Nicotine potentiates proatherogenic effects of oxLDL by stimulating and upregulating macrophage CD36 signaling

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Nicotine potentiates proatherogenic effects of oxLDL by stimulating and upregulating macrophage CD36 signaling. Am J Physiol Heart Circ Physiol 305: H563–H574, 2013. First published June 7, 2013; doi:10.1152/ajpheart.00042.2013.—Cigarette smoking is a major risk factor for atherosclerosis and cardiovascular disease. CD36 mediates oxidized LDL (oxLDL) uptake and contributes to macrophage foam cell formation. We investigated a role for the CD36 pathway in nicotine-induced activation of macrophages and foam cell formation in vitro and in vivo. Nicotine in the same plasma concentration range found in smokers increased the CD36/CD14+ cell population in human peripheral blood mononuclear cells, increased CD36 expression of human THP1 macrophages, and increased macrophage production of reactive oxygen species, PKCδ phosphorylation, and peroxisome proliferator-activated receptor-γ (PPARγ) expression. Nicotine-induced CD36 expression was suppressed by antioxidants and by specific PKCδ and PPARγ inhibitors, implicating mechanistic roles for these intermediates. Nicotine synergized with oxLDL to increase macrophage expression of CD36 and cytokines TNF-α, monocyte chemoattractant protein-1, IL-6, and CXCL9, all of which were prevented by CD36 small interfering (si)RNA. Incubation with oxLDL (50 μg/ml) for 72 h resulted in lipid deposition in macrophages and foam cell formation. Preincubation with nicotine further increased oxLDL-induced lipid accumulation and foam cell formation, which was also prevented by CD36 siRNA. Treatment of apoE−/− mice with nicotine markedly exacerbated inflammatory monocyte levels and atherosclerotic plaque accumulation, effects that were not seen in CD36−/− apoE−/− mice. Our results show that physiological levels of nicotine increase CD36 expression in macrophages, a pathway that may account at least in part for the known proinflammatory and proatherogenic properties of nicotine. These results identify such enhanced CD36 expression as a novel nicotine-mediated pathway that may constitute an independent risk factor for atherosclerosis in smokers. The results also suggest that exacerbated atherogenesis by this pathway may be an adverse side effect of extended use of high concentrations of nicotine independent of their mode of administration.

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SMOKING ACCOUNTS FOR 175,000 cardiovascular (CV) deaths annually in the US. The range of CV diseases exacerbated by cigarette smoke includes diabetic and obesity-related vascular diseases, stroke, myocardial infarction, peripheral vascular, and renal disease (20, 35, 50). Heart attacks are three times more common in smokers than in nonsmokers, and it is estimated that ~30% of deaths from coronary heart disease in the US are attributable to smoking (35). We and others (16, 18) have demonstrated the importance of chemically stable compounds present in the gas phase of cigarette smoke in mediating endothelial injury and atherosclerosis. Nicotine, the principal addictive component of cigarette smoke, is widely used for smoking cessation (2). While short-term exposure to nicotine is considered relatively harmless, there is substantial evidence both in vitro and in vivo (16, 24–25) that long-term nicotine exposure promotes atherosclerosis; yet the mechanism of action of nicotine and its CV consequences are poorly understood. Atherosclerosis is a chronic inflammatory process (48, 56). The current paradigm suggests that CV risk factors such as hypertension and diabetes damage the endothelium, allowing lipoproteins, monocytes, and macrophages to infiltrate the vascular intima and subintima and initiate a chronic inflammatory cascade (47, 53). Macrophages via their scavenger receptors (SR) take up oxidized LDL (oxLDL) and other lipids, undergo activation, and produce cytokines, matrix metalloproteinases, and reactive oxygen species (ROS) (27, 36, 54), while continuing to accumulate lipids and differentiate into foam cells to form the early lesions that mature into atherosclerotic plaques (48, 56).

Both monocytes and macrophages recognize and take up oxLDL via a family of pattern recognition SRs (23, 32). CD36 is a member of the class B SR family (8) that plays a critical role in foam cell formation by mediating recognition and uptake of oxLDL (40). Previous studies have shown that foam cell formation is defective in mice with the CD36 gene deleted globally or in bone marrow derived cells, and atherosclerosis is repressed in double CD36−/− apoE−/− null mice, confirming a dominant proatherogenic role of CD36 (6, 9, 40).

Here we present our novel findings that physiological level of nicotine, acting on human macrophages through nicotinic receptors, selectively increased CD36 gene expression without affecting other related genes including SRA and lectin-like oxidized low-density lipoprotein receptor 1 (LOX1) or the reverse cholesterol transporter ATP-binding cassettes (ABC) A1 and ABCG1. Nicotine synergized with oxLDL to increase macrophage expression of CD36 and production of multiple
proinflammatory cytokines involved in foam cell formation. Importantly, we found that CD36 is required for nicotine-mediated exacerbation of atherosclerotic lesions in apoE<sup>−/−</sup> mice fed a high-fat/cholesterol diet. We believe our studies demonstrate for the first time a link between the CD36 pathway and the proatherogenic properties of nicotine.

**METHODS**

**Materials.** Human THP1 monocytes and supplemental medium were purchased from ATCC (Manassas, VA). Rabbit polyclonal anti-peroxisome proliferator-activated receptor-γ (PPARγ) and PKCδ antibodies were obtained from Cell Signaling Technology (Danvers, MA), and rabbit polyclonal anti-ABCA1 and ABCG1 antibodies were from Novus Biotechnology (Littleton, CO). Rabbit polyclonal anti-tumor necrosis factor (TNF)-α, IL-6, CD36 antibodies, CD36 small interfering RNA (siRNA), scrambled control RNA, and transfection medium were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). oxLDL, acetylated LDL (acLDL), and 125I-oxLDL (15 μg/ml) were purchased from Biomedical Technology (Stoughton, MA). All other chemicals were of the best grade available from commercial sources.

**Cell culture.** Human THP1 monocytes (ATCC TIB 202) were cultured in RPMI 1640 medium supplemented with 10% FBS, 10 mM HEPES, 2 mM l-glutamine, 1 μM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.05 mM/ml 2-mercaptoethanol. The cells were cultured at 37°C, 95% humidity, and 5% CO₂ and used between passages 4 and 12. They were seeded in six-well plates (5 × 10⁵ cells/well) and differentiated into macrophages by preculture with 100 ng/ml phorbol 12-myristate 13-acetate for 36 h. Cells were starved in serum free RPMI 1640 medium for 24 h before the experiments were performed.

**Determination of ROS production.** ROS production was determined by dichlorofluorescein diacetate (DCFDA) assay as described in our previous publications (4). Human THP1 macrophages were incubated for 30 min in culture medium containing 10 μmol/l DCFDA and then incubated with or without nicotine (100 nmol/ml) at 37°C for 15 min. In some experiments the cells were preincubated with a nonselective nicotinic acetylcholine receptor (nAchR) inhibitor, hexamethonium (100 μmol/l), or the α- nAchR blocker bungarotoxin (1 nmol/l, Sigma-Aldrich) for 30 min or incubated with the NADPH oxidase inhibitor diphenyleneiodonium (DPI; 10 μmol/l, Sigma-Aldrich) for 30 min before incubation with nicotine. The cells were scraped into 500 ml 0.2% Triton X-100 and centrifuged. Fluorescence was measured at an excitation wavelength of 485 nm and emission wavelength of 535 nm. The results were expressed as relative light units per milligrams of protein. In addition, ROS production was quantified using lucigenin enhanced chemiluminescence (60).

**Real-time PCR.** Cells were harvested in 1 ml Trizol reagent and total RNA (2 μg) reverse-transcribed using a Superscript II RT First Strand synthesis kit (GIBCO-BRL) according to the manufacturer’s instructions. Real-time PCR was performed with a TaqMan master mix assay kit (ABI). Relative quantities of each transcript were normalized by a housekeeping gene (GAPDH) and expressed as fold increase vs. control. All primers for CD36 (cat no. Hs00354519-m1, Mm01135198-)

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**Fig. 1.** Effect of nicotine on CD36 expression in human THP1 macrophages or isolated human peripheral blood mononuclear cells (PBMCs). A: time course of CD36 protein expression in cells treated with 100 nmol/l nicotine. Nicotine increased protein (β) and mRNA (C) expression of CD36 in a dose-dependent manner. D: nicotine increased cell surface protein expression of CD36 in human PBMCs. Isolated human PBMCs were incubated with 10 nmol/l nicotine for 24 h. Fluorescence-activated cell sorting (FACS) was used to identify cells expressing CD36 (CD36<sup>+</sup>/CD14<sup>−</sup> population) in human monocytes (CD14<sup>+</sup>). E: nonsselective nAchR blocker, hexamethonium (100 μmol/l), or an α7 nAchR blocker, bungarotoxin (1 nmol/l), prevented nicotine-induced CD36 expression. **F:** nicotine did not affect protein expression of lectin-like oxidized low-density lipoprotein receptor 1 (LOX1) and scavenger receptor (SR) A. Data are expressed as means ± SE. *P < 0.05 vs. control; #P < 0.05 vs. nicotine; n = 5–6.
mL), TNF-α (cat no. Hs00171428-m1, Mm00443258-m1), monocyte chemoattractant protein-1 (MCP1; cat no. Hs00234140), IL-6 (cat no. Hs99999032-m1), CXCL9 (cat no. Hs00171065-m1), and IL-1β (cat no. Mm01336189-m1) were obtained from ABI.

**Immunoblot analyses.** The cells were harvested with lysis buffer containing a protein inhibitor cocktail. Protein was quantified by Bio-Rad assay, and 30 μg of total protein were first subjected to SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were incubated with primary rabbit polyclonal anti-PPARγ, PKCδ (Cell Signaling Technology), or rabbit polyclonal anti-TNF-α, IL-6, CD36, SRA, or LOX1 antibodies (Santa Cruz Biotechnology) or rabbit polyclonal anti-ABCAl or ABCG1 antibodies (Novus Biotechnology) followed by incubation with a horseradish-peroxidase-conjugated secondary antibody for 1 h. Equivalence of protein loading and transfer was confirmed by reblotting the samples with an antibody. Immunoreactive bands were detected by chemiluminescence and quantified by densitometry. Relative quantities of each protein were normalized by β-actin and expressed as fold increase vs. control.

**Determination of CD36 expression in human peripheral blood mononuclear cells by flow cytometry.** Peripheral blood (50 mL) was collected from nonsmoking healthy blood donors in 0.109 mol/L sodium citrate. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient density centrifugation (Amersham Bioscience). Isolated PBMCs were plated in RPMI-1640 culture medium containing 1.5 g/L glucose, 10% autologous serum, 1% antibiotic/antimycotic mixture and 2 μmol/L L-glutamine and allowed to attach to the bottom of six-well plates. After 1–2 h, nonadherent cells were removed by raising the cells with PBS. Approximately 1 million cells/mL were incubated with 100 nmol/L nicotine for 24 h. CD36 protein expression on the cell surface was determined by fluorescence-activated cell sorting analysis (FACS; Ref. 57) using anti-CD36-FITC and anti-CD14-PE antibodies (human monocyte marker, eBioscience). A minimum of 10⁵ cells/sample was assessed.

**siRNA for CD36.** Human THP1 macrophages (5 × 10⁵ cells) were cultured in RPMI 1640 medium with 10% FBS and grown to 70% confluence. Twenty hours before transfection, the cells were washed, and the medium was changed to RPMI 1640 with 3% FBS but no antibiotics. The cells were resuspended with trypsin and transfected using transfection medium and 50 nmol/L CD36 siRNA and then incubated for 24 h according to the manufacturer’s instructions (Santa Cruz Biotechnology). A nonspecific, targeted, scrambled RNA was used as a control. Efficiency of siRNA transfection was confirmed by over 70% reduction in CD36 mRNA expression by real-time PCR, compared with the scrambled control RNA. CD36 expression was not affected by the scrambled RNA.

**Macrophage uptake of lipoproteins.** Cellular lipoprotein uptake was assessed by measuring lipoprotein cellular degradation (21). THP1 macrophages (5 × 10⁵ cells) were incubated with or without nicotine (1–1,000 nmol/L) for 24 h followed by incubation with 125I-oxLDL (15 μg/mL) for 4 h at 37°C in serum-free RPMI-1640. 125I oxLDL and oxLDL were obtained from Biomedical Technologies. In some experiments, the cells were transfected with a CD36 siRNA to knock down the CD36 gene before incubation with nicotine (100 nmol/L) increased reactive oxygen species (ROS) production (A–C) and PKCδ phosphorylation (D) in THP1 macrophages. ROS was determined by dichlorofluorescein diacetate (DCFDA) assay (A and B) or by lucigenin chemiluminescence (C). Compared with control (a), incubation with nicotine (b) for 15 min significantly increased ROS production, which was inhibited by preincubation with either the nonselective nAChR blocker hexamethonium (100 nmol/L; c), the α-7 nAChR blocker bungarotoxin (1 nmol/L; d), or the NADPH oxidase inhibitor DPI (10 μmol/L). Nicotine-induced ROS production was also blunted (e). Nicotine increased PKCδ phosphorylation (D), and the antioxidant NADPH oxidase inhibitors DPI (10 μmol/L), apocynin (100 μmol/L), or free radical scavenger N-acetylcyesteine (NAC; 1 mmol/L) significantly inhibited nicotine-induced PKCδ phosphorylation (E). *P < 0.05 vs. control; #P < 0.05 vs. nicotine; n = 4–6.

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Fig. 2. Nicotine (100 nmol/L) increased reactive oxygen species (ROS) production (A–C) and PKCδ phosphorylation (D) in THP1 macrophages. ROS was determined by dichlorofluorescein diacetate (DCFDA) assay (A and B) or by lucigenin chemiluminescence (C). Compared with control (a), incubation with nicotine (b) for 15 min significantly increased ROS production, which was inhibited by preincubation with either the nonselective nAChR blocker hexamethonium (100 nmol/L; c), the α-7 nAChR blocker bungarotoxin (1 nmol/L; d), or the NADPH oxidase inhibitor DPI (10 μmol/L). Nicotine-induced ROS production was also blunted (e). Nicotine increased PKCδ phosphorylation (D), and the antioxidant NADPH oxidase inhibitors DPI (10 μmol/L), apocynin (100 μmol/L), or free radical scavenger N-acetylcyesteine (NAC; 1 mmol/L) significantly inhibited nicotine-induced PKCδ phosphorylation (E). *P < 0.05 vs. control; #P < 0.05 vs. nicotine; n = 4–6.
nmol/l). The cell-mediated hydrolysis of oxLDL protein was assessed by determination of trichloroacetic acid-soluble and chloroform-insoluble radioactivity in the incubation medium. Degradation of oxLDL in the absence of cells was minimal and was subtracted from total oxLDL degradation. The cell layer was incubated overnight with 0.1 N NaOH to assess cell protein level.

Foam cell formation. THP1 macrophages were incubated with or without nicotine in channel slides for 24 h and then incubated with oxLDL (50 μg/ml) for 72 h. Oil red O staining was used to visualize lipid deposits and hematoxylin staining to show the nuclei.

Determination of total cellular cholesterol content. THP1 macrophages in six-well dishes were incubated with or without nicotine for 24 h followed by incubation with oxLDL (50 μg/ml) for 72 h. Total cellular cholesterol was extracted by adding 1 ml hexane/isopropanol (3:2, vol:vol) to the wells (42). The samples were evaporated by using a SpeedVac to remove the solvent and redissolved in 50 ml ethanol. Cholesterol was determined with an enzyChrom AF cholesterol assay kit according to the manufacturer’s instructions (Bioassay Systems, Hayward, CA).

Animal protocols. Six-week-old apoE−/− mice were purchased from Jackson Laboratory (Main, NE); CD36−/−/apoE−/− mice were acquired from laboratory of Dr. R. L. Silverstein at Cleveland Clinic Foundation and bred at the Miami Veterans Affairs Medical Center. After adapting to the new environment, the mice were divided into five groups and received one of the following treatments for 15 wk: apoE−/− mice treated with normal chow (17% of calories from fat and 0.03% from cholesterol), high-fat/high-cholesterol diet (HF; 47% of calories from fat and 0.2% from cholesterol), or HF with nicotine (0.03% from cholesterol), or HF with nicotine (0.03% from cholesterol), or HF with nicotine (0.03% from cholesterol), or HF with nicotine (0.03% from cholesterol), or HF with nicotine (0.03% from cholesterol), or HF with nicotine (0.03% from cholesterol). After adapting to the new environment, the mice were divided into five groups and received one of the following treatments for 15 wk: apoE−/− mice treated with normal chow (17% of calories from fat and 0.03% from cholesterol), high-fat/high-cholesterol diet (HF; 47% of calories from fat and 0.2% from cholesterol), or HF with nicotine (HFN; 100 mg/l in the drinking water), and CD36−/−/apoE−/− mice without nicotine in chamber slides for 24 h and then incubated with oxLDL in the absence of cells was minimal and was subtracted from total oxLDL degradation. The cell layer was incubated overnight with 0.1 N NaOH to assess cell protein level.

Fig. 3. Effects of antioxidants (A), PKC (B), or proliferator-activated receptor-γ (PPARγ) inhibitor (D) on nicotine-induced CD36 expression in human THP1 macrophages. Antioxidant treatment (DPI, apocynin, or NAC) or suppression of PKC by the classic PKC inhibitor calphostin C (0.5 μmol/l) or specific PKCβ inhibitor rottlerin (3 μmol/l) significantly reduced nicotine-induced CD36 expression. Nicotine (100 nmol/l) increased PPARγ expression in a time-dependent manner (C), and inhibition of PPARγ by T0070907 (100 nmol/l) significantly reduced nicotine-induced CD36 expression (D). *P < 0.05 vs. control; #P < 0.05 vs. nicotine; n = 5–6.

The mice were injected intraperitoneally with 2 ml of 3% thiglycolate medium to stimulate the macrophages to enter the peritoneal cavity. On day 5 postinjection, they were anesthetized with sodium pentobarbital (50 mg/kg ip) and injected with 2 ml cold PBS with 10 mmol/l EDTA to rinse the peritoneal cavity. Macrophages from each mouse were plated separately in RPMI/10% FBS overnight. The following morning, nonadherent cells were removed by aspiration and the remaining macrophages washed thoroughly with PBS. Serum cholesterol was determined with a standard assay kit. The Institutional Animal Care and Use Committee at the Miami Veterans Affairs Medical Center approved all animal studies.

Determination of inflammatory monocyte subset by FACS. Peripheral blood was collected from the tail vein into tubes containing 15% EDTA. Red blood cell lysis solution (1.2 ml; Sigma) was added to tubes containing 0.1 ml blood and incubated at 37°C for 4 min, followed by addition of 20 ml PBS and centrifugation to remove the lysed red blood cells. The white cells (106 cells/ml) were incubated with 100 μl protein blocking solution with 1 μl fluorescent FITC-conjugated anti-mice Ly6C antibody (eBioscience) and PE-conjugated anti-mice CD11b antibody (mice monocyte marker; eBioscience) at 4°C for 30 min. Fluorescence on the cell surface was determined by FACS (58) using cells labeled with isotype Ig G as control. A morphological gate was used to exclude granulocytes. Double-positive CD11b+/Ly6Cm inflammatory monocytes were identified at the intersection of two gates.

Quantitative atherogenic lesion area. The aorta was opened along the ventral edge and photographed with a Nikon D40 digital camera. Lesions were identified manually on the digital image. Prevalence maps and lesion extent were calculated using the method of Cornhill et al. (4). Briefly, 28 fiducial points derived from anatomical landmarks were identified on each aortic image. These fiducial points divide the aorta into arch, thoracic, and abdominal segments. Lesion extent in each segment was determined by calculating the fraction of lesion positive pixels in the segment. In addition, an overall value of lesion extent was calculated. These values were logarithmically transformed to better satisfy the assumption of normality when using ANOVA based

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It is known that oxLDLs (and HIV protease inhibitors) induce CD36 expression by increasing ROS production and activating PKCβ (6, 10, 15, 29). To determine whether nicotine also activates the PKC pathway, we measured PKCβ and phospho-PKCβ expression in THP1 macrophages after nicotine stimulation. As shown in Fig. 2D, nicotine significantly increased PKCβ phosphorylation but did not change total PKCβ protein. To examine the effect of antioxidants on nicotine-mediated activation of PKCβ, THP-1 macrophages were incubated with or without the NADPH oxidase inhibitors DPI and apocynin or the free radical scavenger N-acetylcysteine (NAC) for 30 min following nicotine exposure for 24 h. All antioxidant treatments eliminated nicotine-induced PKCβ phosphorylation (Fig. 2E), confirming that nicotine stimulation of PKCβ phosphorylation requires ROS production. We next investigated the effects of antioxidants and PKC inhibition on CD36 expression. As shown in Fig. 3A, DPI, apocynin, and NAC each significantly blunted nicotine-induced CD36 expression. Suppression of PKC by the nonspecific PKC inhibitor calphostin C or the specific PKCβ inhibitor rottlerin also significantly reduced nicotine-induced CD36 expression (Fig. 3B). These results implicate ROS and PKC pathways as intermediates in nicotine-induced CD36 expression.

Previous studies (6, 10) have demonstrated that CD36 expression can be increased through activation of the transcriptional factor PPARγ. To determine whether PPARγ is involved in nicotine regulation of CD36 expression, we again

**RESULTS**

*Increased expression of CD36 in human monocytes/macrophages by nicotine exposure.* CD36 is a proinflammatory, proatherogenic membrane glycoprotein expressed on platelets, monocytes, macrophages, and several other types of cells (8). To investigate the effects of nicotine on CD36 expression, we exposed THP1 macrophages to 100 nmol/l nicotine, a concentration found in the plasma of smokers (19, 52) for 1–48 h. As shown in Fig. 1A, CD36 levels were significantly increased by nicotine during 48 h of exposure, with a maximal effect at 100 nmol/l. To determine whether nicotine enhances CD36 expression in primary human monocytes, PBMCs were isolated from human peripheral blood and incubated with or without nicotine (100 nmol/l) for 24 h. Monocyte cell surface CD36 protein expression was determined by flow cytometry, using CD14 as a marker of human monocytes. As shown in Fig. 1D, nicotine significantly increased the population of CD36+CD14+ cells (1.83 ± 0.23 vs. 0.73 ± 0.07%). To determine the role of nAChR in nicotine-induced CD36 expression, human THP1 macrophages were preincubated with the nonselective nAChR blocker hexamethonium (100 μmol/l) or the α7-nAChR blocker bungarotoxin (1 nmol/l) followed by addition of nicotine (100 nmol/l) for 24 h. Hexamethonium and bungarotoxin significantly prevented the increase in CD36 expression in nicotine (Fig. 1E), supporting intermediary roles for nAChR and possibly α7-nAChR in the pathway of CD36 activation. In contrast to its effects on CD36, we found that nicotine did not affect expression of SRA or LOX1 (Fig. 1F). These results indicate that the effects of nicotine are at least partially selective for CD36. To our knowledge, this is the first evidence that nicotine mediates upregulation of CD36 expression in human monocytes/macrophages, probably through selective nicotinic receptors.

*Nicotine upregulation of CD36 expression involves ROS/PKCβ/PPARγ.* We have also identified nAChRs in human mesangial cells and demonstrated that stimulation of these receptors with nicotine increased ROS production via NADPH oxidase (19). Therefore, we evaluated the effects of nicotine on ROS production in human THP1 macrophages. Nicotine significantly increased ROS production, and this was inhibited by preincubation with hexamethonium, a nonselective nAChR blocker, or bungarotoxin, an α7-nAChR blocker. Inhibition of NADPH oxidase with DPI also blocked nicotine-induced ROS production (Fig. 2H). Increased ROS production by nicotine was further confirmed by lucigenin chemiluminescence assay (Fig. 2H). The results support an intermediary role for nAChR, possibly α7-nAChR, in the nicotine-mediated increase of ROS production by macrophages.

**Fig. 4.** Synergistic effects of nicotine and oxidized LDL (oxLDL) on protein expression of CD36 in human THP1 macrophages. A: nicotine and oxLDL at a high dose (50 μg/ml) but not at a low dose (10 μg/ml) increased protein expression of CD36. The combination of nicotine with high-dose oxLDL further increased CD36 expression. B: CD36 small interfering (si)RNA reduced CD36 protein expression. *P < 0.05 vs. control; †P < 0.05 vs. corresponding combination group; ‡P < 0.05 vs. the corresponding scrambled group; n = 5–6.
exposed macrophages to nicotine and quantified PPARγ protein. As shown in Fig. 3C, nicotine significantly increased PPARγ expression, with a maximal response at 8 h (2-fold). Furthermore, nicotine-induced CD36 expression was blocked by the PPARγ selective inhibitor T0070907 (Fig. 3D).

Effects of combined nicotine and oxLDL on CD36 expression. OxLDL is a major ligand of CD36 and plays an essential role in the pathogenesis of atherosclerotic plaque (49). To investigate whether nicotine and oxLDL interact to upregulate CD36, human THP1 macrophages were treated with nicotine for 24 h, oxLDL for 8 h, or nicotine for 24 h followed by oxLDL for 8 h. Nicotine exposure upregulated CD36 expression; oxLDL at 50 but not 10 μg/ml also significantly increased CD36 expression, and this was further enhanced by the combination of oxLDL at 50 μg/ml and nicotine (Fig. 4A). This suggests an additive relationship between nicotine and oxLDL in regulating CD36 expression. In addition, the cells were pretreated with a CD36-selective siRNA before exposure to oxLDL or nicotine. CD36 siRNA reduced protein expression of CD36 by >70% in the untreated cells, compared with the scrambled RNA control, and prevented the increase in CD36 expression induced by nicotine and/or oxLDL (Fig. 4B).

Nicotine increases oxLDL uptake and foam cell formation through CD36-dependent mechanisms. CD36 is a major mediator of oxLDL uptake by macrophages (39). To determine whether upregulation of CD36 by nicotine increases atherogenic lipid uptake and foam cell formation, we measured oxLDL uptake by a cell-mediated oxLDL degradation assay (21). As shown in Fig. 5A, nicotine exposure mediated in-
creased oxLDL uptake, with a maximal response at 100 nmol/l (25%), and this was prevented by pretreatment with the CD36 siRNA (Fig. 5B). Incubation of cells with oxLDL (50 μg/ml) for 72 h resulted in lipid deposition in macrophages and foam cell formation; preincubation with nicotine further increased oxLDL-induced lipid deposition and foam cell formation, which was also reduced by CD36 siRNA (Fig. 5C). We determined total cellular cholesterol in foam cells by colorimetric cholesterol assay. As shown in Fig. 5D, nicotine significantly increased total cellular cholesterol content in oxLDL loaded cells, which was inhibited by CD36 siRNA. In macrophages lipid homeostasis depends on the balance between influx and efflux of cholesterol; ABCA1 and ABCG1 are two important reverse cholesterol transporters involved in the efflux of cholesterol and phospholipids from macrophages (58). We found that nicotine did not alter macrophage expression of either ABCA1 or ABCG1 (Fig. 6). These results suggest that the increased foam cell formation and macrophage activation caused by nicotine exposure and enhanced expression of CD36 are accompanied by increased lipid uptake; thus efflux pathways are not changed, leading to a progressive increase of intracellular lipids.

Nicotine promotes macrophage activation in response to atherogenic lipid oxLDL uptake in a CD36-dependent manner. There are two major populations of macrophages based on their homeostatic activities and functions, inflammatory (M1) and tissue resident (M2) macrophages (12, 22, 34). M1 macrophages are generated during cell-mediated immune responses. Activated M1 macrophages produce proinflammatory cytokines, including TNF-α, MCP-1, IL-1, IL-6, interferon-γ, CXCL9, CXCL10, CXCL11, and inducible nitric oxide, which are important M1 macrophage markers (34, 48). It has been shown that activation of macrophage CD36 signaling by atherogenic lipid oxLDL can induce proinflammatory and proatherogenic effects (40, 44). Therefore, we investigated the combined effects of nicotine and oxLDL on macrophage activation as determined by M1 inflammatory cytokine expression. As shown in Fig. 7, treatment with nicotine alone increased CD36 mRNA but did not significantly affect expression of TNF-α, MCP-1, IL-6, or CXCL9. OxLDL alone increased mRNA expression of CD36 as well as proinflammatory cytokines (198–377%). The combination of nicotine and oxLDL further augmented expression of both CD36 and inflammatory cytokines (345–712%; n = 5; P < 0.05). Knockdown of CD36 by the siRNA normalized mRNA expression of CD36 and significantly reduced expression of inflammatory genes by the combined nicotine-oxLDL treatment. To confirm a specific role for CD36 in nicotine-mediated macrophage activation and uptake of proatherogenic lipids, human macrophages were treated with acetylated-LDL (AcLDL), a partially selective SRA ligand (5), followed by nicotine. AcLDL increased expression of TNF-α and IL-6 to a level similar to oxLDL-treated cells; however, nicotine did not potentiate AcLDL-induced protein expression of TNF-α or IL-6, and a CD36-selective siRNA also did not prevent the enhanced expression of TNF-α and IL-6 elicited by AcLDL (Fig. 8). These data are consistent with essential intermediary roles for CD36 and oxLDL in the
enhanced production of inflammatory cytokines and foam cell formation of human macrophages.

Effects of nicotine on inflammatory Ly-6C\textsuperscript{hi} monocytes, CD36, and inflammatory cytokines in apoE\textsuperscript{-/-} mice. Ly-6C\textsuperscript{hi} monocytes are markers of inflammation in mice, whereas Ly-6C\textsuperscript{lo} monocytes are considered noninflammatory resident cells. To determine whether nicotine mediates activation of inflammatory monocytes/macrophages in vivo, these marker cells were measured in atherosclerosis-prone apoE\textsuperscript{-/-} mice fed a HF diet or normal chow. A HF diet for 15 wk significantly increased peripheral blood levels of Ly-6C\textsuperscript{hi} monocytes compared with apoE\textsuperscript{-/-} mice on normal chow. Nicotine treatment further increased the Ly-6C\textsuperscript{hi} monocyte population without affecting plasma cholesterol. In contrast, nicotine mediated a small but significant reduction of peripheral blood CD11b\textsuperscript{+}Ly-6C\textsuperscript{lo} monocytes (12 ± 2% in HF vs. 8 ± 1% in HFN; \( P < 0.05 \)). To determine a possible role for CD36 in mediating the enhanced effects of nicotine on the Ly-6C\textsuperscript{hi} monocyte subset, apoE\textsuperscript{-/-} mice with coincident knockout of the CD36 gene (CD36\textsuperscript{-/-} apoE\textsuperscript{-/-}) were fed HF with or without nicotine as described above for 15 wk. Knockout of the CD36 gene eliminated the increased numbers of Ly-6C\textsuperscript{hi} monocytes seen in nicotine-treated apoE\textsuperscript{-/-} mice without affecting plasma cholesterol (Fig. 9, A and B). Nicotine also increased mRNA expression of CD36 as well as the inflammatory cytokines TNF-\( \alpha \) and IL-1\( \beta \) in peritoneal macrophages (Fig. 9, C-E). These effects were significantly attenuated in the CD36\textsuperscript{-/-} apoE\textsuperscript{-/-} mice.

Nicotine exacerbates atherosclerotic lesion formation through upregulation of CD36. It has been reported that nicotine exacerbates atherosclerosis in apoE\textsuperscript{-/-} mice (16, 24). Our initial results predicted that this is mediated by enhanced expression and activation of CD36. To test this, we again subjected apoE\textsuperscript{-/-} mice or CD36\textsuperscript{-/-} apoE\textsuperscript{-/-} mice to HF in the presence or absence of nicotine as described above for 15 wk. Atheromatous lesions were quantified on isolated aortas by en face staining in a double blind manner as described in METHODS. In apoE\textsuperscript{-/-} mice, HF increased both total and segmental lesion areas compared with normal chow as expected, and nicotine exposure caused an additional significant increase (Fig. 10). In contrast, nicotine did not enhance plaque formation in the CD36\textsuperscript{-/-} apoE\textsuperscript{-/-} group. These results are consistent with our data using isolated macrophages and monocytes and suggest a central role for CD36 in the exacerbation of atherosclerotic plaques by nicotine.

**DISCUSSION**

We have shown that nicotine, the addictive component in cigarette smoke, selectively increases CD36 expression in human THP1 macrophages and PBMCs by a mechanism mediated by nicotinic receptors. This promotes macrophage uptake of the proatherogenic lipid oxLDL, resulting in macrophage activation and foam cell formation. These proatherogenic effects of nicotine were inhibited by a CD36 siRNA, implicating a key role of monocyte/macrophage CD36 in mediating nicotine’s proatherogenic effects. To confirm this pathway in vivo, we demonstrated that the effects of nicotine on monocyte/macrophage activation and exacerbation of atherosclerotic plaque were eliminated by simultaneous knockout of the CD36 gene (CD36\textsuperscript{-/-} apoE\textsuperscript{-/-}) with no change in serum cholesterol. Taken together, these results indicate that nicotine-mediated exacerbation of atherosclerosis can be significantly attributed to upregulation of CD36 in monocytes/macrophages.

It has been shown that oxidative stress can increase CD36 expression in macrophages (11, 39). Here we demonstrated that nicotine significantly increased ROS production in THP1 macrophages with a concomitant increase in CD36 expression. Antioxidant treatment reduced ROS production and prevented the increase of nicotine-induced CD36 expression. To identify the target molecules of ROS for nicotine-induced CD36 expression, we investigated the effect of nicotine on PKC\( \varepsilon \) phosphorylation and PPAR\( \gamma \) expression, because both have been demonstrated as signaling molecules that mediate CD36 expression in response to oxLDL and HIV protease inhibitors (6, 15). Our results showed that nicotine stimulated PKC\( \varepsilon \)
phosphorylation and PPARγ expression, while inhibition of PKC or PPARγ reduced nicotine-induced CD36 expression. These results demonstrate that nicotine-induced CD36 expression in human macrophages is mediated by a common signaling pathway dependent on ROS/PKC/PPARγ (6).

In vitro and in vivo studies support the notion that macrophages are key early mediators of atherogenesis, and impairment of their recruitment and activation protects against lesion development (54, 56). CD36 is believed to play a critical role in the initiation of atherosclerotic lesions through its ability to bind and internalize modified LDL trapped in the artery wall, facilitating formation of macrophage foam cells and release of inflammatory cytokines from lipid-loaded macrophages in the arteries (3, 37, 44). CD36 interaction with oxLDL on macrophages triggers a signaling response that is both proinflammatory and proatherogenic (1, 41, 44). Deletion of CD36 in hyperlipidemic mouse models substantially decreased aortic sinus atherosclerosis and arterial lipid accumulation (30). Here we have demonstrated that in human THP1 macrophages nicotine increased atherogenic lipid oxLDL uptake and promoted foam cell formation, a hallmark of atherosclerotic lesions. These proatherogenic effects of nicotine were nullified by a CD36 siRNA, confirming the dialogue between nicotine and CD36 on macrophages. Of note, although there is substantial evidence supporting a role of CD36 in the development of atherosclerotic disease, some aspects continue to be a source of debate (33).

It has been shown that CD36 can modulate inflammatory monocytes and macrophages, promoting their entry into, and containment inside arterial walls, thereby driving chronic inflammation (1, 17, 31, 37, 44). Here we demonstrate that in THP1 macrophages nicotine alone did not affect expression of inflammatory cytokines; however, in the presence of oxLDL, nicotine significantly potentiated oxLDL-induced expression of inflammatory cytokines, including TNF-α, MCP1, IL-6, and CXCL9, which are important markers for M1 macrophage activation (34). Although it has been reported that nicotine inhibits activation of macrophages by LPS (51), we found that in response to oxLDL, nicotine potentiated macrophage activation and release of proinflammatory cytokines through nAchR-CD36-dependent mechanisms. These data suggest nicotine has dual immunomodulatory effects in macrophages that are dependent on the ligand (24).

Recruitment and differentiation of monocytes into macrophages or dendritic cells in the arterial wall play decisive roles in the initiation and progression of atherosclerosis (13, 27–28). Ly-6Chi monocytes have high expression of P-selectin glycoprotein ligand-1 and are better capable of homing to the
atherosclerotic plaque than Ly-6C<sup>hi</sup> monocytes (14, 48). Ly-6C<sup>hi</sup> monocytes selectively adhere to the activated endothelium, accumulate in lesions, and locally differentiate into macrophages, thereby promoting atherosclerosis (45–46). In our in vivo studies, we found that nicotine promoted increased levels of circulating inflammatory Ly-6C<sup>hi</sup> monocytes in apoE<sup>−/−</sup> mice fed a HF diet but not in CD36<sup>−/−</sup>apoE<sup>−/−</sup> mice, implicating CD36 in nicotine induction of inflammatory Ly-6C<sup>hi</sup> monocytes during atherosclerosis in vivo. Furthermore, we demonstrated that nicotine increased mRNA expression of CD36 and proinflammatory cytokines TNF-α and IL-1β in peritoneal macrophages isolated from the same apoE<sup>−/−</sup> mice and this effect was absent in the CD36<sup>−/−</sup>apoE<sup>−/−</sup> mice, confirming that nicotine activates monocytes/macrophages in vivo at least in part through CD36.

Epidemiological studies have established that cigarette smoking (active or passive) is associated with increased risk for atherosclerosis in the coronary and carotid arteries (55). We submit that nicotine is an important component of cigarette smoke that promotes atherosclerosis (7). Here we have shown that nicotine exacerbation of atheromatous lesion formation in atherogenic apoE<sup>−/−</sup> mice is dependent on the presence of CD36, whereas mice with double knockout of CD36<sup>−/−</sup>/apoE<sup>−/−</sup> had reduced overall atherosclerotic lesions and plaque accumulation was entirely unresponsive to nicotine. To our knowledge this is the first evidence to show that CD36 signaling is an essential component of nicotine-mediated exacerbation of atherosclerosis. Because CD36 ligands are generated in vivo by oxidative stress, hyperlipidemia, and inflammation (38, 43), we propose that upregulation of CD36 is the central mechanism for nicotine-mediated exacerbation of atherosclerosis.

In summary, our studies demonstrate that nicotine initiates a cascade of events to promote atherosclerosis. Nicotine, through activation of nAChR, upregulates CD36 expression in monocytes/macrophages, and this causes enhanced oxLDL uptake, foam cell formation, monocyte/macrophage activation, and exacerbated atherosclerosis. The results suggest that this is the major pathway for the proatherogenic effects of nicotine.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


