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Cholinergic stimulation blocks endothelial cell activation and leukocyte recruitment during inflammation

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Endothelial cell activation plays a critical role in regulating leukocyte recruitment during inflammation and infection. Based on recent studies showing that acetylcholine and other cholinergic mediators suppress the production of proinflammatory cytokines via the α7 nicotinic acetylcholine receptor (α7 nAChR) expressed by macrophages and our observations that human microvascular endothelial cells express the α7 nAChR, we examined the effect of cholinergic stimulation on endothelial cell activation in vitro and in vivo. Using the Shwartzman reaction, we observed that nicotine (2 mg/kg) and the novel cholinergic agent CAP55 (12 mg/kg) inhibit endothelial cell adhesion molecule expression. Using endothelial cell cultures, we observed the direct inhibitory effects of acetylcholine and cholinergic agents on tumor necrosis factor (TNF)–induced endothelial cell activation. Mecamylamine, an nAChR antagonist, reversed the inhibition of endothelial cell activation by both cholinergic agonists, confirming the antiinflammatory role of the nAChR cholinergic pathway. In vitro mechanistic studies revealed that nicotine blocked TNF-induced nuclear factor–κB nuclear entry in an inhibitor κB (IκB)α- and IκBε-dependent manner. Finally, with the carrageenan air pouch model, both vagus nerve stimulation and cholinergic agonists significantly blocked leukocyte migration in vivo. These findings identify the endothelium, a key regulator of leukocyte trafficking during inflammation, as a target of anti-inflammatory cholinergic mediators.

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Abbreviations used: ACh, acetylcholine; α-BGT, α-bungarotoxin; CAP55, cholinergic agonist P55; HRP, horseradish peroxidase; HuMVECs, human microvascular endothelial cells; ICAM-1, intercellular adhesion molecule 1; IκB, inhibitor κB; MCP-1, monocyte chemoattractant protein 1; MIP-1α, macrophage inflammatory protein 1α; MIP-1β, macrophage inflammatory protein 1β; MIP-2, macrophage inflammatory protein 2; nAChR, nicotinic acetylcholine receptor; VCAM-1, vascular cell adhesion molecule 1; VNS, vagus nerve stimulation.

Endothelial cells play a critical role in host immune responses during inflammation and infection. Proinflammatory molecules produced during infection, sepsis, and other inflammatory disease states activate the endothelium (for review see reference 1). When activated, the endothelium expresses multiple adhesion molecules and additional proinflammatory mediators. A critical function of the activated endothelium during inflammation is to coordinate the migration of peripheral blood leukocytes to sites of inflammation/infection. Cell-associated adhesion molecules, such as E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1), and chemokines expressed by the endothelium facilitate the rolling, adhesion, activation, and emigration of circulating leukocytes across the endothelial cell barrier to the site of infection or inflammation. Endothelial cell activation and leukocyte recruitment are critical for the effective elimination of invading pathogens. However, excessive leukocyte accumulation during infection and inflammation mediated by the overexpression (or sustained expression) of adhesion molecules by the endothelium can lead to tissue damage. By contrast, insufficient endothelial cell activation and subsequent impaired immune cell trafficking can result in host immunosuppression. Thus, the regulation of endothelial cell activation must be controlled precisely. Numerous therapeutic agents attenuate the excessive activation of the endothelium. Interestingly, dexamethasone inhibits LPS-induced endothelial cell adhesion molecule expression but not the cellular activation induced by TNF (2).

The recently described cholinergic anti-inflammatory pathway is a physiological mechanism that modulates host inflammatory responses

R.W. Saeed and S. Varma contributed equally to this work.
†Dr. Varma died on 5 January 2004.

via cholinergic mediators or by electrical stimulation of the vagus nerve (for review see reference 3). Studies describing the cholinergic antiinflammatory pathway show that acetylcholine (ACh), the primary neurotransmitter released by the vagus nerve, and nicotinic acetylcholine receptor (nAChR) agonists block TNF production by LPS-stimulated macrophages (4) via the α7 nAChR (5). Stimulation of the vagus nerve releases ACh, leading to suppressed TNF production in vivo (4) via the α7 nAChR (5). Like ACh, nicotine, a nicotinic cholinergic agonist, binds and activates nAChRs (6) and exerts antiinflammatory activities in vitro and in vivo (4, 7–9). Nicotine also has been shown to be an effective treatment in experimental animal models of ulcerative colitis (9, 10) and sepsis (11). Recent studies by Wang and coworkers (5) revealed that the antiinflammatory activity of nicotine after LPS treatment is mediated via the α7 nAChR expressed by macrophages. Unfortunately, the use of nicotine as a therapeutic agent is limited by its toxicity.

Endothelial cells express nAChRs (12–16). Previously it was unknown whether ACh and cholinergic agonists modulate endothelial cell activation and leukocyte recruitment. Herein, we report that human microvascular endothelial cells (HuMVECs) express α7 nAChR, and that nicotine, a novel cholinergic agonist (CAP55), and vagus nerve stimulation (VNS) inhibit leukocyte recruitment during local inflammation via the cholinergic pathway. This effect is mediated, in part, by blocking endothelial cell activation via NFκB, suggesting that the endothelium is a target of the cholinergic antiinflammatory pathway.

RESULTS

nAChR agonists suppress endothelial cell activation in vivo

Activation of the endothelium, characterized by increased cell surface adhesion molecule expression, is a critical component for leukocyte recruitment during inflammation. The localized Shwartzman reaction is characterized by endothelial cell activation, with increased and sustained expression of adhesion molecules (17). We observed that nicotine significantly decreased both VCAM-1 mRNA and E-selectin mRNA expression by the endothelium (Fig. 1 A), as determined by quantitative real-time RT-PCR methods. Immunostaining methods showed that treatment with nicotine (2 mg/kg) reduced VCAM-1 and E-selectin protein expression by the endothelium (Fig. 1, B and C) when compared with vehicle-treated animals. Enumeration of VCAM-1 and E-selectin staining revealed that nicotine-treated sections had ~5 (±2) and 3 (±2) positive capillaries/vessels per field, respectively, whereas saline-treated animals had 10 (±3) and 8 (±3), respectively. These data show that the cholinergic agonist nicotine significantly reduced endothelial cell activation in vivo.

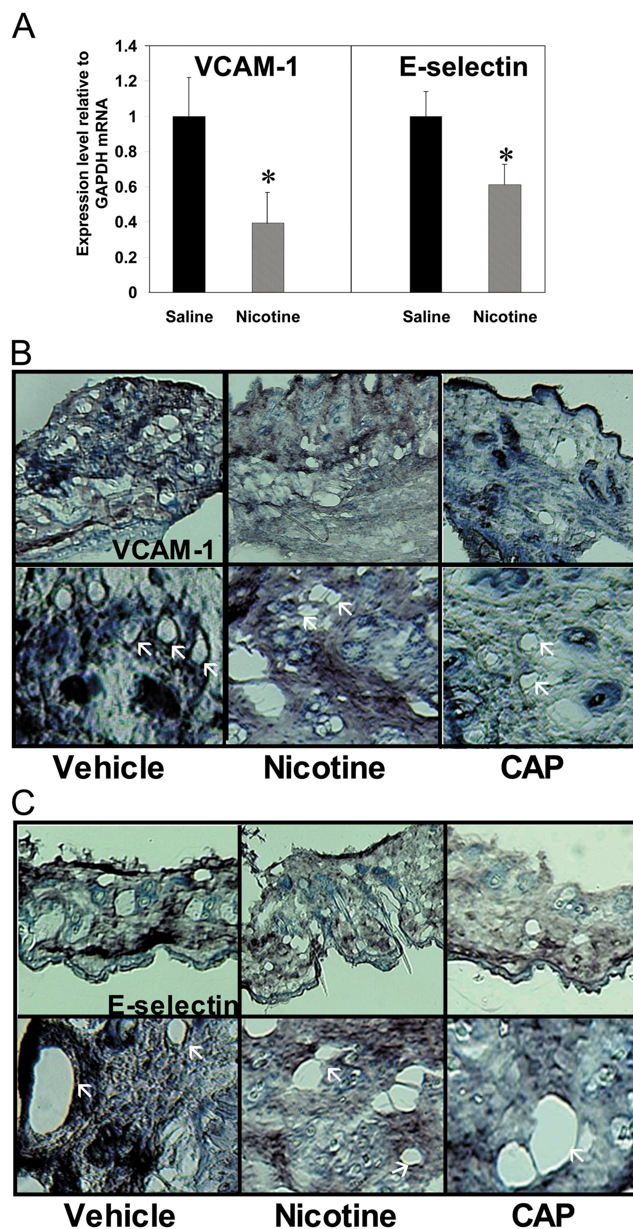

Figure 1. Cholinergic agonists block endothelial cell activation in vivo. Mice previously injected with a preparatory dose of LPS in the ear received vehicle (saline), nicotine (2 mg/kg), or CAP55 (12 mg/kg) 15 min before systemic LPS challenge (Shwartzman reaction model). Five h after LPS challenge, mouse ears were analyzed for (A) VCAM-1 and E-selectin mRNA expression by quantitative RT-PCR. Data are shown as the average relative expression of VCAM-1 and E-selectin mRNA copy number (normalized to GAPDH) in samples obtained from saline- versus nicotine-treated mice. *, $P < 0.05$ comparing nicotine treated vs. saline treated using the Student's *t* test. (B) VCAM-1 and (C) E-selectin expression within the ears by immunostaining methods (arrows indicate vessels).

Chemical structure and characterization of CAP55

One of the significant disadvantages of nicotine as a therapeutic agent is its toxicity. Therefore, a library of compounds was generated to produce novel cholinergic agonists. When the inhibition of TNF production by LPS-stimulated macrophages was used a screening assay, CAP55 emerged as a lead cholinergic compound. The chemical structure of nicotine and CAP55 are shown in Fig. 2 A. Both nicotine and

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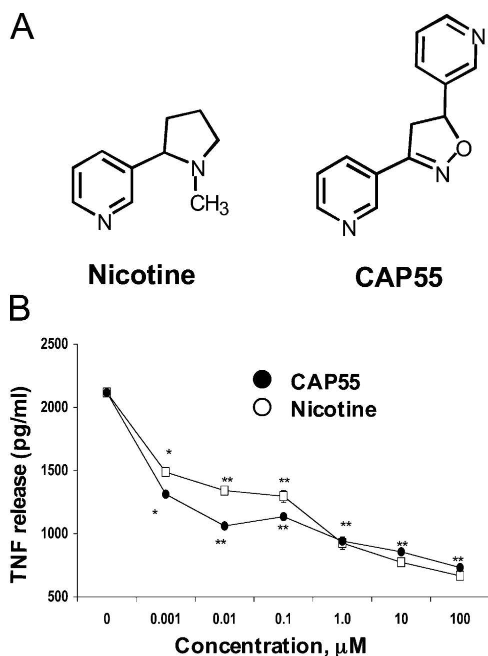

Figure 2. Structure and antiinflammatory activity of CAP55 in vitro. (A) Chemical structure of nicotine and CAP55. (B) CAP55 and nicotine inhibit TNF production by LPS-stimulated macrophages in vitro. Experiments were performed as described in Materials and methods. *, $P < 0.05$; **, $P < 0.001$ comparing CAP55 + LPS- or nicotine + LPS-treated cells versus LPS-treated cells alone, as determined using the Student's *t* test.

CAP55 significantly inhibit TNF production by LPS-stimulated macrophages (Fig. 2 B). Based on the antiinflammatory activity of this cholinergic compound, we investigated the effect of CAP55 (12 mg/kg) on endothelial cell activation in vivo using the local Schwartzman reaction. Similar to our results with nicotine, CAP55 inhibited both VCAM-1 and E-selectin expression by the endothelium in vivo by ~50% (Fig. 1, B and C).

ACh and nAChR agonists inhibit TNF-induced adhesion molecule expression by HuMVECs

To determine the direct effect of cholinergic mediators on endothelial cell activation, we treated HuMVECs with vehicle, ACh, nicotine, or CAP55 before stimulation with TNF. Treatment of HuMVECs with TNF alone significantly induced adhesion molecule expression over control (Fig. 3 A). Treatment of endothelial cell cultures with ACh significantly abrogated TNF-induced expression of E-selectin, ICAM-1, and VCAM-1, in a dose-dependent manner (up to 60–70%). Similarly, nicotine reduced TNF-mediated adhesion molecule expression by HuMVECs by 60–70% (Fig. 3, B and D). The novel nAChR agonist, CAP55, blocked TNF-induced ICAM-1 expression by the endothelium by ~40–50%, (Fig. 3 C). No cytotoxicity was observed with ACh or the cholinergic agonists at the concentrations used (unpublished data), indicating that the effect was specific. In addition, these cholinergic agonists did not induce endothelial cell adhesion molecule expression in the absence of TNF (unpublished data).

HuMVECs express the cell surface α7 nAChR

The α7 nAChR expressed by macrophages mediates the antiinflammatory effects of cholinergic stimulation in vitro and in vivo (5). Using RT-PCR and Western blotting methods, we observed α7 nAChR mRNA and protein expression by cultured HuMVECs (Fig. 4, A and B). Further RT-PCR studies revealed the expression of α5 and α9 nAChR mRNA (unpublished data). We confirmed the surface expression of the α7 nAChR by HuMVECs using FITC-labeled α-bungarotoxin (α-BGT), a selective α7 nAChR antagonist (Fig. 4 C). Competition studies using nicotine and unlabeled α-BGT significantly reduced FITC–αBGT binding by HuMVECs.

Mecamylamine blocks the effects of cholinergic agonists on adhesion molecule expression

To test whether the inhibitory effect of nicotine and CAP55 on endothelial cell activation in vitro was mediated through the cholinergic pathway, we used mecamylamine, a nonselective nAChR antagonist. Addition of mecamylamine attenuated the effects of nicotine and CAP55 on TNF-induced ICAM-1 expression (Fig. 5, A and B). These data suggest that the suppressive effect of nicotine and CAP55 on TNF-mediated endothelial cell activation is mediated, in part, through the nAChR pathway.

ACh and nAChR agonists reduce TNF-induced chemokine production by endothelial cells

The endothelium is not only a target of proinflammatory mediators; it also is a source of proinflammatory molecules such as chemokines. Treatment of HuMVEC monolayers with TNF induced the production of IL-8, monocyte chemoattractant protein 1 (MCP-1), and regulated on activation, normal T cell expressed and secreted (RANTES) (Fig. 6, A and B), but not macrophage inflammatory protein 1α (MIP-1α) or MIP-1β (not depicted). ACh and the nAChR agonist, nicotine, suppressed MCP-1, RANTES, and IL-8 production (≥50%) by TNF-induced HuMVECs (Fig. 6, A and B). Similarly, the novel nAChR agonist, CAP55, blocked IL-8 production by TNF-treated HuMVECs by ~50% (Fig. 6 C).

Treatment of endothelial cells with nicotine blocks leukocyte binding in vitro

To confirm that impaired adhesion molecule expression observed with cholinergic agonists reduced leukocyte adhesion, we performed in vitro binding assays. Treatment of the HuMVECs with TNF increased the binding of previously labeled monocytes and neutrophils by approximately fivefold (Fig. 7, A and B) when compared control cultures. Nicotine pretreatment significantly abrogated TNF-induced monocyte and neutrophil binding (Fig. 7). These data suggest that nicotine reduced the adhesion of leukocytes to HuMVECs and support the hypothesis that cholinergic agonists decrease endothelial cell activation.

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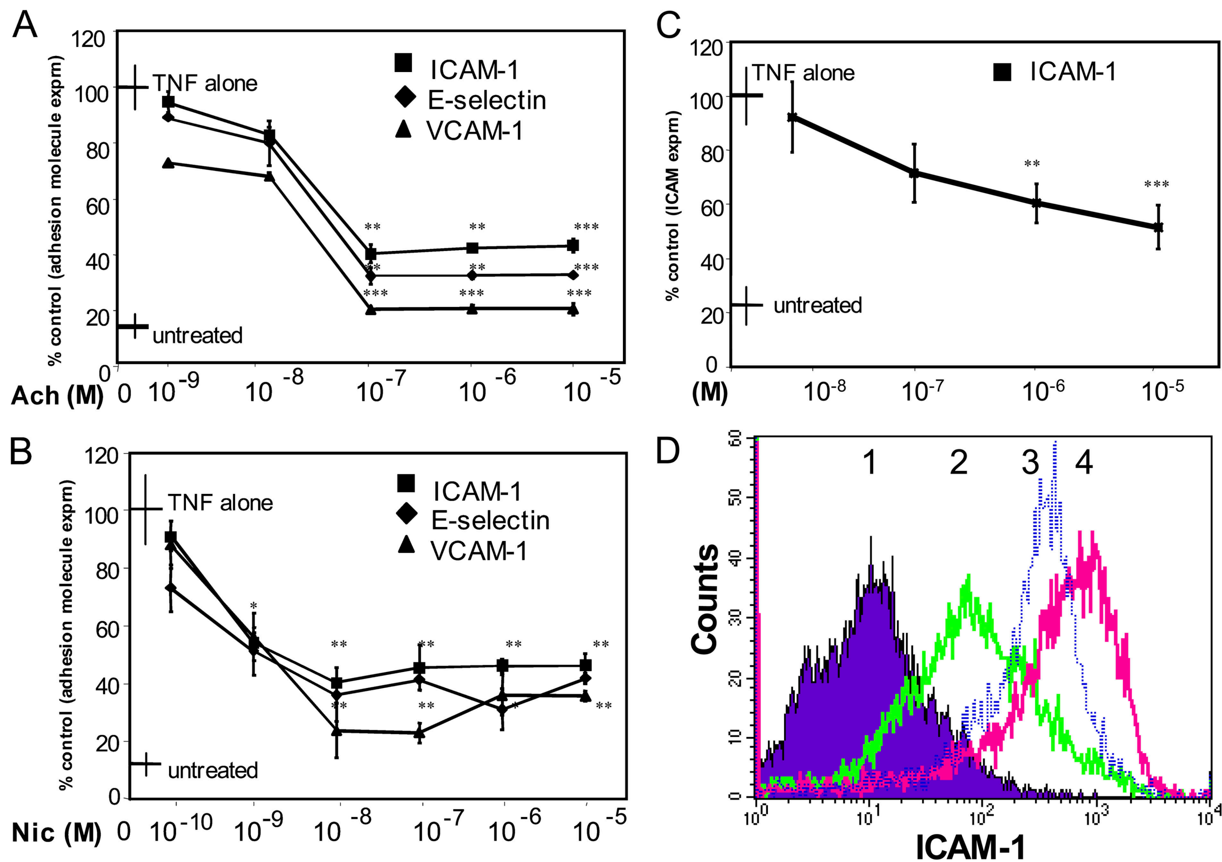

Figure 3. ACh and cholinergic agonists block adhesion molecule expression by TNF-treated endothelial cells in vitro. Confluent monolayers of HuMVECs were untreated, treated with TNF (1 ng/ml) alone or were treated with (A) ACh, (B) nicotine, or (C) CAP55 before TNF stimulation (1 ng/ml). Cell surface expression of ICAM-1 (■), E-selectin (◆), or VCAM-1 (▲) was determined using a cell-based ELISA method. 100% represents 0.55 (0.04), 1.12 (0.05), and 0.864 (0.03) OD (±SD) at 450 for E-selectin, ICAM-1, and VCAM-1, respectively. *, $P < 0.05$, **, $P < 0.01$ comparing treatment + TNF versus TNF alone using the Student's *t* test. Data are shown as the percent control (±SD), with TNF alone as control. (D) ICAM-1 expression was assessed using flow cytometry methods (mean fluorescence intensity, MFI) using ICAM-1–specific antibodies: (1) untreated cells (isotype control), MFI = 29.6; (2) untreated cells, MFI = 164; (3) nicotine (10^{-6} M) + TNF (1 ng/ml), MFI = 380; and (4) TNF (1 ng/ml) alone, MFI = 677.

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Nicotine inhibits NF-κB nuclear localization

Because the NF-κB pathway regulates many genes involved in TNF-mediated endothelial cell activation, we examined the effect of nicotine on NF-κB and inhibitor κB (IκB) family members. We found that nicotine (10^{-6}–10^{-7}M) reduced the nuclear translocation of NF-κB in HuMVECs after a 15-min TNF treatment (Fig. 8 A). Further studies showed that nicotine increased IκBα (Fig. 8 B) and IκBε (Fig. 8 C) levels in the cytoplasm, when compared with control cells.

Cholinergic stimulation blocks leukocyte migration during inflammation in vivo

Based on our findings that cholinergic agonists blocked endothelial cell activation and leukocyte binding, we next examined the effect of cholinergic agonists on leukocyte recruitment in vivo. Using the air pouch model of leukocyte migration, we administered either vehicle or cholinergic agonists before carrageenan challenge. We observed that nicotine (2 mg/kg) significantly reduced leukocyte trafficking by ~60% compared with vehicle treatment (Fig. 9 A). CAP55 (4 or 12 mg/kg, i.p.) blocked leukocyte recruitment induced by carrageenan by 55% and 62%, respectively, when compared control (Fig. 9 B).

Administration of mecamylamine, a negative allosteric modulator of the nAChR, significantly attenuated the accumulation of leukocytes induced by cholinergic agonists (Fig. 9, A and B, insets). These data demonstrate that both nicotine and CAP55 block leukocyte migration during acute inflammatory responses, predominantly via a cholinergic pathway.

Previous studies describe the antiinflammatory effects of VNS mediated through the cholinergic pathway, specifically through the α7 nAChR (5). Like cholinergic agonists, VNS reduced leukocyte recruitment by 60% compared with sham-operated animals (Fig. 9 C). These data further suggest that cholinergic activation suppresses host inflammatory responses, (i.e., leukocyte migration across the endothelium).

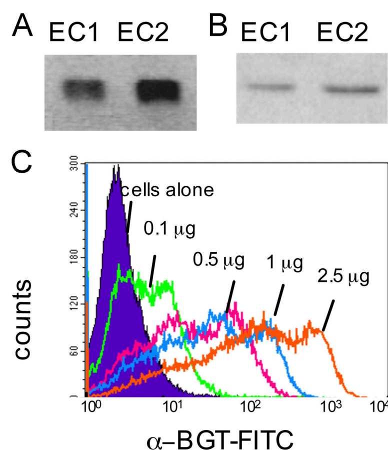

Figure 4. HuMVECs express the α7 nAChR. The expression of α7 nAChR by HuMVECs (EC1, EC2) was assessed by (A) RT-PCR and (B) Western blotting methods. (C) HuMVECs bind α-BGT-FITC (0–2.5 μg), a specific α7 nAChR antagonist, as determined by flow cytometry.

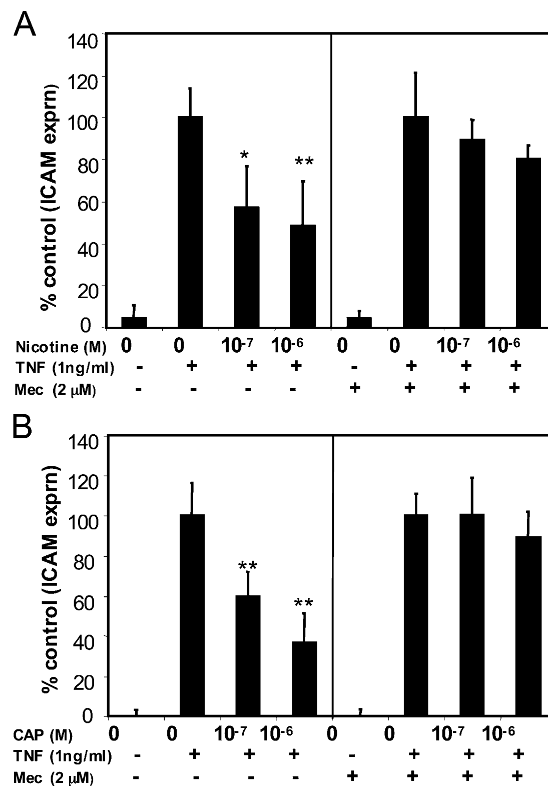

Figure 5. Nicotine and CAP55 block TNF-induced adhesion molecule expression in vitro via the nAChR pathway. HuMVECs were untreated, treated with TNF (1 ng/ml) alone, or treated with mecamylamine (Mec, 2 μM) before (A) nicotine (10^{-6}–10^{-7} M) or (B) CAP55 (CAP55, 10^{-6}–10^{-7} M) plus TNF (1 ng/ml) for 18 h. ICAM-1 expression (black bars) was determined using a cell-based ELISA technique. Data are shown as the percent control (±SD), with TNF alone as control. 100% represents 0.574 (0.04) and 0.497 (0.04) OD 450 for the nicotine and CAP55 sets, respectively. *, $P < 0.05$; **, $P < 0.01$, comparing treatment plus TNF versus treatment plus mecamylamine plus TNF using the Student's *t* test.

Cholinergic agonists suppress production of inflammatory mediators found in the pouch fluid

The synthesis of inflammatory mediators by immune cells within the pouch is a critical step in promoting leukocyte recruitment to inflamed tissues. Because cholinergic agonists inhibited the accumulation of leukocytes in the air pouch model, we next analyzed the pouch fluids for inflammatory mediators. Pouch TNF levels peak 2 h after carrageenan challenge and then decline to baseline 24–48 h after challenge (18). Administration of nicotine blocked TNF production in the pouch by 30% when compared with vehicle-treated control animals. CAP55 reduced TNF levels by 40% when compared with vehicle-treated controls (100 ±7% control vs. 60 ±11%). Neutrophils and monocytes, the predominant inflammatory cells within the pouch, express chemokine receptors that mediate their recruitment by specific chemokines (MIP-2 and MCP-1, respectively). We observed that nicotine (2 mg/kg) and CAP55 (12 mg/kg) significantly reduced the pouch fluid levels of MCP-1 by 20% and 30%, respectively. By contrast, MIP-2, MIP-1α, and MIP-1β levels were only slightly decreased by nicotine and CAP55 treatments.

DISCUSSION

Herein we report that cholinergic signals inhibit endothelial cell inflammatory responses in vitro and in vivo. Using the localized Shwartzman reaction model, characterized by sustained E-selectin and VCAM-1 expression (17), we showed that nicotine (2 mg/kg) significantly suppresses endothelial cell activation in vivo (Fig. 1). Although nicotine treatment had no effect on complete blood cell (wet) and differential (absolute) counts when compared with saline treatment in these studies, this therapeutic dose is close to the LD_{50} of nicotine in mice (3–9 mg/kg). Therefore, we examined the effect of a novel cholinergic agonist with antiinflammatory activity, CAP55 (LD_{50} ≥40 mg/kg), on endothelial cell activation in this model (Fig. 2). At 12 mg/kg, CAP55 was very effective in inhibiting endothelial cell activation in vivo (Fig. 1). Because of its reduced toxicity, CAP55 offers therapeutic advantages over nicotine.

To confirm the direct effects of ACh and cholinergic agents on endothelial cell activation in vitro, we used HuMVECs. ACh, nicotine, and CAP55 significantly blocked TNF-induced adhesion molecule expression (Fig. 3) and chemokine expression (Fig. 6) by HuMVECs in a dose-dependent manner. Consistent with these observations, we found that nicotine treatment of the endothelium reduced leukocyte/endothelial cell adhesion (Figs. 7, A and B). It has been postulated that, as leukocytes roll across the vasculature, inflammatory mediators present on the surface of the endothelium, such as chemokines, progressively activate them (19) and that, upon binding to adhesion molecules, neutrophils receive signals and become activated (20, 21). Neutrophil activation allows their firm adhesion required for successful trans-

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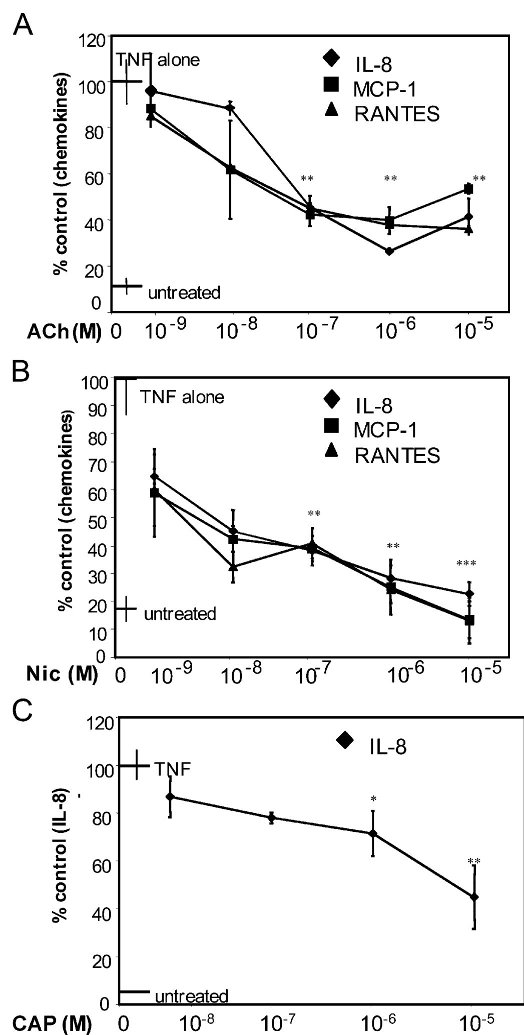

Figure 6. ACh and cholinergic agonists block chemokine production by TNF-treated HuMVECs. HuMVECs were untreated, treated with TNF (1 ng/ml) alone, or treated with (A) ACh, (B) nicotine, or (C) CAP55 before TNF stimulation (1 ng/ml) for 18 h. The production of IL-8 (◆), MCP-1 (■), or RANTES (▲) was determined by ELISA. Data are shown as the percent control (±SD), with TNF alone as control. 100% represents 18,848 (430), 63,436 (9,194), 3,122 (64) pg/ml (±SD) for IL-8, MCP-1, and RANTES, respectively. *, $P < 0.05$; **, $P < 0.01$ comparing treatment plus TNF versus TNF alone using the Student's *t* test.

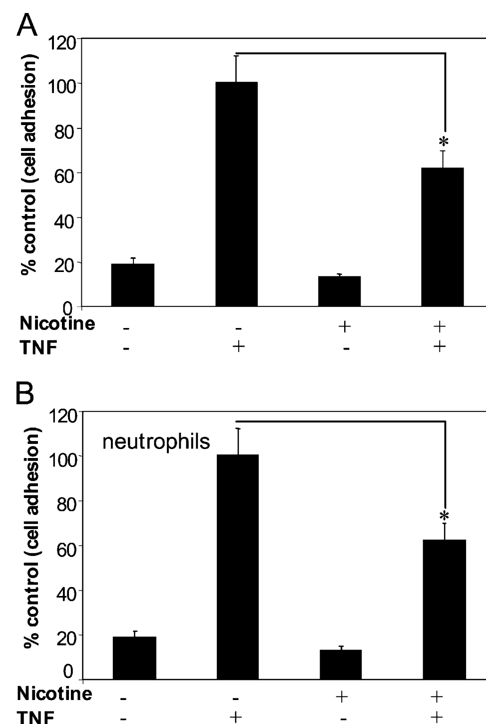

Figure 7. Nicotine blocks monocyte and neutrophil adhesion to HuMVECs. HuMVECs were untreated, treated with TNF alone, or treated with nicotine (Nic, 10^{-7} M) before TNF stimulation (1 ng/ml) or no TNF stimulation. HuMVECs were washed and incubated with either (A) human monocytes or (B) neutrophils (previously labeled with Calcein AM) for 0.5 h. After washing, bound leukocytes were quantified using a cytofluorescence assay method. The data are shown as the number of cells bound to the HuMVEC monolayers expressed as percent control (±SD), with the control being TNF-treated HuMVECs alone. 100% represents ~16% (or 3.2×10^4) and 27% (or 5.4×10^4) of input monocytes and neutrophils, respectively (based on a standard curve of labeled cells). *, $P < 0.05$ nicotine plus TNF versus TNF alone using the Student's *t* test.

migration during inflammation (22). Therefore, if cholinergic agonists inhibit the binding of immune cells to the endothelium, the subsequent steps of emigration and activation should be impaired. We chose 1 ng/ml TNF for our in vitro assays because this dose is below the saturating dose (10 ng/ml) that induces maximal adhesion molecule expression in our in vitro assays. This dose allows the detection of an increase or a decrease in endothelial cell activation by specific agonists. In addition, this is a biologically relevant dose of TNF, i.e., serum TNF levels of 1 ng/ml are fairly high but are achievable.

To examine the effect of nicotine on TNF-induced adhesion molecule expression in vivo, mice were injected with rTNF (1 μg s.c.) into the pinnae of the ear, and 2 h after treatment E-selectin expression was examined by quantitative RT-PCR. We observed that nicotine reduced E-selectin mRNA expression by 50% (relative to GAPDH). These data are consistent with our in vitro results using TNF and support the hypothesis that nicotine acts on endothelial cells to down-regulate TNF-induced inflammatory responses in vivo. The antiinflammatory activities of nicotine have been previously reported: nicotine (a) inhibits cytokine/chemokine production (4, 9, 23), (b) inhibits NF-κB activation (24), (c) abrogates T cell development and maturation (7), and (d) inhibits neutrophil and monocyte killing function (25). In animal models, nicotine suppresses the progression of experimental ulcerative colitis (9, 10) and cutaneous inflammation (26) and improves survival during endotoxemia and sepsis (11). In addition, nicotine has been used successfully in the treatment of human ulcerative colitis (27–29). However, the precise mechanism by which nicotine inhibits inflammation in these models is not completely understood.

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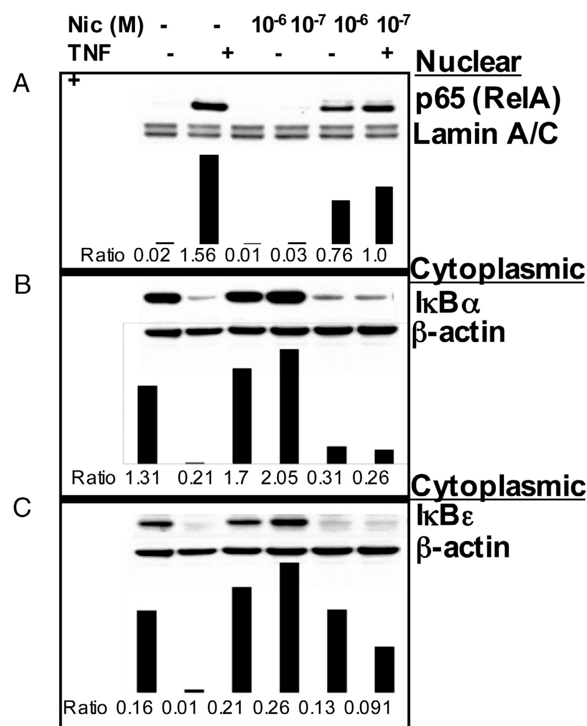

Figure 8. Nicotine blocks NF-κB nuclear translocation in an IκBα- and IκBε-dependent manner. HuMVECs were either untreated or treated with nicotine (10^{-6}–10^{-7} M) before TNF stimulation or no stimulation. Cytoplasmic and nuclear fractions were isolated 15 min after TNF addition. Samples were electrophoresed, transferred, and Western blotted using (A) NF-κB, (B) IκBα, or (C) IκBε antibodies. Lamin A/C and β-actin were used as controls for loading the nuclear and cytoplasmic fractions, respectively. Data are also shown as the ratio of NF-κB, IκBα, or IκBε to control (nuclear or cytoplasmic) protein.

Wang and coworkers (5) recently identified the α7 nAChR expressed by macrophages as the target of antiinflammatory cholinergic mediators, including nicotine. Interestingly, the α7 nAChR receptor has been implicated in inflammation in chronic inflammatory pain transmission in vivo (30). Numerous studies have identified the expression of nAChRs (including α2, α3, α4, α5, α7, β2, and β4 subunits) by several endothelial cell types, including cerebral (12), aortic (13, 14), umbilical vein (15), and coronary microvascular (16) endothelial cells. In this report, we show the expression of α7 nAChR mRNA and protein by HuMVECs (Fig. 4, A and B) and binding of the α7-nAChR selective antagonist, α-BGT, by HuMVECs (Fig. 4 C). Nicotine exerts diverse and numerous activities on resting endothelial cells, including proliferative (31) and apoptotic effects (32). Interestingly, the α7 nAChR expressed by the endothelium plays a role in nicotine-mediated angiogenesis (15). This report is the first report to describe the effects of ACh and cholinergic agonists on endothelial cell inflammatory responses.

To demonstrate that nicotine and CAP55 blocked endothelial cell activation via nAChRs, we used mecamy-

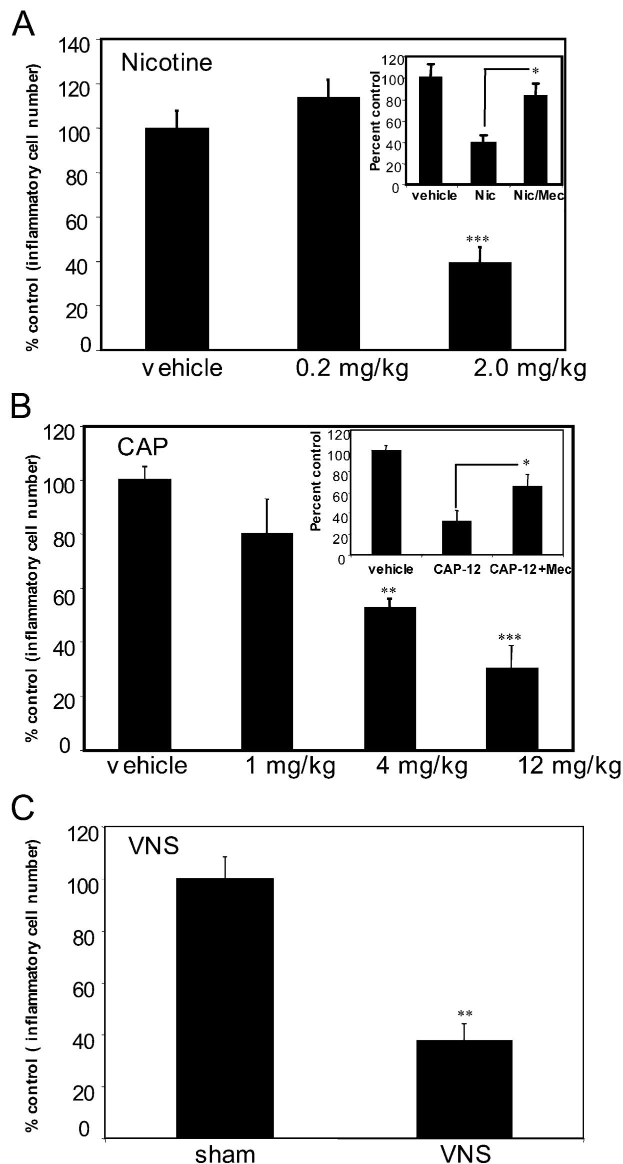

Figure 9. Cholinergic stimulation blocks leukocyte recruitment in vivo. (A) The effect of vehicle (saline) or nicotine (0.2 and 2 mg/kg, i.p.) on leukocyte recruitment was examined using the carrageenan air pouch model. (B) The effect of saline or CAP55 (1, 4, or 12 mg/kg, i.p.) on leukocyte recruitment. (A, inset) Mice were pretreated with mecamylamine (Mec, 200 μg/mouse) before treatment with nicotine (2 mg/kg) or (B inset) CAP55 (12 mg/kg). (C) VNS blocks leukocyte recruitment (compared with sham surgery treatment) using the carrageenan air pouch model. The data are shown as the average number of inflammatory cells per pouch (± SEM), presented as percent control vehicle-treated animals as the control. 100% represents 2.7×10^6, 1.4×10^6, and 1.8×10^6 cells per pouch in the groups of animals treated with nicotine, CAP55, and VNS, respectively. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, using the Student's *t* test comparing treatment versus vehicle or treatment versus treatment plus mecamylamine (insets).

lamine, a nonselective and noncompetitive nAChR blocker that occupies the ion channel and not the nicotine-binding site. The in vitro and in vivo nAChR signaling by nicotine

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and other cholinergic agonists can be reversed by mecamylamine (16, 33, 34). Mecamylamine reversed the inhibitory effects of both nicotine and CAP55 on endothelial cell activation in vitro (Fig. 5, A and B), and in vivo (Fig. 9, insets), suggesting that nicotine and CAP55 exert their antiinflammatory effects on the endothelium via the nAChR pathway.

The NF-κB/Rel family of transcription factors regulates the expression of many genes that control endothelial cell activation during inflammation. The effect of nicotine on NF-κB activation in the U937 monocytic cell line was previously identified (24). We found that nicotine reduced the nuclear import of NF-κB (p65 Rel A) after TNF stimulation (Fig. 8 A) and increased cytoplasmic levels of the IκB proteins (α and ε) that bind and retain NFκB in the cytoplasm (Fig. 8, B and C). IκBε, a relatively newly identified member of the IκB family (35, 36), was recently shown to play a functional role in endothelial cell activation (37). IκB levels were assessed at 15 min after TNF addition (before the resynthesis of IκBs), suggesting that nicotine reduces IκB degradation. Interestingly, nicotine-treated endothelial cells (in the absence of TNF) had increased cytoplasmic levels of IκB proteins. Future studies will focus on the effect of cholinergic stimulation (by agonists and electrical stimulation) on NF-κB activation in vivo. Another proposed mechanism by which cholinergic agonists inhibit endothelial cell activation is by inducing the shedding of TNF receptors. We found that nicotine did not down-regulate TNF receptor (I and II) surface expression in the presence or absence of TNF.

Although human endothelial cells respond to cholinergic mediators in vitro, this finding does not establish that similar responses occur in vivo or occur to the same degree. Therefore, we next examined whether the effect of cholinergic agents and cholinergic stimulation on endothelial cell activation impaired in vivo leukocyte trafficking. Using the carrageenan air pouch model, we observed that cholinergic agonists (nicotine and CAP55) suppress leukocyte migration during inflammation in vivo (Fig. 9, A and B). Mecamylamine antagonizes many of the effects of nicotine (38–40) and of epibatidine, another potent nAChR agonist shown to suppresses inflammatory pain in animals induced by kaolin and carrageenan (41). Administration of mecamylamine blocked the inhibitory actions of nicotine and CAP55 (Fig. 9, A and B, insets), suggesting that this effect, in part, is mediated through the nAChR cholinergic pathway. Further studies using VNS, a method previously shown to activate the cholinergic antiinflammatory pathway in rodents and to suppress proinflammatory cytokine production in vivo (4, 5), revealed that VNS significantly reduces leukocyte recruitment in vivo (by ∼60%) (Fig. 9 C).

These studies demonstrating the suppressive effects of nicotine and CAP55 on endothelial cell activation and leukocyte recruitment highlight the potential therapeutic use of cholinergic agonists in cases of excessive inflammatory responses by the endothelium. In addition to immunomodulation by cholinergic agonists, recent studies in experimental animals reveal that electrical stimulation of the efferent vagus nerve (which releases the neurotransmitter acetylcholine) modifies host inflammatory responses (for review see reference 3). Activation of this neural cholinergic antiinflammatory pathway via vagus nerve stimulation reduces both systemic and local inflammation in vivo (4, 42). Our studies demonstrate the antiinflammatory effects of VNS and cholinergic agonists on endothelial cell inflammatory responses in vivo and identify the endothelium as a potential cholinergic target.

MATERIALS AND METHODS

Reagents

ACh, pyrostigmine bromide (acetylcholinesterase inhibitor), λ carrageenan (type IV), LPS from *Escherichia coli* (0111:B4), and nicotine were purchased from Sigma-Aldrich. Mecamylamine hydrochloride was purchased from ICN Biomedicals. CAP55, a novel cholinergic agonist, was provided by Y. Al-Abed (Institute for Medical Research at North Shore-LIJ).

Animal models of leukocyte recruitment and endothelial cell activation

All experimental procedures using laboratory animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Institute for Medical Research at North Shore-LIJ.

Localized Shwartzman reaction model. The localized Shwartzman reaction was performed as previously described (17). Briefly, female BALB/c mice (22–26 g, Taconic) were injected with i.p. saline, nicotine, or CAP55 (i.p., at the indicated doses), and 20 μg LPS (preparatory dose) was injected s.c. into the pinnae of the ear. One day later, four mice per group were injected with vehicle (saline), nicotine, or CAP55 (i.p. at the indicated doses) 15 min before challenge with LPS (150 μg, i.p.) to generate a localized vasculitic reaction. Five hours later, mice were killed by carbon dioxide asphyxiation. The ears were excised, flash-frozen in liquid nitrogen, and stored at −80°C for immunostaining and quantitative RT-PCR analyses. Each experiment was repeated twice.

Carrageenan air pouch model. The carrageenan air pouch model was performed as previously described (18). To generate dorsal air pouches, Swiss Webster mice (26–33 g, Taconic) were anesthetized (ketamine/xylazine) on days zero and 3, and 6 ml of sterile air was injected s.c. to form a cavity. On day 6, animals received either vehicle (saline) or nAChR agonist (nicotine or CAP55 diluted in saline) i.p. at the indicated concentrations, and 15 min later 1% carrageenan was injected into the preformed air pouch. In one series of experiments, mecamylamine (200 μg/mouse, PBS) was injected into the pouch 5 min before saline, nicotine, or CAP55 injection. In another series of experiments, the effect of VNS (performed as described in reference 5, except that electrical stimulation was applied for 2 min) on leukocyte recruitment was compared with sham surgery. The animals were killed by carbon dioxide asphyxiation 6 h later, and the cellular infiltrate and fluid exudates were collected as previously described (43). TNF, prostaglandin E2, MCP-1, MIP-2, MIP-1α, and MIP-1β levels in the pouch fluids were determined by ELISA. The collected cells (RBC-free) were counted by hemocytometer and by using a flow cytometry method (44). Each experiment was repeated at least twice. Data showing the inhibitory effect of cholinergic agonists on leukocyte recruitment to the pouch are shown as percent control (mean ± SEM, with vehicle-treated as control). In one series of experiments, blood was drawn from killed mice by cardiac puncture and analyzed for complete blood cell (wet) and differential (absolute) counts by Ani Lytics, Inc.

Histological analyses of ears for inflammatory cell accumulation and VCAM-1 and E-selectin expression (Shwartzman reaction)

Ear sections (5 μ) from the Shwartzman reaction were stained using VCAM-1 and E-selectin antibodies, according to the manufacturer's rec-

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ommendations (BD Biosciences), using anti–mouse IgG-horseradish peroxidase (HRP) and diaminobenzidine substrate and counterstained with hematoxylin-eosin. Three fields per section (three sections per sample) were assessed and scored as the average number (±SD) of VCAM-1– or E-selectin–positive capillaries/vessels. Representative slides were photographed at magnifications of 20 and 100.

Analysis of VCAM-1 and E-selection mRNA expression by quantitative RT-PCR

Frozen ear tissues were homogenized. Total RNA was isolated using an RNeasy kit and QIAshredder mini spin columns (QIAGEN) and treated with DNase to remove genomic contamination (QIAGEN). The relative expression of VCAM-1 and E-selectin mRNA was determined by quantitative real-time PCR using TaqMan technology using the Eurogentec quantitative RT-PCR mastermix and the Prism 7700 sequence detection system (Applied Biosystems). Optimal concentrations of primers, probes, and the RNA were standardized. VCAM-1 primers, forward, 5′-CTGCTCAAGTGATGGGATACCA-3′, reverse, 5′-AGGCTGCAGTTCCCCATTATT, and the VCAM-1 TaqMan probe, 5′-TCCCAAAATCCTGTGGAGCAGACA-3′ were added at final concentrations of 900 nM and 150 nM, respectively. E-selectin primers, forward, 5′-CCGTCCCTTGGTAGTTGCAC-3′, reverse, 5′-CAAGTAGAGCAATGAGGACGATGT-3′ and the E-selectin TaqMan probe, 5′-TTCTGCGGCAGGAACCTCACTCCT-3′ were added at final concentrations of 500 nM and 100 nM, respectively. Mouse GAPDH was used as an internal control gene; mouse GAPDH primers, forward, 5′-TGTGTCCGTCGTGGATCTGA-3′, reverse, 5′-CCTGCTTCACCACCTTCTTGA-3′ and the GAPDH TaqMan probe, 5′-CCGCCTGGAG-AAACCTGCCA-3′ were added at final concentrations of 500 nM and 100 nM, respectively. The thermal cycler conditions were 35 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s Data were analyzed using Sequence Detection System software, version 1.9.1. Relative expression of VCAM-1 and E-selectin gene in ears obtained from mice treated with nicotine was calculated in comparison with vehicle-treated control samples using the Δ-Δ-Ct method (User Bulletin 2, Applied Biosystems). All samples were run in duplicate.

Cells and cell culture

Macrophage studies. Human macrophages were isolated, cultured, and used as previously described (5). Macrophages were treated with nicotine, CAP55, or left untreated before stimulation with 100 ng/ml LPS. After a 4-h stimulation period, culture supernatants were collected and assayed for TNF levels using standard ELISA methods (R&D Systems and J. Han [Institute for Medical Research of North Shore-LIJ]). Data are shown as pg/ml TNF ±SD.

Endothelial cells. Primary cultures of adult dermal HuMVECs were obtained from Clonetics (division of BioWhittaker) and were maintained in complete growth media (Clonetics). Experiments were initiated using synchronized confluent monolayers (passages 4–8). Parallel sets of cells were assessed using (3-4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolrum bromide and neutral red staining techniques as previously described (45, 46). TNF receptor expression by HuMVECs was assessed using the TNFR Fluorokine kit (R&D Systems).

α7nAChR expression and α-BGT binding by HuMVECs

Analysis of α7 nAChR mRNA expression. Total RNA was extracted from confluent monolayers of HuMVECs using RNAzol (Teltest Inc.). The cDNA was prepared from 1 μg of RNA using 0.25 ng of oligo-$(dT)_{12-18}$ and moloney murine leukemia virus RT (Grand Island Biological Company). 2 μl aliquots of cDNA were amplified by PCR using Supermix (Grand Island Biological Company) in a thermal cycler (model 9600, Perkin-Elmer) using specific primers for human α7 nAChR. The primers for human α7 nAChR were 5′-CCTGGCCAGTGTGGAG-3′(forward); 5′-TACGCAAAGTCTTTGGACAC-3′ (reverse). Amplified fragments of expected size (414 bp) were analyzed using a 2% agarose gel and photographed over UV light.

Analysis of α7nAChR protein expression. Confluent monolayers of HuMVEC cell lysates (20 μg/lane) were separated by electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were probed with rabbit primary antibody specific for human α7 nAChR, followed by incubation with HRP-conjugated goat anti–rabbit IgG and revealed using ECL (Amersham Biosciences).

α-BGT-FITC binding assays. HuMVECs were harvested using Enzyme Free Dissociation Buffer (Grand Island Biological Company), and $\sim 2 \times 10^5$ cells per condition were incubated with FITC–α-BGT (0–2.5 μg; Molecular Probes, Inc.) for 45 min in FACS buffer (PBS containing 2% BSA and 0.1% sodium azide) at 4°C in the dark. Washed cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences). Data were collected and analyzed using CellQuest software (BD Biosciences). Each experiment was repeated twice, with similar results.

Analysis of adhesion molecule expression

Endothelial cell surface expression of ICAM-1, VCAM-1, and E-selectin was determined using a cell-based ELISA, as previously described (47). In brief, G_0/G_1 synchronized HuMVECs grown in 96-well plates were preincubated with either ACh (plus pyrostigmine bromide), an acetylcholinesterase inhibitor at 50 μg/ml) or nAChR agonists (as indicated in the figure legends) for either 5 min (ACh) or 30 min (nAChR agonists) before the addition of 1 ng/ml TNF. After an overnight culture, adhesion molecule expression was determined using ICAM-1 (Chemicon International, Inc.), VCAM-1 (BD Biosciences), and E-selectin (BD Biosciences) antibodies ($n \geq 4$ per condition). In one series of experiments, the HuMVECs were pretreated with 2 μM mecamylamine before the addition of nicotine or CAP55 plus 1 ng/ml TNF. In another set of experiments, HuMVECs were grown and treated in six-well plates and assessed for ICAM-1 expression by flow cytometry using an isotype control IgG_1 or anti-ICAM-1 monoclonal antibody (Chemicon International, Inc.), followed by anti–mouse IgG_1-FITC (BD Biosciences). Each experiment was repeated at least twice.

Analysis of chemokine production by endothelial cell cultures

Cell-free culture supernatants from the adhesion molecule assays ($n = 4$ per condition) were assayed for chemokines. MIP-1α and -1β were analyzed by ELISA as previously described (48). RANTES and MCP-1 were analyzed by ELISA with antibodies (RANTES: MAB678 and AF478; MCP-1: MAB679 and AF279) or ELISA kits (R&D Systems).

Assay for monocyte and neutrophil adhesion to HuMVECs in vitro

Immune cell isolation and labeling. Monocytes from human peripheral blood were isolated by density-gradient centrifugation, followed by adherence. Human neutrophils were isolated from fresh blood after the sedimentation of erythrocytes using dextran, followed by centrifugation through Ficoll/Hypaque (Amersham Biosciences). Remaining RBCs were removed by hypotonic lysis. Monocytes and neutrophils were labeled with Calcein AM (Molecular Probes, Inc.) according to the manufacturer's directions. Endothelial cell adhesion assays: HuMVECs grown in 96-well plates (as described in Analysis of adhesion molecule expression) were either untreated or treated for 1 h with nicotine (10^{-7} M) before stimulation with 1 ng/ml TNF or no stimulation. Labeled monocytes or neutrophils (2×10^5 cells/well) were added 5 h later to washed HuMVECs ($n = 4$–6 replicates per sample). After 0.5 h, nonadherent leukocytes were removed by washing three times with media. A standard curve was made using known concentrations of labeled cells/well. Leukocytes/well were determined by cytofluorescence using a microplate reader (CytoFluor II, PerSeptive Biosystems). Assays were repeated twice.

Western blotting to assess NF-κB nuclear translocation and IκBα and IκBε levels

G_0/G_1 synchronized HuMVECs were treated with nicotine (10^{-6}–10^{-7} M) or left untreated for 0.5–1 h before the addition of TNF (1 ng/ml). Nuclear and cytoplasmic extracts were prepared 0.25 h after TNF addition using the

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NE-PER kit (Pierce Chemical Co.). Cell lysates (~10 μg/lane) were electrophoresed, transferred to polyvinylidene difluoride membranes, and probed with antibody to NF-κB (p65 (Rel A); Cell Signaling Technology, Inc.), IκBα, and IκBε (Santa Cruz Biotechnology, Inc.) or with antibodies to control nuclear (Lamin A/C; Santa Cruz Biotechnology, Inc.) and cytoplasmic (β-actin; Chemicon International, Inc.) proteins. After incubation with HRP-conjugated secondary antibody, specific proteins were revealed using ECL (Amersham Biosciences). Band densities were determined using the National Institute of Health Image Program, and the ratios of the specific/control bands are shown.

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K.J. Tracey and Y. Al-Abed are inventors on patents related to cholinergic agonists as antiinflammatory agents and K.J. Tracey is a consultant to Critical Therapeutics, Inc. The authors have no other potential conflicting financial interests.

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