Nicotine Induces Proinflammatory Responses in Macrophages and the Aorta Leading to Acceleration of Atherosclerosis in Low-Density Lipoprotein Receptor\(^{-/-}\) Mice

Paul P. Lau, Lan Li, Aksam J. Merched, Alan L. Zhang, Kerry W.S. Ko, Lawrence Chan

**Objective**—We investigated the molecular mechanism of nicotine-accelerated atherosclerosis in a hyperlipidemic low-density lipoprotein receptor\(^{-/-}\) mouse model.

**Methods and Results**—Low-density lipoprotein receptor \(^{--}\) mice received time-release nicotine or placebo pellets for 90 days. Aortic lesion size was 2.5 times larger in nicotine-treated than in placebo-treated mice \((P<0.001)\). A mild increase in lipids was seen in treated mice. We quantified 18 different serum cytokines and found a significant increase of tumor necrosis factor \(\alpha\), interleukin 1\(\beta\), and keratinocyte-derived chemokine in nicotine-treated mice. Among 107 nuclear factor \(\kappa\B (NF-\kappa B)\) target genes screened from the aorta, we found that nicotine treatment upregulated only 4 atherogenic genes including vascular adhesion molecule 1 and cyclooxygenase 2 on day 60 and platelet-derived growth factor \(B\) and platelet 12-lipoxygenase on day 90. At the cellular level, nicotine induced tumor necrosis factor \(\alpha\) and inducible nitric oxide synthase expression in RAW264.7 cells via the nicotinic acetylcholine receptors. Induction was confirmed in peritoneal macrophages isolated from nicotine-treated mice. Finally, we showed that preconditioned medium from nicotine-treated RAW264.7 cells activated NF-\(\kappa B\) in human smooth muscle cells and vascular endothelial cells as evidenced by nuclear localization and electromobility shift assay.

**Conclusions**—Chronic nicotine exposure augments atherosclerosis by enhancing the production of proinflammatory cytokines by macrophages, which, in turn, activate atherogenic NF-\(\kappa B\) target genes in the aortic lesions. (*Arterioscler Thromb Vasc Biol. 2006;26:143-149.*)

**Key Words:** nicotine ■ atherosclerosis ■ inflammation ■ macrophages ■ NF-\(\kappa B\)

Cigarette smoking has been implicated in numerous diseases, including coronary artery disease.\(^1,2\) Nicotine is a predominant chemical among >100 constituents in cigarette smoke and has been suspected to be a causative agent for atherosclerosis for decades. The mechanism of the proatherogenic action of nicotine is still largely speculative. Although nicotine has been reported to cause an unfavorable lipoprotein profile in some animal models, there is no direct correlation between dyslipidemia and smoking in humans when lifestyle differences were corrected. Researchers have been searching for other potential proatherogenic effects of nicotine. A few in vitro studies suggest that nicotine damages cultured lung and aortic endothelial cells through overstimulation or desensitization of the nicotinic acetylcholine receptors \(\alpha 4\beta 2\) (nAChR).\(^3-6\) Prolonged exposure to nicotine lead to nAChR desensitization, leading to cell bulking\(^7\) and, eventually, detachment, implicating that nicotine effects are caused by direct injury to the arterial wall. Although neuronal nAChR exists in many cell types, including endothelial cells, smooth muscle cells, and macrophages, there has been no direct evidence for nicotine-mediated injury on endothelial cells in any in vivo studies reported to date.

Other than its potential role in initiating injury, nicotine was found to promote angiogenesis in apolipoprotein E-null mice.\(^8\) In this model, nicotine enhanced lesion growth in association with an increase in lesion vascularity. The effect of nicotine, a 184% increase in en face aortic lesion size, was abrogated by a cyclooxygenase (COX)-2 inhibitor. However, absence of an effect of nicotine on angiogenesis obtained from several in vitro and in vivo studies in rats and in humans has also been reported.\(^9-11\) Some authors speculate that nicotine stimulates angiogenesis only in pathological settings.\(^8\)

Inflammation plays a key role in atherosclerosis, but nicotine has often implicated to be anti-inflammatory. For example, among several atherogenic factors found upregu-
imated by nicotine, endothelial nitric oxide synthase mRNA synthesis was induced in cultured human coronary artery endothelial cells. 12 It is known that endothelial nitric oxide synthase may generate a low concentration of nitric oxide with antiatherosclerotic properties. Moreover, by cDNA microarray analysis, the same group13 found that nuclear factor κB (NF-κB) was downregulated by nicotine in cultured endothelial cells. More recently, the α7 subunit of the nicotinic receptor was reported to be required for acetylcholine inhibition of tumor necrosis factor (TNFα) release from isolated C57 macrophages, implicating vagus nerve involvement in inhibiting the release of macrophage TNFα via the cholinergic anti-inflammatory pathway.14

Because there are seemingly conflicting and nonreconcilable findings in different cell and animal models on the mechanism of action of nicotine, there was a call for additional studies to clarify how nicotine affects the interacting effects of different molecular players involved in nicotine-induced angiogenesis and atherogenesis.15

Because nicotine indeed augments atherosclerotic lesion, and inflammation is known to promote atherosclerosis, we hypothesized that the major effects of nicotine on atherosclerosis are not those that pertain to the short-term, acute direct effects examined in most in vitro studies performed with cultured cells, but the long effects induced by chronic exposure. Herein, focusing on the effect of chronic nicotine exposure in low-density lipoprotein (LDL) receptor (LDLR)−/− mice, we demonstrate that nicotine produces a proinflammatory state that augments atherosclerosis via interacting factors produced by different cell types in this model of familial hypercholesterolemia.

Methods

Animal Treatments

We fed male LDLR−/− mice (6 to 8 weeks of age) on a C57BL/6 background (Jackson Laboratory; n = 51) an atherogenic diet (Harlan Teklad) throughout the course of the experiment. Nicotine (90-day release, 5 mg) or placebo time-release pellets of 3 mm in size were implanted subcutaneously on the side of the neck with a 10-gauge stainless steel reusable precision trochar (Innovative Research of America). After 30, 60, and 90 days of treatments, we killed the mice for measurement of aortic lesion size and biochemical analysis. Details are available online at http://atvb.ahajournals.org.

Electrophoretic Mobility-Shift Assay

We prepared nuclear protein extracts from cultures of human aortic endothelial cells (HAECs), vascular smooth muscle cell (VSMC), and RAW cells and from isolated aorta essentially as described by Dignam et al.16 The biotinylated probe set including a cold probe (Panomics, Inc) for NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C) was used. A DNA binding reaction containing 10 µg of proteins in 10 mM ethylenediaminetetraacetic acid (EDTA) and 5% glycerol was carried out in the presence of 1 µg of poly (d-I-C) and 10 ng of the biotinylated probe in 10 µL at 22°C for 30 minutes. Reacted products were separated on a 6% nondenaturing polyacrylamide gel in 0.5× TBE at 4°C. After electrophoresis, we transferred the gel by electroblotting to a nitrocellulose filter in 0.5× TBE for 30 minutes at 300 mA at 4°C and exposed the nitrocellulose membrane to a UV crosslinker oven for 3 minutes. The signal was detected by ABC-elite (Vector Labs) system and enhanced chemiluminescence (Amersham).

Immunohistochemistry and Quantitative Real-Time RT-PCR

We performed immunohistochemical analysis on serial aortic sections (4 µm) to investigate the localization of TNF-α, COX-2, vascular cell adhesion molecule (VCAM)-1, inducible nitric oxide synthase (iNOS), macrophages (Mac 3), and P65/RelA.17 For quantitative real-time RT-PCR, we extracted total RNA from the isolated aorta or from cultured cells and performed quantitative PCR using transferrin as a housekeeping gene. Details are available online at http://atvb.ahajournals.org.

Bioplex Mouse Cytokine Immunoassays and NF-κB Target Gene Arrays

We used 96-well microplate-format Bio-Plex assays (BioRad) to quantify the serum levels of cytokines in 2 plates of either 8 (8-plex) or 10 (10-plex) cytokines and chemokines in a single well per plate and a Bio-Plex Suspension Array System to read the Bio-Plex cytokine assays as described by the manufacturer. Serum samples were stored at −80°C before the assays to avoid protein degradation. Total RNA samples from nicotine- and placebo-treated mice of the different time points were extracted and pooled in groups from their isolated aortas. We synthesized cDNAs from RNA pooled from 5 mice per group and labeled them with biotinylated dUTP according to the manufacturer’s protocol (the Mouse TranSignal NF-κB Target Gene Array kit, Panomics). The biotin-labeled cDNA probes were then hybridized individually to the mouse TranSignal NF-κB Target Gene Array membranes with cDNA prepared from the placebo and nicotine-treated mice. The chemiluminescence signals on the arrays were compared after exposure to the BioMax x-ray films.

Results

Nicotine Augments Aortic Lesion Formation

Male LDLR−/− mice, 6 to 8 weeks of age, were allowed to enter the 90-day treatment protocol. Chronic nicotine treatment using the 90-day time-release pellets produced a steady-state blood nicotine level of 56.86±22.9 ng/mL, which overlaps values that are reported in the literature for heavy smokers.18 The blood nicotine and its metabolite, cotinine, remained at similar levels from day 30 up to day 90. The mice were fed an atherogenic diet (Harlan Teklad) throughout the entire period.

Nicotine had no effects on the body weight of these LDLR−/− mice. However, on day 90, we found that nicotine treatment increased en face aortic lesion size by 240% compared with that of the placebo-treated group, whereas at earlier time points on day 60 and day 30, aortic lesion areas were not significantly different in nicotine- or placebo-treated mice (Figure 1). The finding of profound nicotine augmentation of atherosclerosis corroborates that observed with the oral nicotine-treated apolipoprotein E knockout mice at the age of 40 weeks (2-fold increase).8

Total serum cholesterol was similar in nicotine- and placebo-treated mice from day 30 to day 60. On day 90, there was a 25% increase in plasma cholesterol in nicotine-treated over placebo-treated mice. Analysis of plasma lipoprotein profiles of the nicotine-treated mice showed that there was a modest elevation (25%) in very LDL cholesterol on day 90 in the nicotine-treated mice compared with the placebo-treated mice. Nicotine had no effect on the intermediate-density lipoprotein or LDL fractions during the entire course of treatment (please see online http://atvb.ahajournals.org). We note that nicotine was reported to induce an atherogenic lipoprotein profile in several animal models.19–21 However,
Keratinocyte-Derived Chemokine

our data showed that nicotine affects plasma lipids only in a modest manner in these dyslipidemic mice. MAC3 immunostaining showed similar macrophage infiltrations in the lesions at different stages of treatment (data not shown).

Nicotine Increases Serum Inflammatory Cytokines TNFα, Interleukin 1β, and Keratinocyte-Derived Chemokine

We examined inflammatory cytokines by measuring the serum concentration of cytokine nicotine- and placebo-treated mice on day 90 (Figure 2). Among the 18 cytokines tested [RANTES, macrophage inflammatory protein (MIP-1α), keratinocyte-derived chemokine (KC), colony stimulating factor (CSF), interleukin (IL)-17, IL-12 P70, IL-12 P40, IL-6, IL-3, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-10, granulocyte-macrophage-CSF, interferon (IFN)-γ, and TNF-α], only 3 showed significant changes (P<0.05); TNF-α increased 100% (P=0.028), IL-1β increased 50% (P=0.02), and KC increased 75% (P=0.004) over the placebo controls. TNF-α, IL-1β, and chemokine KC are cytokines that mediate and regulate innate immunity. In addition, CSF (P=0.17) and MIP-1α (P=0.09) also showed a trend toward higher levels, although the change was not statistically significant (Figure 2).

Nicotine Upregulates NF-κB Target Genes, VCAM-1, COX-2, and Platelet-Derived Growth Factor β in the Aorta

NF-κB is a transcription factor that mediates transcriptional responses to changes in the environment. TNF-α and IL-1β are ligands for signaling to NF-κB via the TNF receptor and the Toll/IL-1 receptor. Oxidative stress and binding of cytokines to TNF receptor and Toll-like receptor, which are present in macrophages, and smooth muscle cells and endothelial cells initiate a signaling pathway leading to the activation of NF-κB. All of these molecular effectors could lead to increased inflammatory reaction at the site.

We next performed a screen of NF-κB target gene cDNA array (Panomics, 107 mouse targets) using RNA isolated from the aorta of control and nicotine-treated LDLR−/− mice. Only 5 [glutathione S-transferase, Wilms’ tumor 1, VCAM-1, PTGS-2 (COX-2), and platelet-derived growth factor (PDGF)β] of 107 genes tested displayed any significant change; all were found to be upregulated in the nicotine-treated group. We found no downregulation of any genes examined. Although the upregulated detoxifying enzyme, glutathione S-transferase, and Wilms’ tumor 1 are interesting, we focused on the 3 proinflammatory factors and their possible effects on atherosclerosis. We verified the upregulation of the 3 NF-κB proinflammatory target genes by quantitative RT-PCR. We analyzed RNA extracted from the aorta of the nicotine- and placebo-treated LDLR−/− mice at day 30, day 60, and day 90 of treatment (Figure 3). Nicotine treatment increased the expression of VCAM-1, COX-2, and PDGFβ at all of the time points tested with the increase being statistically significant for VCAM-1 (330%) and COX-2 (540%) on day 60 and PDGFβ (166%) and for platelet-12-LO (200%) on day 90 (Figure 3A). We also examined the expression of vascular endothelial growth factor c, another NF-κB target gene that is an angiogenesis regulator, and found no apparent effect of nicotine on the level of vascular endothelial growth factor c transcripts in these mice (data not shown).

In addition to the quantitative assessment of the transcripts of these proinflammatory genes, we also corroborated the expression of these NF-κB target genes at the protein level by immunohistochemistry in the aorta using antibodies to...
Nicotine Activation of NF-κB by Macrophage Exudates

Because some NF-κB target genes were upregulated in macrophages, we investigated the effects of nicotine on NF-κB activation in the aorta of nicotine-treated mice. Normally, uninduced NF-κB is sequestered in the cytoplasm by binding to inhibitory κB proteins. Activation involves degradation of IκB and subsequent translocation of induced NF-κB to the nucleus. IL-1, TNF-α, and reactive oxygen intermediates are among the numerous agents that can activate NF-κB. In the aortic lesions of placebo-treated mice, the staining of most of the cells in the intima with antibodies against P65/RelA appeared to be diffuse (background) or exclusively cytoplasmic, whereas in the nicotine-treated lesion, P65/RelA staining occurred mostly inside the nucleus (Figure 5A). In a parallel experiment, we preincubated RAW 264.7 cells in DMEM in the presence or absence of nicotine for 17 hours, then transferred the preincubated medium to the test cells in separate dishes and continued the incubation for another 17 hours. After the second incubation, the test cells were subjected to an electrophoretic mobility-shift assay (EMSA; Figure 5B) and a nuclear localization assay (Figure 5C).

Nicotine-induced iNOS and other cytokine expression in macrophages in vitro, we next investigated the molecular crosstalk between macrophages and smooth muscle cells and endothelial cells, the other major players in atherosclerotic lesion formation.
activation of their own NF-κB, causing P65/RelA to assume a nuclear localization (Figure 5C).

By EMSA, a shifted band corresponding to the P65/RelA DNA probe complex was readily detectable when the target NF-κB DNA was incubated with nuclear extracts isolated from VSMC, RAW, and HAEC cells (Figure 5B, left panel, lanes 1 to 2; right panel, lanes 2 to 4) that were exposed to DMEM preconditioned by prior incubation of RAW 264.7 cells in the presence of nicotine (P-RAW/N) but not with medium preconditioned in the absence of RAW264.7 cells (P-RAW/0; Figure 5B, right panel, lane 1).

Nuclear extracts isolated from the aorta of mice treated with nicotine in vivo also showed more P65/RelA complex than placebo controls (Figure 5B, right panel, lanes 5 and 6). In the placebo control mice, the basal minor NF-κB activation was likely because of lipidemia in this mouse model.

**Discussion**

We have corroborated previous reports on the proatherogenic effects of nicotine in vivo by demonstrating that nicotine has an enhancing effect on atherosclerosis in the LDLR-/- mouse model. The postulated molecular mechanism of nicotine action on atherogenesis has ranged from initiation to angiogenesis. In this study, we have discovered that nicotine augments the synthesis and secretion of proinflammatory cytokines and iNOS and the generated oxidative stress by monocytes and macrophages and their secondary effects on endothelial and smooth muscle cells in the aortic lesion. This is a novel finding. This sequence of events deviates significantly from a popular theory built on earlier work by Villablanca, whose studies concentrated on the in vitro effects of nicotine on endothelial cells. In addition, we also found that the greatly increased atherosclerosis of 240%

**Figure 4.** Quantitative real-time RT-PCR of cytokine genes expressed in peritoneal macrophages (A) and RAW 264.7 cells (B) after exposure to 10⁻⁶ mol/L nicotine for 17 hours. Placebo in B means PBS incubation. C, RAW cells preincubation with the α-nAChR antagonist α-bungarotoxin reverses nicotine effects. Nic indicates nicotine; BT, bungarotoxin. *P<0.05 between placebo and nicotine-treated groups.

**Figure 5.** NF-κB activation. A, Nuclear localization of NF-κB P65/RelA in VSMC (human coronary arterial smooth muscle cells), RAW (mouse monocyte/macrophage RAW 264.7 cells), and HAEC. Cells were incubated with DMEM, DMEM+N for 17 hours, or incubated with the RAW cell-preconditioned medium in the absence of nicotine (P-RAW/0) or in the presence of nicotine (P-RAW/N). FITC images were detected by deconvolution microscopy. B, Aortic lesion of placebo and nicotine-treated mice, stained with anti-P65/RelA antibodies. C, EMSA of nuclear extracts from VSMC, RAW, and HAEC cultured cells and isolated aorta tissues from nicotine and placebo-treated mice. P, no protein was added. Left: lane 1, nuclear extract from VSMC incubated with P-RAW/0 (with no nicotine); lane 2, incubated with P-RAW/N (in the presence of 10⁻⁶ mol/L nicotine); lane 3, same as lane 2, added cold NF-κB DNA probe. Right: lane 1, RAW 264.7 incubated with DMEM; lane 2, RAW 264.7 cells incubated with P-RAW/N; lane 3, HAEC cells incubated with P-RAW/0; lane 4, HAEC incubated with P-RAW/N; lane 5, nuclear extracts from aorta isolated from placebo-treated mice; lane 6, nuclear extract from aorta isolated from nicotine-treated mice.
in nicotine compared with placebo-treated mice was completely out of proportion to the very minor difference in lipid and lipoprotein profiles of these 2 groups of mice. The fact that the markedly increased lesion size cannot be accounted for by the slight difference in lipid/lipoproteins is reminiscent of a similar situation in humans in which the observed increased serum cholesterol in smokers has been estimated to account for only 9% of the excess coronary artery disease risk.1

The novel finding that nicotine augments proinflammatory cytokine synthesis in macrophages and stimulates their plasma concentration is the key entry point for understanding the molecular mechanism of nicotine-augmented atherosclerosis in this mouse model. The molecular crosstalk between macrophages and endothelial and smooth muscle cells, which occur in the lesion, was demonstrated by in vitro studies using cultured cells. The nicotine-conditioned medium (P-RAW/N) collected from preincubation with RAW264.7 cells activated NF-κB in VSMC and HAEC as demonstrated by both P65/RelA nuclear localization and in EMSA. Our finding could explain the previously reported noneffects of nicotine on the endothelial cells and smooth muscle cells. In many of these in vitro studies, nicotine could only cause an effect when exogenous factors (eg, PDGFβ) or serum was added to the medium.23–25

Based on the total data collected in this study, we propose the following scenario (Figure II, available online at http://atvb.ahajournals.org) depicting the mechanism of how nicotine augments atherosclerosis. Nicotine directly induces iNOS and TNF-α expression in monocytes and macrophages via the nicotinic acetylcholine receptors. In turn, the activated macrophages, after they infiltrate the lesion, activate the NF-κB transcriptional factor in macrophages, smooth muscle cells, and endothelial cells with their secreted proinflammatory cytokines and generated oxidative stress. Of the NF-κB target genes stimulated by nicotine, activation of VCAM-1 and COX-2 occurs first, followed by PDGFβ at a later stage. The nicotine-activated expression of VCAM-1 on day 60 helps promote macrophage transendothelial migration. The concurrent increase of COX-2, which facilitates the progression of atherosclerosis, enhances vascular permeability, cell proliferation, chemotaxis, and influx of white blood cells, including phagocytes and lymphocytes, from the circulation into the tissue.26,27 Subsequently, nicotine additionally stimulates PDGFβ synthesis in the lesion, which induces 12-LO in response to inflammation and promotes enhancement of angiogenesis28 and thrombosis.29 Interestingly, PDGF A- and B-chain mRNA levels are increased in monocytes from hypercholesterolemia patients.30 The growth factor can additionally promote smooth muscle cell migration, as well as enhance nicotine-induced proliferation of endothelial cells at the later stages (day 90).31 Whether this mechanistic pathway applies to other animal models or to human remains to be determined. Obviously, other genetic and environmental considerations need to be taken into consideration.

In conclusion, our study has enhanced our understanding of the molecular steps leading to nicotine augmentation of atherosclerosis in a mouse model and provides additional rationale for application of anti-inflammatory therapeutic approaches for atherosclerosis prevention and treatment in smokers and individuals with long-term nicotine usage.

Acknowledgments

This work was supported by National Institutes of Health grants HL56668 and HL51586 and Betty Rutherford Chair from St. Luke’s Episcopal Hospital and Baylor College of Medicine (L.C.).

References


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Arterioscler Thromb Vasc Biol. 2006;26:143-149; originally published online October 27, 2005; doi: 10.1161/01.ATV.0000193510.19000.10
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/26/1/143
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Nicotine Induces Proinflammatory Responses in Macrophages and the Aorta
Leading to Acceleration of Atherosclerosis in LDLR-/− Mice

ONLINE SUPPLEMENT

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Condensed abstract:

We investigated the molecular mechanism of nicotine-accelerated atherosclerosis in hyperlipidemic LDLR-/− mouse model. Our findings reveal a novel mechanism of nicotine in atherogenesis. Nicotine directly activates macrophages via the nicotinic acetylcholine receptors, inducing multiple downstream events, subsequently resulting in NFκB-mediated inflammation in the arterial wall and accelerated atherosclerosis.

1. Equal contributions.

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Lau et al Macrophage Responses to Nicotine in Atherosclerosis 1
Materials and Methods

Biochemical measurements:
Serum nicotine level was determined by high-performance liquid chromatography (HPLC).\(^1\) Pooled mouse serum at each time-point (Day 30, Day 60, and Day 90) was acidified with 1 N acetic acid (pH 4.0) before the nicotine assays. Disposable C\(_{18}\) SepPak cartridges (Waters) were conditioned with acetonitrile and equilibrated with water before applying the acidified serum into the column. The eluted and dried samples were analyzed with a Beckman HPLC system (Gold 126). The mice were anesthetized with isoflurane before we collected blood from the retro-orbital plexus in EDTA-containing tubes after a 4-h fast. Aortic en face lesions were evaluated by quantitative morphometry as previously described.\(^2\) We performed histological and immunohistochemical studies were performed on fresh-frozen OCT-embedded proximal aortic sections (5 \(\mu\)m thick). Lipid and FPLC (Amersham Pharmacia Biotech) analyses were performed as previously described.\(^2\) Statistical analyses were made with SigmaStat (SPSS) and statistical significance was assigned at P<0.05 where pair-t tests as suggested by SigmaStat were used to compare the placebo and nicotine treated groups. We injected each mouse with 1 mL 3% thioglycolate solution and collected peritoneal macrophages by washing the peritoneum with 8 mL FBS-free RPMI medium (Mediatech).
Immunohistochemistry and Quantitative Real-time Reverse Transcription QRT-PCR

We performed immuno-histochemcial analysis on serial aortic sections (4 µm) to investigate the localization of TNFα, COX-2, VCAM-1, iNOS, macrophages (Mac 3) and P65/RelA. Slides were treated and stained as described(3) fixed in acetone, and then blocked by 3% (w/v) hydrogen peroxide, followed by Avidin/Biotin blocking (Vector) and 0.1% (w/v) BSA (Vector) blocking for 1 hr. The primary antibodies used were anti-PDGF rabbit (Spring Bioscience), anti-mouse TNFα rat (BD Bioscience), anti-mouse COX-2 rabbit (Cayman Chemical), anti-Mac-3 rat (Santa Cruz Biotechnology), anti-VCAM-1 rabbit (Santa Cruz Biotechnology), for anti-NOS2 rabbit (Santa Cruz Biotechnology), and anti-NFkB P65/RelA(A) rabbit antibodies (Santa Cruz Biotechnology). A Zeiss microscope equipped with AxioVison camera and software was used for image capturing. For QRT-PCR, we extracted total RNA from the isolated aorta or from cultured cells with an Absolutely RNA RT-PCR miniprep kit (Stratagene) and synthesized cDNA from 1 µg total RNA by using the iScript cDNA kit (Biorad). Then we performed quantitative PCR (Q-PCR) as described(3) using transferrin as a house keeping gene. Synthetic gene-specific primer sets used were designed by Primer3 (MIT). Primer sequences are detailed in Table I.
References


Online figure legend.

**Figure I. Plasma lipids:** Total plasma cholesterol (upper panel) and FPLC profiles (lower panel) of the nicotine and placebo treated mice (30, 60 and 90 days). Plasma was pooled from 8 mice of each group.

**Figure II. Schematic depiction of nicotine-augmented atherosclerosis.** Nicotine first activates monocytes/macrophages, via the nicotinic acetylcholine receptors, generating TNFα and iNOS, the latter of which leads to the production of reactive oxygen species such as nitric oxide, peroxide, and their product, peroxynitrite. The generated oxidative stress together with the inflammatory cytokines, such as TNFα, IL-1 and KC, activate the NFκB transcription factor in the aortic cells. The first group of activated NFκB target genes are VCAM-1 and COX-2 and the second set are PDGFb and 12-LO (platelet). Lesion growth and expansion are then accelerated and augmented.
Fig II. Lau et al

Nicotine

Monocytes/macrophages

IL-1  TNF-α
cytokines

NO + H₂O₂  NO₃⁻Na  Oxidative stress

TLR  TNFR

IKK

IKB  (Ub)n

Proteosome

NfkB  p65/p50

P65/p50

Nucleus

VCAM-1  COX-2  PDGFβ
Lesion expansion  Smooth muscle cell migration
Atherosclerosis progression
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