Mecamylamine Suppresses Basal and Nicotine-Stimulated Choroidal Neovascularization

Katsuji Kiuchi,1,2,3 Masato Matsuoka,1,2,5 Jenny C. Wu,3,4 Raquel Lima e Silva,1,2 Muralitharan Kengatharan,5 Mary Vergbese,5 Sbinji Ueno,1,2 Katsutoshi Yokoi,1,2 Nau Htee Khu,1,2 John P. Cooke,4 and Peter A. Campochiaro1,2

PURPOSE. Nicotinic acetylcholine receptors (nAChR) are best known for their role in neurotransmission, but they have recently been demonstrated on vascular endothelial cells. Acetylcholine is their endogenous ligand, but they are also stimulated by nicotine. By stimulating nAChR, nicotine promotes tumor angiogenesis as well as atherosclerotic plaque neovascularization. In this study, the authors investigated the role of nAChR in the pathogenesis of choroidal neovascularization (CNV).

METHODS. The effect of the nonselective nAChR antagonist mecamylamine was tested on human retinal and choroidal endothelial cells in vitro and in a murine model of CNV.

RESULTS. Several nAChR isoforms were identified in retinal and choroidal microvascular endothelial cells, and the ability of these cells to form tubules when grown in growth factor-reduced basement membrane matrix and supplemented with VEGF was suppressed by the nAChR antagonist mecamylamine. Supplementation of the drinking water of mice with nicotine increased the size of CNV lesions at Bruch membrane rupture sites, an effect that was blocked by subcutaneous administration of mecamylamine (50 mg/kg/d) by an osmotic pump. In the absence of nicotine, CNV formation was suppressed by the infusion of 50 mg/kg/d mecamylamine or by topical application 0.1 or 1% mecamylamine to the cornea.

CONCLUSIONS. These data suggest that endogenous activation of nAChR promotes CNV and that activation of nAChR by nicotine may contribute to the increased incidence of CNV seen in smokers with age-related macular degeneration (AMD). Topically administered mecamylamine could provide an appealing new treatment approach for CNV. (Invest Ophtalmol Vis Sci. 2008;49:1705–1711) DOI:10.1167/iovs.07-0089

Age-related macular degeneration (AMD) is a complex group of diseases in which genetic susceptibility combined with environmental exposure results in a disease phenotype that consists of deposits of material, referred to as drusen, along Bruch membrane, atrophy of photoreceptors and retinal pigment epithelial (RPE) cells (geographic atrophy), and enhanced risk for choroidal neovascularization (CNV). Polymorphisms associated with increased or reduced risk for AMD have been identified.1–4 The strongest environmental risk factor for AMD is cigarette smoking, which is associated with substantially higher incidences of geographic atrophy and CNV.5,6 Nicotine is a major component of cigarette smoke that activates nicotinic acetylcholine receptors (nAChRs), which have acetylcholine as their endogenous ligand. Neuronal nAChRs mediate the stimulatory and addictive effects of nicotine.7 Recently, it has been shown that nAChRs are expressed by vascular endothelial cells8 and that endothelial cells synthesize, store, and release acetylcholine.9 Increasing evidence suggests that such nonneuronal nAChRs are involved in the regulation of vital cell functions, such as mitosis, differentiation, organization of the cytoskeleton, cell-cell contact, locomotion, and migration.10 Thus, acetylcholine, originally identified as a neurotransmitter, also functions as an autocrine factor that modulates endothelial cell functions involved in angiogenesis. Nicotine mediates the central nervous system effects of smoking, and it may contribute to other effects because it induces endothelial cell proliferation in vitro8 and promotes neovascularization in atherosclerotic plaques and tumors.11 Recently, it has been shown that nicotine enhances the size and severity of experimental CNV.12 This raises the question, do nAChRs and their endogenous ligand, acetylcholine, participate in the pathogenesis of CNV? In this study, we investigated the role of nAChRs in a murine model of CNV.

MATERIALS AND METHODS

Expression of mRNA for nAChRs in Human Endothelial Cells

In three types of cultured human microvascular endothelial cells—dermal (HDMVEC; Vec Technologies, Inc; Rensselaer, NY), choroidal (HEC; gift of Mary Elizabeth Hartnett, Department of Ophthalmology, University of North Carolina), and retinal (HRECs; Cell Systems Corporation, Kirkland, WA)—mRNA levels for nAChR isoforms were measured using a real-time reverse transcription-polymerase chain reaction (RT-PCR) assay. Total RNA was extracted from cultured cells using purification kit (RNAeasy Mini Kit; Qiagen, Valencia, CA) according to the protocol provided by the manufacturer. Primers and probes (Assays-on-Demand; Applied Biosystems, Foster City, CA) for the human genes of the nAChR subunits α1, α2, α3, α4, α5, α6, α7, α9, α10, β1, β2, β3, and β4 were used for one-step real-time RT-PCR. The nucleotide databases were searched to confirm gene specificity. To avoid
amplification of contaminating genomic DNA, the primers were located at an exon/intron junction. For each assay, we used no-template and no-reverse transcriptase (RT) controls, which produced insignificant signals, suggesting that primer–dimer formation and genomic DNA contamination effects were negligible.

All PCR reactions were performed using a sequence detection system (ABI Prism 7500; Applied Biosystems) and reagent (TaqMan Multiscribe & RNase Inhibitor Mix; Applied Biosystems) according to the manufacturer’s protocol. Briefly, 2 μL diluted total RNA (100 ng) was added to 0.5 μL of the 40 μL PCR master mix and 1 μL 20 μL gene expression mix (Assays-on-Demand; Applied Biosystems). The amplification included a 30-minute, 48°C step required for reverse transcription, and a denaturation step for 10 minutes at 95°C, followed by 40 cycles consisting of 15 seconds at 95°C and 1 minute at 60°C. Data from triplicate samples were analyzed with the sequence detector software (Applied Biosystems) and expressed as mean (± SD) of mRNA relative to that of 18S RNA. Fold induction over control was determined by normalizing treated samples to the control.

Detection of nAChR Proteins in Human Endothelial Cells by Immunoblotting

Western blot analyses were performed for a subset of nAChRs with available antibodies directed against them. Cell lysates of HDMVEC, HCEC, and HREC were prepared in lysis buffer with 50 mM Tris (pH 6.8), 2% (wt/vol) SDS, 5% (vol/vol) glycerol, 1% (vol/vol) 2-mercaptoethanol, 50 mM NaF, 2.5 mM EDTA, 2.5 mM EGTA, 5 mM β-glycerophosphate, 1% (vol/vol) phosphatase inhibitor cocktail 2 (Sigma), and 1× complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN). Protein was quantified with the use of an assay kit (Quant-It Protein; Invitrogen, Carlsbad, CA). Each sample (20 μg protein) was subjected to SDS-PAGE and immunoblotted with antibodies against nAChR subunits α5, α7, β2, and β3 (Santa Cruz Biotechnology, Santa Cruz, CA).
Mecamylamine and Choroidal Neovascularization

Mountain View, CA) using a handheld coverslip as a contact lens to view the retina. Burns were performed in the 9, 12, and 3 o’clock positions of the posterior pole of the retina. Production of a bubble at the time of laser, which indicated rupture of Bruch membrane, was an important factor in obtaining choroidal neovascularization; therefore, only burns in which a bubble was produced were included in the study.

Three independent experiments were performed to investigate the effect of mecamylamine on laser-induced CNV. In the first experiment, the effect of continuous subcutaneous administration of 50 mg/kg/d mecamylamine using osmotic pumps (Alzet; Diurect Corp., Cupertino, CA) was investigated in nicotine-treated mice. In the second experiment, the effect of continuous subcutaneous administration of 50 mg/kg/d mecamylamine using osmotic pumps (Alzet; Diurect Corp.) was examined in mice with laser-induced rupture of Bruch membrane in the absence of nicotine stimulation. Finally, a masked experiment was conducted to investigate a topical formulation of mecamylamine.

Three treatment samples containing 0.1% mecamylamine, 1% mecamylamine, or vehicle alone were prepared and coded at CoMentis and shipped to Johns Hopkins University. Mice were divided into three groups that received twice-daily topical administration to the cornea of 10 μL solution A, B, or C, starting 2 hours before laser-induced rupture of Bruch membrane and continuing for either 1 or 2 weeks, when the mice were perfused with fluorescein labeled-dextran and the area of the CNV was measured. After all measurements of the CNV area were completed, the code was broken.

Systemic Administration of nAChR-Active Drugs

Mecamylamine (50 mg/kg/d) in phosphate-buffered saline (PBS), vehicle alone, or both were loaded into osmotic mini-pumps (Alzet model 2002; Diurect Corp.) with internal volumes of 200 μL and mean pumping rate of 0.5 μL/h. Pumps were implanted beneath the skin of the back. After 2 days, the mice had laser-induced rupture of Bruch membrane at three locations in each eye. In some animals, nicotine or vehicle (2% saccharine) was administered for 2 weeks orally (100 μg/mL in the drinking water ad libitum). This amount of nicotine administration achieves serum cotinine levels similar to those observed in moderate smokers.14

Histologic Assessment of CNV

The mice were perfused with 1 mL PBS containing 50 mg/mL fluorescein-labeled dextran (2 × 10⁶ average molecular weight; Sigma-Aldrich, St. Louis, MO), and choroidal flat mounts were prepared as previously described.14 Briefly, the eyes were removed and were fixed for 1 hour in 10% phosphate-buffered formalin, and the cornea and lens were removed. The entire retina was carefully dissected from the eyecup. Radial cuts were made in eyecups, and they were flat-mounted in aqueous mounting medium (Aquamount; BDH, Poole, UK). Flat mounts were examined by fluorescence microscopy (Axioskop; Carl Zeiss Meditec, Thornwood, NY), and images were digitized with a three charge-coupled device (CCD) color video camera (IK-TU40A; Toshiba, Tokyo, Japan) and a frame grasper. Image analysis software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD) was used to measure the area of each CNV lesion.

Measurement of Mecamylamine in Plasma

After 14 days, blood was drawn and plasma was isolated and frozen. Plasma levels of mecamylamine were analyzed in the animals receiving the drug subcutaneously through miniature infusion pumps (Alzet; Diurect Corp.) using the following LC/MS/MS (HPLC system [Shimadzu VP System, Columbia, MD]); MDS SCIEX API 3000 mass spectrometer [Applied Biosystems]) conditions: mobile phase; A: 0.2% formic acid in water, B: 0.18% formic acid in methanol; column, 2 × 20 mm HPLC sorbent (Phalanx C18; Higgins Analytical, Mountain View, CA) guard cartridge; injection volume, 25 μL; gradient, 5% B for 0.5 minutes, then 5% to 95% B in 2 minutes; flow rate, 400 μL/min; interface, heated
ion spray source (TurboIonSpray; Applied Biosystems) (electrospray ionization) at 400°C; polarity, positive ion; Q1/Q3 ions, 168.2/137.2 for mecamylamine, 256.2/167.2 for diphenhydramine (internal standard), 272.1/215.2 for dextromethorphan (internal standard).

Statistical Analysis
Data are expressed as mean ± SEM. Statistical comparisons were made using one-way analysis of variance (ANOVA). Probabilities for comparison of treatments were adjusted for multiple comparisons by the Dunnett method. \( P < 0.05 \) was considered statistically significant.

RESULTS
Characterization of nAChRs in Human Ocular Endothelial Cells
HDMVECs express endothelial markers, including factor VIII-related antigen, and they incorporate acetylated LDL. We observed that HRECs and HCECs expressed endothelial markers, specifically von Willebrand factor and CD31, and they manifested acetylated LDL uptake (data not shown). In growth factor–reduced basement membrane matrix (Matrigel; BD Biosciences), each cell type formed tubule networks characteristic of endothelial cells.

We identified the nAChR subunits on HDMVECs, HRECs, and HCECs by performing nAChR subunit-specific RT-PCR analysis of subunits \( \alpha_1 \) to \( \alpha_{10} \) and \( \beta_1 \) to \( \beta_4 \). All mammalian \( \alpha \) and \( \beta \) nAChR subunits were expressed in each of the endothelial cell types, except for the \( \alpha_4 \) subunit. However, there were differences in the level of expression of each isoforms among the cell types (Fig. 1). Expression of selected \( \alpha \) and \( \beta \) nAChR isoforms was confirmed by Western blot analysis (Fig. 2).

Effect of Mecamylamine on Endothelial Tube Formation
Mecamylamine is a broad-spectrum nAChR antagonist that blocks most heteromeric and homomeric forms of the nAChR and provides a useful tool to explore the role of nAChR in biological processes. After seeding the cells on growth factor–reduced basement membrane matrix (Matrigel; BD Biosciences), HDMVECs manifested tube formation within 24 hours in control medium. Notably, mecamylamine (1–1000 nM) dose dependently attenuated endothelial network formation (Fig. 3). A reduction of greater than 50% in tube formation was observed at a mecamylamine concentration of 0.1 \( \mu \)M. HRECs and HCECs also formed tubelike networks in growth factor–reduced basement membrane matrix (Matrigel; BD Bio-

Sciences). For both cell types, the tube length was increased by VEGF (10 ng/mL; Fig. 4), and the effect of VEGF to increase tube length was abolished by 1 \( \mu \)M mecamylamine.

Stimulation of nAChRs Contributes to Choroidal Neovascularization
Previous studies have shown that nicotine, an agonist for nACh receptors, stimulates tumor neovascularization and CNV.\(^{11,12}\) We found that mice given nicotine in their drinking water had an increase in the area of CNV at Bruch membrane rupture sites (Fig. 5). The nicotine-induced stimulation of CNV was blocked by infusion of 50 mg/kg/d of mecamylamine by a subcutaneously implanted osmotic pump, confirming that the stimulation of CNV by nicotine occurs through nACh receptors. In a separate experiment, administration of 50 mg/kg/d mecamylamine was found to suppress the development of CNV at Bruch membrane rupture sites in the absence of nicotine, indicating that stimulation of nACh receptors by acetylcholine, the endogenous ligand of nACh receptors, contributes to CNV (Fig. 6). The mean plasma level of mecamylamine associated with the suppression of CNV was 295 ± 23 ng/mL, and the mean level in retina-choroid was 34 ± 3 \( \mu \)g/g tissue.

Topical Delivery of Mecamylamine to the Eye Suppresses CNV
The suppression of CNV by mecamylamine suggests that it could potentially be used as a therapeutic agent; however, administration by a subcutaneous pump is not ideal. We therefore sought to determine whether CNV could be inhibited by topical administration of mecamylamine to the eye. In preliminary studies, it was found that topical delivery of 0.1% or 1% mecamylamine resulted in significant levels in retina-choroid with no detectable levels in plasma (data not shown). In a masked experiment, mice were treated with three topical formulations given twice daily starting 2 hours after the rupture of Bruch membrane. Analysis of the area of CNV was performed 7 and 14 days after rupture of Bruch membrane. When the code was broken, it was found that 0.1% and 1% mecamylamine had caused significant suppression of CNV compared with vehicle alone at both time points (Fig. 7).

DISCUSSION
Epidemiologic studies have demonstrated that smoking is the most consistent and strongest environmental risk factor for the development of neovascular AMD.\(^{5,6}\) Previous studies have
demonstrated that mice exposed to cigarette smoke or hydroquinone, an oxidant known to be present in cigarette smoke, show an increase in sub-RPE deposits and diffuse thickening of Bruch membrane. Given that diffuse thickening of Bruch membrane is the most consistent pathologic risk factor for neovascular AMD, it is likely that oxidative stress contributes to tobacco-induced CNV. However, recent studies have demonstrated that nicotine can directly stimulate neovascularization in tumors and in atherosclerotic plaques by acting on nAChR on vascular endothelial cells.

In this study, we have demonstrated that nAChR is expressed on retinal and choroidal endothelial cells in addition to dermal endothelial cells. Furthermore, the ability of these cells to form tubes in growth factor–reduced basement membrane matrix (Matrigel; BD Biosciences), an in vitro assay of angiogenesis, is blocked by mecamylamine, a nonselective antagonist of nAChR. Mammalian nAChRs are composed of homopentamers derived from subunits α9, α10, and α11 or heteropentamers derived from six α subunits(α1-α6) and four β subunits(β1-β4). In

**Figure 5.** Mecamylamine blocks nicotine-induced stimulation of choroidal neovascularization. Six-week-old C57BL/6 mice were given 100 µg/ml nicotine in their drinking water, and each underwent implantation of an osmotic pump that released mecamylamine or vehicle subcutaneously. After 2 days, these mice and a control group of three mice that did not receive any nicotine experienced rupture of Bruch membrane at three locations in each eye. Fourteen days after laser, the mice were perfused with fluorescein-labeled dextran, and the area of choroidal neovascularization at Bruch membrane rupture sites was measured by image analysis. Compared with choroidal neovascularization lesions in untreated mice that did not receive nicotine and underwent implantation of pumps that released only vehicle (B) but not in mice that received nicotine and mecamylamine (C). Measurement of the area of choroidal neovascularization by image analysis confirmed that nicotine caused a significant increase in lesion size that was completely blocked by mecamylamine (D). **P = 0.006470142; ***P = 0.007367749; ANOVA with Dunnett adjustment for multiple comparisons.

**Figure 6.** Mecamylamine suppresses choroidal neovascularization in the absence of nicotine. Each of 6-week-old C57BL/6 mice underwent plasma draw and implantation of an osmotic pump that released mecamylamine subcutaneously. After 2 days, the mice experienced rupture of Bruch membrane at three locations in each eye; after 14 days, plasma samples were drawn, mice were perfused with fluorescein-labeled dextran, and each area of choroidal neovascularization at Bruch membrane rupture sites was measured by image analysis. Compared with control mice, there was a significant reduction in the area of CNV at rupture sites in mice treated with mecamylamine. **P = 0.0048; by ANOVA with Dunnett adjustment for multiple comparisons.

**Mecamylamine**
Mecamylamine delivered topically to the eye suppresses CNV. Six-week-old C57BL/6 mice were divided into three groups and were started on twice-daily administration of eye drops containing 0.1% or 1% mecamylamine or vehicle, with investigators masked with respect to treatment groups. Two hours after the first drop, Bruch membrane was ruptured with laser photocoagulation in three locations in each eye. After 1 or 2 weeks of treatment, mice were perfused with fluorescein-labeled dextran. When the code was broken, it was found that mice perfused after 1 week of treatment with 0.1% (A) or 1% (C) mecamylamine had smaller areas of CNV than mice treated with vehicle (B). Image analysis confirmed that compared with mice treated with vehicle, the area of CNV (mm²) was significantly smaller in mice treated with either dose of mecamylamine (D; \( P < 0.0159; \{**P = 0.0127\); ANOVA with Bonferroni correction for multiple comparisons). In mice treated with drops for 2 weeks after laser and then perfused (E-H), there was a significant reduction after treatment with 0.1% mecamylamine compared with placebo (\( **P = 0.0224 \)) but not after treatment with 1% mecamylamine (\( P = 0.1753 \)).

The mechanism by which stimulation of nAChR stimulates neovascularization is not yet completely clear, but recent studies indicate that the nAChR and VEGF-mediated pathways for angiogenesis are interdependent. There is a striking concordance between the transcriptomes of endothelial cells stimulated by nicotine and those stimulated by VEGF. Furthermore, the VEGF-induced migration of endothelial cells is partially blocked by mecamylamine. In a model of inflammatory angiogenesis, neovascularization of subcutaneously implanted polyvinyl sponge discs is inhibited by mecamylamine, even in the absence of exogenous nicotine. In this study, we found that VEGF-induced formation of tubes by retinal and choroidal endothelial cells is suppressed by mecamylamine. Therefore, VEGF-induced effects on ocular endothelial cells and some endothelial cells from extraocular tissues are modulated by endogenous stimulation of nAChR. Additional work is needed to determine the details of cross-talk between VEGF receptor and nAChR signaling.

Intraocular injections of the VEGF antagonists ranibizumab and bevacizumab have become the first-line treatment for CNV and have provided tremendous benefit to patients. How ever, repeated intraocular injections carry small but definite risk. Development of topical agents that suppress CNV and can be self-administered by patients is an important goal. Clinical trials investigating topical mecamylamine in patients with CNV are now under way.

References


