Nicotine Enhances Angiotensin II-Induced Mitogenic Response in Vascular Smooth Muscle Cells and Fibroblasts

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Objective—The pathogenetic mechanism of tobacco-related cardiovascular diseases is still not well defined. We examined the potential possibility of an interaction between nicotine, a major component of cigarette smoke, and angiotensin II (Ang II), which plays an important role in the pathogenesis of cardiovascular diseases characterized by Ang II type 1 (AT₁) receptor-mediated abnormal growth of vascular smooth muscle cells (VSMC) and fibroblasts.

Methods and Results—Nicotine or Ang II-stimulated [3H]thymidine incorporation and c-fos expression in adult rat aortic VSMC and adventitial fibroblast. The nicotine-induced DNA synthesis was not affected by valsartan, an AT₁ receptor-specific blocker, or PD123319, an Ang II type 2 (AT₂) receptor-specific antagonist. Nicotine or Ang II stimulation rapidly increased extracellular signal-regulated kinase (ERK) activation, tyrosine- and serine-phosphorylation of signal transducer and activator of transcription (STAT)1 and STAT3, and p38 mitogen-activated protein kinase (p38 MAPK), in both cell types. Interestingly, co-administration of nicotine and Ang II at lower doses, which did not affect cell growth, induced DNA synthesis and c-fos expression accompanied by enhancement of ERK, STAT, and p38MAPK activity. PD98059, a mitogen-activated protein kinase/ERK kinase inhibitor, or SB23058, a p38MAPK inhibitor, significantly attenuated the vasotrophic effect of nicotine and Ang II.

Conclusions—These results suggest that nicotine exerts a growth-promoting effect on vascular cells and enhances the Ang II-induced vasotrophic effect, which is at least partly mediated by the activation of ERK, STAT, and p38MAPK.

Key Words: angiotensin II ■ nicotine ■ ERK ■ STAT ■ vascular smooth muscle cell ■ vascular adventitial fibroblast
present study, we investigated the possibility that nicotine enhances Ang II-induced vasotrophic effects and examined the cellular and molecular mechanisms of potential cross-talk of nicotine and Ang II in arterial VSMC and fibroblasts.

**Methods**

**Cell Culture and Measurement of DNA Synthesis by [3H]Thymidine Incorporation**

Adult rat aortic VSMC and adventitial fibroblasts were prepared from adult Sprague-Dawley rat thoracic aortae (Clea Japan Inc, Tokyo, Japan) as previously described.14,15 Cells at passage 3 to 8 were used for the following experiments. Cells were serum-starved for 48 hours to induce a quiescent state. Subconfluent and quiescent cells cultured in 24-well plates (Techno Plastic Products AG, Trasadingen, Switzerland) were stimulated with various stimuli for 12 hours and pulsed with 1 μCi/mL [3H]-thymidine specific activity (Du Pont NEN Research Products) for an additional 24 hours. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and subsequently incubated with ice-cold 5% trichloroacetic acid for 20 minutes at 4°C. The cells were washed twice with ice-cold 5% trichloroacetic acid, then with ice-cold PBS, and lysed with 0.5% NaOH. The radioactivity of the cell lysate was determined using a liquid scintillation counter. AT1 or AT2 receptor binding was measured as previously described.14 Briefly, the cells were incubated for 1 hour at 37°C with 0.1 nmol/L $^{125}$I-[Sar1, Ile8]Ang II (Du Pont NEN Research Products) in the absence or presence of 1 μmol/L valsartan (donated by Novartis AG) or 1 μmol/L PD123319 (Research Biochemical International). AT1 receptor binding was calculated as the difference between the total count and the count from samples incubated with valsartan. AT2 receptor binding was determined by subtracting the count of samples incubated with PD123319 from the total count.

**Transfection and c-fos Promoter Assay**

VSMC were seeded in 6-well plates and transfected with c-fos-luciferase reporter gene (p2FTL, 1 μg) using LipofectAMINE PLUS reagent (GIBCO-BRL) according to the manufacturer’s instructions. The fos-luciferase reporter gene p2FTL consists of two copies of the c-fos 5′-regulated enhancer element (-357 to -276), the herpes simplex virus thymidine kinase gene promoter (-200 to +70), and the luciferase gene.16 At 48 hours after transfection, the transfected cells were incubated with a serum-free medium for 24 hours. Then the cells were treated with Ang II (10⁻⁷ mol/L) and/or nicotine (10⁻⁸ mol/L) for 4 hours, washed with PBS, and lysed with a 200-μL cell lysis buffer (Promega Corp) at room temperature. Then, 10 μL of the cell extract was mixed with a 100-μL luciferase assay reagent (Promega Corp), and luciferase activity was measured.

**Western Blot Analysis**

Total proteins were prepared from the cultured VSMC and adventitial fibroblasts, and Western blot was performed as previously described.14 Immunoblotting was done using anti-extracellular signal-regulated kinase (ERK), anti-phospho-ERK, anti-signal transducers and activators of transcription (STAT)1, anti-phospho-STAT1 and 3, anti-phospho-p38 MAPK, anti-p38 MAPK (New England Biolabs), and anti-STAT3 (Santa Cruz Biotechnology).

**Statistical Analysis**

Values are expressed as mean±SEM in the text and figures. The data were analyzed using ANOVA. If a statistically significant effect was
Effect of Nicotine and Ang II on Vascular Cells

Results

Dose-Dependent Effects of Nicotine on DNA Synthesis of VSMC and Adventitial Fibroblasts

To determine the effect of nicotine on growth of vascular cells, we first investigated the effect of nicotine on [3H]thymidine incorporation as a marker of DNA synthesis using cultured VSMC and adventitial fibroblasts. We observed that nicotine at 10^{-7} - 10^{-4} mol/L induced a 20% to 40% increase in [3H]thymidine incorporation in both VSMC and adventitial fibroblasts; however, it inhibited DNA synthesis at 10^{-3} mol/L (Figure 1A). Therefore, the pattern of DNA synthesis in response to nicotine was bimodal, with stimulation at low nicotine concentrations (<10^{-4} mol/L) and inhibition at high nicotine concentrations (>10^{-3} mol/L). We observed that addition of nicotine (10^{-4} mol/L) increased the cell number of both VSMC and fibroblasts (Figure 1B). Cytotoxic effects of high concentration of nicotine (>10^{-3} mol/L) assessed by decrease in number of attached cells, increase in the number of floating cells, and increase in LDH release into culture medium was observed (data not shown). Radioligand binding assay showed that rat aortic VSMC and adventitial fibroblasts exclusively expressed the AT_{1} receptor (10.02±0.85 fmol/10^6 cells and 10.65±0.22 fmol/10^6 cells, respectively, mean±SEM, n=4) and no detectable level of the AT_{2} receptor. Treatment with nicotine (10^{-4} and 10^{-3} mol/L) for 48 hours did not affect the expression of the AT_{1} receptor or the AT_{2} receptor (VSMC: 11.07±1.05 fmol/10^6 cells; 10^{-4} mol/L nicotine, 9.85±0.82 fmol/10^6 cells; 10^{-3} mol/L nicotine; mean±SEM, n=4; fibroblasts: 9.47±0.85 fmol/10^6 cells; 10^{-4} mol/L nicotine, 10.80±0.64 fmol/10^6 cells; 10^{-3} mol/L nicotine; mean±SEM, n=4).

Effect of Nicotine and Ang II on DNA Synthesis and c-fos Transcriptional Activity in VSMC and Adventitial Fibroblasts

To examine the possible interaction of nicotine and Ang II in the regulation of vascular cell proliferation, we stimulated the cells with nicotine (10^{-4} or 10^{-3} mol/L) and Ang II (10^{-7} mol/L), and measured [3H]thymidine incorporation as well as c-fos expression. As shown in Figure 1, nicotine treatment enhanced Ang II-induced [3H]thymidine incorporation in both cultured rat VSMC and adventitial fibroblasts. The Ang II-mediated increase in [3H]thymidine incorporation was abolished by the addition of valsartan (10^{-3} mol/L), a specific AT_{1} receptor antagonist, but not by PD123319 (10^{-3} mol/L), a specific AT_{2} receptor antagonist, although nicotine-induced [3H]thymidine incorporation was not affected by either valsartan or PD123319 (Figure 1). Next, we examined the effect of Ang II and/or nicotine on c-fos promoter activity using p2FTL-transfected cells. We observed that treatment with nicotine (10^{-4} mol/L) increased c-fos transcription in both cell types, and moreover that the Ang II-induced increase in c-fos promoter activity was enhanced by nicotine treatment (Figure 2). Furthermore, we examined the possibility of whether Ang II and nicotine could synergistically activate the cell growth and observed that co-administration of lower doses of nicotine (10^{-12} mol/L) and Ang II (10^{-10} mol/L) increased [3H]thymidine incorporation (Figure 1) and c-fos promoter activity (Figure 2) both in VSMC and fibroblasts, whereas Ang II or nicotine at these doses did not affect cell growth.

Cellular Mechanism of Interaction of Nicotine and Ang II in Vascular Cell Proliferation

To examine the signaling mechanism of the enhancement of AT_{1} receptor-mediated proliferation of VSMC and adventitial fibroblasts by nicotine, we focused on ERK activity, because the ERK pathway, which is activated by the AT_{1} receptor and various growth factors, is critical for cell proliferation, differentiation, and hypertrophy. We also examined whether nicotine increased AT_{1} receptor-mediated activation of STAT, because STAT seems to be involved in AT_{1} receptor-mediated c-fos expression in VSMC. Nicotine (10^{-4} mol/L) stimulation rapidly increased ERK activity, reaching a peak at 2 minutes, and declining to near basal level at 30 minutes in both cell types (Figure 2). Nicotine-induced ERK activation was clearly observed at 10^{-7} mol/L, and became more prominent at approximately 10^{-6} mol/L, whereas ERK activ...
activation was hardly observed at $10^{-5}$ mol/L nicotine (Figure 2). In addition, nicotine stimulation induced tyrosine- and serine-phosphorylation of STAT1 and STAT3, with a similar pattern to ERK activation (Figure 2). Ang II ($10^{-7}$ mol/L) stimulated phosphorylation of ERK and STAT in both cell types, and this AT1 receptor-mediated activation of ERK and STAT was enhanced by nicotine treatment (data not shown). We observed that nicotine activated p38 MAPK time- and dose-dependently both in VSMC and fibroblasts (Figure 2). Furthermore, we observed that co-administration of lower doses of nicotine ($10^{-12}$ mol/L) and Ang II ($10^{-10}$ mol/L) activate the ERK, STAT, and p38MAPK both in VSMC and fibroblasts (Figure 3). Total protein levels of ERK, STAT, and p38MAPK were not changed in all these experimental conditions. Moreover, we observed that nicotine ($10^{-10}$ mol/L), or Ang II ($10^{-7}$ mol/L)-induced [H]thymidine incorporation was inhibited by treatment with a mitogen-activated protein kinase/ERK kinase (MEK) inhibitor, PD98059, or p38MAPK inhibitor, SB23058, in a dose-dependent manner (Figure 4A and B). Increase in [H]thymidine incorporation induced by lower doses of nicotine ($10^{-12}$ mol/L) and Ang II ($10^{-10}$ mol/L) was also inhibited by PD98059 or SB23058. We did not observe any cytotoxic effects, such as cell death or a decrease in cell number with PD98059 at concentrations of 5 $\mu$mol/L to 50 $\mu$mol/L, or SB203580 at concentration of 1 $\mu$mol/L to 10 $\mu$mol/L, although even the basal level of [H]thymidine incorporation was inhibited by PD98059 at higher concentrations (> 25 $\mu$mol/L). (See online Figures I and II, available at http://atvb.ahajournals.org.)

**Discussion**

Increasing evidence has indicated that nicotine plays a potential role in the pathogenesis of tobacco-related cardiovascular diseases; however, there is little information on the intracellular signal pathways of nicotine-mediated actions in vascular cells. To elucidate the cellular and molecular mechanisms involved in nicotine-induced effects on the vasculature, we investigated the impact of nicotine on proliferation of cultured arterial VSMC and adventitial fibroblasts. This study provided the possibility that activation of ERK, STAT, and p38MAPK is involved in the nicotine-mediated proliferation of vascular VSMC and fibroblasts. Moreover, we demonstrated that nicotine enhanced the mitogenic effects of Ang II on both cell types at least partially via increases in activity of ERK, STAT, and p38MAPK. These results suggest that the interaction of nicotine and Ang II may contribute to the pathogenesis of smoking-related cardiovascular diseases.

Exposure of VSMC and adventitial fibroblasts to nicotine at a concentration of $10^{-7}–10^{-8}$ mol/L, which is similar to the plasma concentration in cigarette smokers, increased DNA synthesis by 20% to 40% with increased c-fos promoter activity, as previously reported. The apparently nicotine-mediated mitogenic effects in vitro and the more prominent actions of nicotine in vivo (eg, promoting tumor growth and enhancing atherosclerotic lesions) led us to hypothesize that nicotine may interact with other systems, thereby contributing to the development of smoking-related cardiovascular diseases. Indeed, we demonstrated that nicotine enhanced Ang II-induced DNA synthesis as well as c-fos promoter activity, with co-administration of nicotine and Ang II at lower doses, which did not affect cell growth. Because nicotine-induced DNA synthesis was not affected by either an AT1 receptor blocker, valsartan, or an AT2 receptor antagonist, PD123319, it is possible that nicotine potentiates Ang II-induced activation of growth-promoting signaling molecules such as ERK and STAT, leading to the enhancement of Ang II-induced proliferation, without influencing the transactivation of AT1 and AT2 receptors. Consistent with this notion, we observed that pretreatment of VSMC and adventitial fibroblasts for 48 hours with nicotine ($10^{-4}$ and $10^{-5}$ mol/L) did not affect AT1 and AT2 receptor expression, suggesting that the nicotine-mediated proliferation of VSMC and adventitial fibroblasts is independent of AT1 receptor expression.

Our data showed that nicotine stimulated phosphorylation of ERK in VSMC and adventitial fibroblasts. Moreover, we observed that nicotine enhanced Ang II-induced ERK activation in both cell types. The nicotine- and/or Ang II-mediated DNA synthesis was strongly attenuated by inhibition of ERK

**Figure 3.** Effects of nicotine and Ang II on ERK phosphorylation, p38 MAPK phosphorylation, and tyrosine- and serine-phosphorylation of STAT1 and STAT3 in VSMC and adventitial fibroblasts. Subconfluent and quiescent VSMC and adventitial fibroblasts were treated with Ang II ($10^{-10}$ mol/L) and/or nicotine ($10^{-12}$ mol/L) for 2 minutes. Immunoblotting was performed using anti-ERK, anti-phospho-ERK, anti-p38 MAPK, anti-phospho-p38 MAPK, anti-STAT1, anti-phospho-STAT1, anti-STAT3, and anti-phospho-STAT3 antibodies. Figures show representative data from three separate experiments. VAF indicates vascular adventitial fibroblasts.
activation with an MEK inhibitor. These results suggest that ERK is involved in nicotine-mediated vascular cell growth. Expression of the c-fos gene is regulated by the net interaction of different transcriptional factors. Activation of ERK may also result in increased production of serum response factor, and this may act in concert with the activation of STAT, a component of the nuclear sis-inducing factor-complex, thereby resulting in an increase of c-fos transcription. STAT is now known to be activated by many different extracellular signaling proteins, including cytokines, growth factors such as epidermal growth factor (EGF), PDGF, and Ang II via the AT1 receptor. Recent evidence has revealed that serine-phosphorylation as well as tyrosine-phosphorylation is required for maximal activation of STAT, and that activation of ERK has been reported to be involved in the serine-phosphorylation of STATs. We demonstrated that in response to AT1 receptor stimulation, tyrosine- and serine-phosphorylated STAT1 and STAT3 accumulated in the nuclei of VSMC and became a component of the nuclear sis-inducing factor-complex, resulting in enhancement of c-fos promoter activity. In this study, we demonstrated that nicotine increased tyrosine- and serine-phosphorylation of STAT1 and STAT3, thereby increasing c-fos promoter activity. These results suggest that nicotine-mediated activation of STAT may contribute to its enhancing actions on Ang II-induced growth-promoting effects on the vasculature, although the role of nicotine-mediated STAT activation in cell growth has to be clarified in more detail in future experiments. Moreover, we demonstrated that p38MAPK is involved in Ang II- and nicotine-mediated VSMC and vascular adventitial fibroblast proliferation. Ushio-Fukai et al. reported that p38MAPK is a critical component of the oxidant stress-sensitive signaling pathways activated by Ang II in VSMC and indicate that it plays a crucial role in vascular hypertrophy. Taken together, our results demonstrated that nicotine directly increased AT1 receptor-mediated signaling and proliferation of VSMC and adventitial fibroblasts without affecting Ang II receptor expression. The role of Ang II in nicotine-mediated actions in vivo remains to be further investigated, and could provide new insights into the pathogenesis of smoking-related cardiovascular disease.

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