al. 2007). Once entering the circulation, nicotine is extensively metabolized. 70-80% of the nicotine is metabolized to cotinine (Benowitz 1997; Kilaru et al. 2001; Yildiz 2004)), 4-7% is converted to nicotine-1´-N-oxide and 1-2% to norcotinine (Benowitz and Jacob 1997). Cotinine is further metabolized, mainly to trans-3´-hydroxycotinine, but also to cotinine-N-oxide and norcotinine, among others (Benowitz and Jacob 1997; Zevin et al. 1998). Trans-3´-hydroxycotinine is the most abundant metabolite found in urine (Benowitz and Jacob 1997; Benowitz et al. 1994). In tobacco users, the plasma concentrations of the major metabolites; cotinine and trans-3´-hydroxycotinine are considerably higher than nicotine (Benowitz and Jacob 2001).

The renin-angiotensin system (RAS) is an important system for maintaining normal homeostasis. Angiotensin-converting enzyme (ACE), a key enzyme in RAS, degrades bradykinin and converts angiotensin I to the vasoactive peptide angiotensin II (ang II). Ang II induces several physiological actions involved in the atherosclerotic process, such as vasoconstriction, proliferation and migration of smooth muscle cells and formation of foam
cells (Weir and Dzau 1999). ACE is located on the luminal surface of the vascular endothelium, but is also present in high amounts in a soluble form in plasma (Wei et al. 1991). It has been suggested that the circulating ACE is important for short-term regulation of the cardiovascular homeostasis, whereas the vascular ACE plays a central role in long-term regulation of the cardiovascular homeostasis (Dzau 1993) and appears to be an important pathophysiologic factor involved in hypertension and atherosclerosis (Dzau 1993; Ruiz-Ortega et al. 2001).

There is genetic variation in the ACE gene i.e. an insertion/deletion polymorphism, consisting of the presence or absence of a 278 bp long fragment located in intron 16 on chromosome 17 (Rigat et al. 1992; Tiret et al. 1992). This polymorphism generates three different genotypes (II, ID, DD) which are strongly connected to the level of ACE in plasma, where II have low levels, ID medium and DD high levels (Rigat et al. 1990). Epidemiological studies have indicated that the DD-genotype is associated with increased risk for cardiovascular diseases (Cambien et al. 1992).

The aim of this study was to investigate the effect of nicotine and the nicotine metabolites; cotinine, cotinine-N-oxide, nicotine-1’-N-oxide, norcotinine and trans-3’-hydroxycotinine, on ACE human endothelial cells. Furthermore, this study investigates if the effect of nicotine and nicotine metabolite is influenced by ACE genotype.
MATERIALS AND METHODS

Experimental design

This study was approved by the regional ethics committee at the Faculty of Health Sciences, Linköping, Sweden (03-602). Confluent HUVECs were subcultured into 96-well plates for analysis of ACE activity and cellular viability or in 24-well plates for ACE expression assay or toxicity testing. 1-2 ml cell suspension was transferred to a tube and stored at -20°C for analysis of ACE genotype. Before starting the experiments, the cell culture medium was replaced with cell culture medium without fetal bovine serum and endothelial cell growth factor (ECGF). ACE activity was analysed after incubation with nicotine and nicotine metabolites (0.1-10 µM) for 10 min up to 24 h. PBS and ethanol (0.01%) were used as controls. To study if effects on ACE activity are due to increased protein synthesis, the expression of ACE in endothelial cells after treatment with nicotine and nicotine metabolites were analysed. Furthermore, ACE activity was analysed in endothelial cells after preincubation with a protein synthesis inhibitor (cycloheximide 10 µM) alone or in combination with nicotine. To further analyse the mechanism of ACE regulation, ACE activity was analysed in serum from 3 healthy volunteers after incubation with nicotine. ACE genotype was analysed to evaluate if the effect of nicotine and nicotine metabolites is dependent on ACE genotype. All treatments and analyses (except ACE genotyping) were performed in duplicate.

Isolation of endothelial cells from human umbilical cords

Human umbilical cords were collected from the delivery ward at Linköping University Hospital, Sweden. Endothelial cells (HUVEC) were isolated from umbilical veins and
cultured according to a method first described by Jaffe et al. (Jaffe et al. 1973). In brief, the umbilical vein was treated with collagenase (0.5 mg/ml) for 25 min at 37°C. Collagenase and cell perfusate were collected in a tube and washed twice by centrifugation for 5 min at 600 g. The cells were resuspended in culture medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with endothelial cell growth factor (ECGF, 30 μg/ml), heparin (20 U/ml), HEPES (10 mM), insulin (0.24 IE/ml), nonessential amino acids (1X), oxalacetic acid (1.2 mM), penicillin (5 U/ml), streptomycin (0.5 μg/ml), and 17% inactivated fetal bovine serum.

Cell culture

The cell solution was transferred to a 25 cm² culture flask coated with gelatine (0.2%) and incubated at 37°C, 5% CO₂. After 24 h, non-adherent cells were removed by washing 4-5 times in Hanks' Balanced Salt Solution (HBSS) and then again incubated in culture medium at 37°C. Medium was replaced every 48-72 h. At confluence, cells were trypsinised and subcultured in new 25 cm² culture flasks.

Preparation of serum

Whole blood was collected in silicone coated tubes (Venoject Terumo Medical Products, Somerset, NJ, USA) and was left in room temperature for 2 h. The tubes were centrifuged at 210 g for 10 min. Serum was transferred to a new tube and stored at -20°C until analysis.
**Angiotensin converting enzyme activity assay**

ACE activity was analysed using a commercial radioenzymatic assay (ACE-direct REA, Bühlmann Laboratories, Schönenbuch, Switzerland). The assay was performed according to the manufacturer’s protocol for analysis of ACE in serum. For analysis of ACE in HUVEC the assay was performed with a few modifications; 1) 10 µl of the calibrator, “zero calibrator” and “control normal” were each added to two wells in a 96-well plate containing 90 µl medium without fetal bovine serum and ECGF. 2) the synthetic substrate $^3$H-hippuryl-glycyl-glycine was added directly to each well and the plate was incubated at 37°C for 2 h. 3) After incubation, 150 µl of the content in the wells was transferred to scintillation vials containing 50 µl 1 M HCl. 1.5 ml scintillation liquid was added to all samples and each sample was counted for 5 min in a beta counter (LKB Wallac, 1217 Rackbeta, Turku, Finland).

**Angiotensin converting enzyme expression assay**

HUVECs were lysed using Cellytic M and the cell lysate was collected and transferred to eppendorf tubes and stored at -20°C until analysis. ACE expression was analysed using Quantikine, Human ACE Immunoassay (R&D Systems, Minneapolis, USA) and the analysis was performed according to the manufacturer’s instructions.

**Determination of ACE genotype**

Genomic DNA was isolated from HUVECs using QIAmp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacture’s protocol with a few modifications; 1) HUVECs were detached from the culture flask using trypsin and resuspended in cell culture medium. The cell suspension was transferred to tubes and stored at -20°C. 200 µl cell
suspension was then used for isolation of genomic DNA. 2) DNA was eluted from the spin column using 100 µl elution buffer. ACE genotype was determined using a protocol described by Cheon et al. (Cheon et al. 2000). Three primers were used; 5´-CTGCAGGTGTCTGCAGCATGTGC-3´, 5´-GATTACAGGCGTGATACAGTCACTTTT-3´ and 5´-GCCATCACATTTCGTAGATCTGGTAG-3´ (Invitrogen Ltd., Paisley, UK). For the deletion gene, the primers will allow detection of a 237 bp fragment, and for the insertion gene, two fragments (525 bp and 155 bp) will be detected. For the PCR reaction a “ready-to-use” reaction mixture was used in a total volume of 20 µl. Each primer was used at a concentration of 0.4 µM and for each reaction 4 µl DNA solution was added to the reaction mixture. The DNA amplification started with an initial denaturation at 94°C for 5 min, followed by 30 cycles of the following thermal profile: denaturation at 94°C for 45 sec, primer annealing at 65°C for 45 sec and primer extension at 72°C for 45 sec, followed by 1 cycle of final extension at 72°C for 5 min. The amplified DNA was separated by gel electrophoresis in a 1.5% agarose gel stained with ethidium bromide and visualised by UV-light. ACE genotype was determined based on the length of the DNA fragments.

**Cellular viability and toxicity testing**

Cellular viability was analysed using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega; Madison, WI, USA). The variation in number of viable cells in each well was calculated to ensure that there was no influence of the test substances on the cell count. Toxicity was analysed by counting viable cells after treatment with Trypan Blue (0.05%).
Statistical analysis

Data are expressed as mean ± S.E.M. Statistical analysis was performed using one-way ANOVA for repeated measures followed by Dunnett’s post-hoc test. One unit ACE is defined as the amount of enzyme required to produce 1 µmol hippuric acid per minute and liter. Statistical significance is described with: * = p < 0.05 and ** = p < 0.01.

Drugs and chemicals

DMEM, fetal bovine serum, gentamicin, HBSS, HEPES, nonessential amino acids, penicillin-streptomycin, and trypsin-EDTA (10x) (GIBCO™) were all bought from Invitrogen Ltd (Paisley, UK). Cellytic M, collagenase, cotinine, cycloheximide, ethidium bromide, gelatine, nicotine, oxalacetic acid, REDExtract-N-Amp Blood PCR ReadyMix and tryphane blue were obtained from Sigma-Aldrich (St. Louis, MO, USA). Insulin (Actrapid®) was purchased from Novo Nordisk (Bagsværd, Denmark), ECGF from Roche Applied Science (Basel, Switzerland) and Heparin (Heparin Leo®) from Leo Pharma A/S (Ballerup, Denmark). Nicotine-1’-N-oxide, cotinine-N-oxide, norcotinine, trans-3-Hydroxycotinine were obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). Certified Molecular Biology Agarose and PCR molecular ruler were bought from Bio-Rad Laboratories (Hercules, CA, USA). The nicotine and nicotine metabolites were dissolved in distilled H2O except from cotinine, which was dissolved in ethanol (99.5%). The drugs were then diluted in sterile PBS. For cotinine, a fixed concentration of ethanol (0.01%) was used.
RESULTS

Effect of nicotine and nicotine metabolites on ACE activity

Incubation of endothelial cells with nicotine, cotinine oxide, norcotinine and trans-3’-hydroxycotinine (0.1-10 µM) for 1 h induced an increase in ACE activity (figure 1A-D). Treatment with nicotine-1’-N-oxide for 1 h showed a tendency to increase the ACE activity, but the effect was not statistically significant (figure 1E). No effect could be seen after stimulation with cotinine (figure 1F). A marked variation in response to the drugs could be observed between endothelial cells from different individuals (figure 2A). For individuals showing an increased ACE activity after incubation with nicotine and nicotine metabolites, most of them (6 of 9) had the greatest effect after 30-60 min (data not shown). A few individuals showed an increased ACE activity still after 24 h (data not shown).

Effect of nicotine and nicotine metabolites on ACE expression

The expression of ACE in endothelial cells increased slightly during incubation with nicotine, cotinine-N-oxide, and nicotine-1’-N-oxide (figure 3A-C). Treatment with norcotinine and trans-3’-hydroxycotinine for 1 h showed a tendency to increase the expression of ACE, but the effect was not statistically significant (figure 3D-E). However, the effect on the expression was not as obvious as the effect on the ACE activity. Incubation with cotinine had no effect on the expression of ACE (figure 3F). The individual difference seen in ACE activity, could also be observed in the expression of ACE (fig 2B).
FIG. 1. ACE activity in cultured endothelial cells treated with A) nicotine, B) cotinine-N-oxide, C) norcotinine, D) trans-3-hydroxycotinine, E) nicotine-1´-N-oxide and F) cotinine for 1 h. Values are mean ± S.E.M. Statistical significance is described by * p < 0.05, ** p < 0.01, n = 11-16

Effect of cycloheximide on nicotine induced increase in ACE activity

Preincubation of endothelial cells with cycloheximide for 10 min, followed by incubation with nicotine, showed that nicotine could still increase the ACE activity. However,
the effect was slightly reduced at 1 µM and 10 µM nicotine (figure 4). Treatment with cycloheximide alone had no effect on ACE activity.

**FIG. 2. Individual differences in the response to treatment with trans-3-hydroxycotinine. A) ACE activity in 3 individuals, B) ACE expression in 2 individuals.**

**Effect of nicotine on ACE activity in serum**

Incubation of serum from 3 healthy individuals with nicotine (0.1-10 µM) for 1 h showed no effect on ACE activity (figure 5).

**Effect of ACE genotype on nicotine induced increase in ACE activity and expression**

To examine if the effect of nicotine and nicotine metabolites on ACE is dependent on genetic variations in the ACE gene, analysis of ACE genotype was performed in endothelial cells from a number of subjects. No difference in the effect of nicotine or nicotine metabolites could be observed between individuals with different ACE genotype (data from the nicotine experiment is shown in figure 6).
**FIG. 3.** Expression of ACE in cultured endothelial cells treated with A) nicotine, B) cotinine-N-oxide, C) nicotine-1’-N-oxide, D) norcotinine, E) trans-3-hydroxycotinine, and F) cotinine for 1 h. Values are mean ± S.E.M. Statistical significance is described by * p < 0.05, ** p < 0.01, n = 9-13

**Cellular viability and toxicity testing**

The cellular viability analysis showed that the maximal variation in the number of endothelial cells in each well was; mean ± 6% (S.D. 3.6%) for untreated cells. For cells
treated with nicotine or nicotine metabolites the mean variation in cell number per well was less than 6%. Treatment with nicotine or nicotine metabolites showed no visible signs of toxicity.

**FIG. 4.** ACE activity in cultured endothelial cells treated with nicotine alone or pre-incubation with cycloheximide [10 µM] and treated with nicotine. A) 4 representative individuals that respond to nicotine. Values are mean ± S.E.M. expressed as % of control. B) An example of 1 individual where nicotine induced an increase in ACE activity.

**FIG. 5.** ACE activity in serum after in vitro treatment with nicotine for 1h. Values are mean ± S.E.M. n = 3
Nicotine, a major compound of tobacco, has been suggested as an important factor for development of atherosclerosis in smokers (Benowitz 1997). Studies have shown that nicotine can cause endothelial dysfunction (Neunteufl et al. 2002; Rohani and Agewall 2004), which is an early marker of atherosclerosis. In addition, Lou et al. (2006) showed a protective effect of the ACE-inhibitor captopril on nicotine-induced endothelial dysfunction in vivo and in vitro. These results indicate that ACE may be important for the pathogenesis of atherosclerosis in smokers.

Nicotine undergoes extensive liver metabolism (Benowitz and Jacob 1997) and most of the metabolites have longer physiologic half life than nicotine (Moyer et al. 2002). According to Benowitz and Jacob (2001), smokers have considerably higher plasma levels of cotinine and trans-3′-hydroxycotinine than nicotine. Therefore, it is important to study the impact of the nicotine metabolites on the vascular system. This study is, to our knowledge, the first to study the impact of the metabolites on the vascular system.
We choose to examine the effect of nicotine and the 5 most abundant nicotine metabolites found in plasma; cotinine, cotinine-N-oxide, nicotine-1’-N-oxide, norcotinine and trans-3’-hydroxycotinine. The concentrations of nicotine and nicotine metabolites used in this study are similar to those observed in plasma from smokers (Benowitz and Jacob 2001).

This study examined the effect of nicotine and nicotine metabolites on ACE in human endothelial cells. We found that nicotine and nicotine metabolites can increase both activity and expression of ACE. The effect on the ACE activity was quite clear and most of the metabolites induced a dose-dependent increase. The effect on the expression of ACE was, however, not as obvious. We could see that nicotine and a few of the metabolites induced a slight increase in ACE expression at some concentrations, but there was no dose-dependent effect.

There are 2 previous \textit{in vitro} studies investigating the effect of nicotine on ACE. Zhang et al showed that incubation of human coronary artery endothelial cells with nicotine for 24 h induced an increase in the gene expression of ACE (Zhang et al. 2001). On the other hand, Saijonmaa et al. could not see any effect of nicotine alone, but together with VEGF, nicotine induced a dose-dependent increase in ACE activity and expression in HUVECs after 24 h incubation. In our study we found the greatest effect of nicotine and nicotine metabolites after 30-60 min. Sajinioma et al. (2005) and Zhang et al. studied the effect after incubation with nicotine for 4 h or more, which may explain the discrepancies in the results.

A few \textit{in vivo} studies have been performed investigating the effect of smoking and nicotine on serum ACE activity. Results from these studies suggest that the acute effect of smoking and nicotine infusion is an increased serum ACE activity (Mizuno et al. 1982; Sugiyama et al. 1986), which is consistent with our results. Chronic smoking has, however, been shown to decrease the serum ACE levels (Haboubi et al. 1986; Ninomiya et al. 1987). However, the majority of the ACE in serum is formed in the vascular endothelium of the
lungs. Since smoking is known to harm the lung tissue, the reduction in ACE activity may reflect damage to the endothelia rather than an actual effect on the enzyme. In addition, these studies measured the serum ACE activity, whereas we measured the endothelial ACE, which is proposed to be more relevant concerning the development of atherosclerosis (Dzau 1993).

Furthermore, none of these in vivo studies have determined the ACE-genotype for the individuals included in the study. Since the I/D polymorphism has been shown to account for 47% of the variation in serum ACE, the ACE genotype should be taken into consideration when comparing ACE levels in different individuals. Therefore, conclusions drawn from these studies may not be entirely accurate.

Thus, the results from the present study are in concordance with previous studies, but methodological differences, such as time, cell type, presence of growth factors, tissue or serum ACE, makes it difficult to compare the results with previous studies.

To further investigate the underlying mechanism for elevation in ACE activity, the endothelial cells were pre-incubated with a protein synthesis inhibitor, and then treated with nicotine. If the increase in ACE activity was due to an increased expression of the enzyme, incubation with a protein synthesis inhibitor would abolish the effect of the drugs. However, nicotine could still induce an elevation in ACE activity, although the effect was slightly reduced at the highest concentrations. Thus, it appears like nicotine and nicotine metabolites increases the activity of ACE by affecting both synthesis and activity of the enzyme. In addition, we were also interested in examining if nicotine had any effect on circulation ACE in serum. Serum from 3 healthy volunteers was incubated with nicotine for 1 h in the same concentrations as the HUVEC, and ACE activity was analysed. The results showed no effect of nicotine on serum ACE. Thus, it appears like nicotine can regulate ACE only when it is located in the endothelial cell and does not influence ACE circulating in the plasma.
In the present study, we also found a great variation between different individuals, in the response to nicotine and nicotine metabolites. In endothelial cells from some individuals the drugs induced a clear dose-dependent increase in ACE activity, whereas in endothelial cells from other individuals the activity was not affected. Furthermore, we could see that in cells from individuals responding with increased ACE activity, nicotine and all metabolites except cotinine induced an increase.

Since the genetic variation in the ACE gene has been shown to regulate levels of ACE in plasma, we hypothesized that the individual variation we had found might be explained by the individuals’ ACE-genotype. Unfortunately, we could not see any association between ACE-genotype and effect of the drugs. There were cells from individuals in all three ACE genotype groups who responded to the drugs, and there were cells from individuals in all three groups who did not respond. Furthermore, the effect of nicotine was not dependent on the basal levels of ACE activity. Since the donors of the umbilical cords were anonymous we have no information concerning the individuals’ life style (tobacco use, hormonal status, diet or salt intake) which perhaps may explain the individual variation seen in your results.

The fact that several of the nicotine metabolites can induce an increase in endothelial ACE is an important finding. Nicotine has been shown to affect a number of cardiovascular factors, and it is possible that these systems are affected by nicotine metabolites as well. Since some of the metabolites are present in higher concentrations than nicotine in plasma (i.e. cotinine and trans-3’-hydroxycotinine) (Benowitz and Jacob 2001), we believe that the effects of the metabolites are at least equally important as the effects of nicotine. Therefore, we claim that, when studying effects of tobacco use and nicotine, the effects of the major metabolites also must be considered.

To understand how smoking is involved in the development of hypertension and atherosclerosis, several aspects have to be considered. Tobacco contains numerous chemical
compounds, and the underlying cause of atherosclerosis in smokers is probably multifactorial. Our findings could explain one cellular mechanism by which smoking exerts negative effect on the vascular system.

In conclusion, this study presents evidence that nicotine and nicotine metabolites increase the activity of ACE in human endothelial cells. Nicotine has previously been shown to increase endothelial ACE (Zhang et al. 2001), but in contrast to earlier studies, we could see an immediate effect, as ACE was affected already after 1 h. This effect could be observed in activity, as well as expression of the enzyme. We also showed a significant individual variation in the response to the drugs, which was not due to the individuals’ ACE genotype. The findings from this study could explain one cellular mechanism behind increased risk of cardiovascular diseases in smokers.

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