Modulation of cerebral microvascular permeability by endothelial nicotinic acetylcholine receptors

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Modulation of cerebral microvascular permeability by endothelial nicotinic acetylcholine receptors. Am J Physiol Heart Circ Physiol 289: H212–H219, 2005. First published February 11, 2005; doi:10.1152/ajpheart.01210.2004.—Nicotine increases the permeability of the blood-brain barrier in vivo. This implies a possible role for nicotinic acetylcholine receptors (nAChRs) expressed on the cerebral microvascular endothelium in the pathogenesis of brain edema (46–48). These in vivo effects of nicotine on cerebral microvascular barrier permeability, however, are not well understood.

MATERIALS AND METHODS

Animals, radioisotopes, antibodies, and chemicals. All animal protocols used in this study were approved by the University of Arizona Institutional Animal Care and Use Committee and conform to National Institutes of Health (NIH) guidelines. Female Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN), housed under standard 12:12-h light-dark conditions, and given food and water ad libitum throughout the study.

[14C]sucrose was purchased from ICN Pharmaceuticals (specific activity 462 nCi/mmol; Irvine, CA). Rabbit polyclonal antisera against nAChR subunits α3, α4, α5, α7, β2, β3, and β4 and specific blocking peptides were purchased from Research and Diagnostics (Benicia, CA). Alexa Fluor 488-conjugated anti-rabbit IgG was purchased from...

NICOTINE BINDS WITH HIGH AFFINITY to various forms of the nicotinic acetylcholine receptor (nAChR), where it acts as an agonist (40). As such, nAChRs are likely mediators of the effects of nicotine throughout the body (29, 38), although non-receptor-mediated effects of nicotine have been observed (55). Recently, we demonstrated that nicotine increases the permeability of the blood-brain barrier (BBB) via a redistribution of tight junction proteins in cerebral microvessels (26). These cytostructural and functional effects of nicotine on the BBB indicate a role for nAChRs in the regulation of cerebral microvascular permeability. It is not immediately clear, however, how nicotine could lead to changes in the neurovascular unit.

nAChRs are found throughout the brain, on both neurons and astrocytes (25). Given the extensive communication that occurs between the microvascular endothelium and the parenchyma (6, 8, 14, 60), it is possible that central nAChRs could mediate the release of a vasoactive factor or factors that would affect BBB structure and function, such as VEGF (11) or bradykinin (7). Some evidence suggests that nAChRs might be expressed on cerebral microvessels (25, 32). Functional nicotinic receptors have been characterized in peripheral endothelial cells (42, 47) and epithelial cells (57) and are thought to be involved in the regulation of cell shape and barrier integrity in surface-lining tissues (15).

nAChR subunits are expressed in cultured brain endothelial cells, and nicotine-induced hyperpermeability in this model system is attenuated by the α7 nAChR antagonist α-bungarotoxin (1). It is therefore possible that nAChRs expressed on the abluminal or luminal endothelial membranes could mediate a direct effect of nicotine on cerebral endothelial cells. However, care must be taken in extrapolating expression studies in cell culture to the native tissue. For example, in vitro studies originally indicated that cerebral endothelial cells did not express N-methyl-D-aspartate (NMDA) receptors (48), but subsequent studies in isolated microvessels indicated that NMDA receptors are expressed at the BBB (54). Furthermore, expression of receptor subunit proteins does not necessarily imply the assembly of functional receptors (56). Thus the expression and function of nAChRs at the BBB need to be examined in a whole animal model.

In this study, expression of neuronal-type nAChR subunits (α3, α4, α5, α7, β2, β3, and β4) was investigated in isolated cerebral microvessels by immunofluorescence microscopy. Assessment of BBB permeability was performed by in situ brain perfusion with the paracellular permeability marker [14C]sucrose (30, 50) after nicotine treatment with and without nicotinic antagonists. The nicotinic antagonist mecamylamine, which acts at most neuronal-type nAChRs and crosses the BBB (58), was coadministered with nicotine to establish whether the effect of nicotine on BBB permeability (26) is nAChR mediated. Hexamethonium, a nonspecific nAChR antagonist that does not cross the BBB under normal circumstances (45), was coadministered to determine whether the effect of nicotine on BBB permeability is mediated by central nAChRs.

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Molecular Probes (Eugene, OR). All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.

**Immunofluorescence of nAChRs in cerebral microvessels.** Naïve female Sprague-Dawley rats (240–300 g) were anesthetized with 1 ml/kg of ketamine (78.3 mg/ml, 3.1 mg/ml xylazine) and decapitated. The brain was removed from the skull, the meninges and choroid plexuses were removed, and the brain stem and cerebellum were dissected away from the cerebral hemispheres. The hemispheres were homogenized in a fivefold volume of buffer (in mM: 103 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 15 HEPES, 25 NaHCO₃, 10 glucose, and 1 sodium pyruvate, with 10 g/l 64K dextran), suspended in an equal volume of 26% dextran, and centrifuged for 10 min at 5800 g and 4°C. The pellet was resuspended and passed through a 100-μm mesh. The filtrate was centrifuged for 10 min at 1500 rpm and 4°C, and the resulting pellet was resuspended in 0.25 ml of buffer, smeared onto microscope slides, and heat fixed at 95°C for 10 min. The vessels were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS, and blocked for 1 h in 1% BSA in PBS. Slides were incubated with rabbit polyclonal antisera against nAChR subunits α₃, α₄, α₅, α₆, and β₆, diluted 1:10 in PBS with 1% normal goat serum for 30 min, rinsed with 1% BSA in PBS, and incubated with Alexa Fluor 488-conjugated anti-rabbit IgG (2 μg/ml) for 30 min. After placement and sealing of coverslips, digital photographs were taken with ×100 oil-immersion objectives on a Nikon TE300 fluorescent microscope with a fluorescein filter. Both brightfield and fluorescence images were taken of each vessel in these experiments to confirm the presence or absence of positive immunoreactivity. Preincubation (1 h, 4°C) of antisera with specific blocking peptides (Table 1) for each subunit was used to test for antibody specificity.

Positive antibody controls were performed in 20-μm-thick coronal slices taken from the hippocampal region. Slices were mounted onto gelatin-coated slides and stored at −80°C until use. After being brought to room temperature, slices were fixed with ice-cold 2% paraformaldehyde for 10 min. They were then washed twice for 10 min with SSC buffer (30 mM Na₃C₆H₅O₇, 300 mM NaCl, pH 7.0), with ice-cold 50–50 methanol-acetone for 7 min, and three times for 10 min with SSC + 0.05% Tween 20. Endogenous peroxidase activity was quenched with freshly prepared 1% H₂O₂ for 20 min before blocking with 1% BSA in PBS for 40 min. Slices were incubated overnight at 4°C in nAChR antisera diluted 1:10 in 1% BSA in SSC, rinsed three times for 10 min with SSC + 0.05% Tween 20, incubated 1 h in Alexa Fluor 488-conjugated anti-rabbit IgG (2 μg/ml in 1% BSA in SSC), and rinsed twice for 10 min with PBS before coverslip mounting.

**Co-administration of nicotine with mecamylamine and hexamethonium.** Female Sprague-Dawley rats (240–300 g) were implanted with osmotic pumps (Alzet 2ML2; Durect, Cupertino, CA) for continuous subcutaneous delivery of nicotine (4.5 mg·kg⁻¹·day⁻¹), nicotine and mecamylamine (4.5 mg·kg⁻¹·day⁻¹), nicotine and hexamethonium (3 mg·kg⁻¹·day⁻¹), or 0.9% saline for 7 days as previously described (26). Blood pressure (BP) and heart rate (HR) were measured with a noninvasive tail-cuff monitor (Columbus Instruments NIBP-8) before implantation and on day 7 of treatment. Animals were habituated to handling and the measurement apparatus during at least three separate sessions the week before implantation. Animals were placed in a holding tube for at least 5 min but no more than 15 min before the beginning of measurement. All measurements were performed between 1200 and 1500 to minimize the effect of any circadian variation in BP or HR. Measurements from the final preimplant session were used as baseline.

**In situ brain perfusion.** Animals were anesthetized as above and heparinized by intraperitoneal injection (10,000 U/kg). A ventral midline incision was made at the neck, and the common carotid arteries were exposed. The arteries were cannulated with silicone tubing, and the jugular veins were cut. The perfusion medium consisted of a mammalian Ringer solution [in mM: 117 NaCl, 4.7 KCl, 0.8 MgSO₄, 24.8 NaHCO₃, 1.2 KH₂PO₄, 2.5 CaCl₂, and 10 glucose, with 39 g/l dextran (mol wt 70,000) and 10 g/l BSA, pH 7.4] with Evans blue that was oxygenated with 95% O₂-5% CO₂. The perfusate was passed via peristaltic pump through a heating coil (37°C) and a bubble trap. Once the desired perfusion pressure and rate were achieved (~100 mmHg and 3.1 ml/min), [¹⁴C]sucrose (10 μCi/ml Ringer) was infused with a slow-speed syringe pump (0.5 ml·min⁻¹·hemisphere⁻¹; model 22, Harvard Apparatus, South Natick, MA). The animal was perfused for 5, 10, or 20 min (n = 4–6 per time point per treatment), after which the animal was decapitated and the brain was removed. Samples of the radioactive perfusate were collected from each carotid cannula as a reference. The choroid plexuses were excised, the meninges were removed, and the cerebral hemispheres were sectioned and homogenized. Brain tissue and 100-μl samples of perfusate were prepared for liquid scintillation counting by incubation in 1 ml of tissue solubilizer (TS-2, Research Products) were added, and the samples were counted by incubation in 1 ml of tissue solubilizer (TS-2, Research Products) were added, and the samples were counted by liquid scintillation spectrometry (model LS 5000 TD Counter; Beckman Instruments, Fullerton, CA). Results were reported as the ratio (Rₛₑ) of radioactivity in the brain (Cᵢ) to that in the perfusate (Cₓ):
For statistical comparison of \( K_{in} \) and \( V_{D2} \) between two treatment groups, the pooled estimate of variation around the regression lines \( (s_{in \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot \cdot 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Fig. 1. Expression of nicotinic acetylcholine receptor (nAChR) subunits in rat brain microvessels. Positive immunoreactivity was observed for nAChR subunits α3, α5, α7, and β2. In all cases, preincubation of the primary antibody with the corresponding blocking peptide attenuated or abolished immunoreactivity. No immunoreactivity was observed in cerebral microvessels for nAChR subunits α4, β3, or β4. Second and fourth columns are brightfield images of the fields presented in the first and third columns, respectively. All images are ×100 in oil immersion. Ab, antibody; BP, blocking peptide; ft, fluorescent; bf, brightfield. Scale bar = 1 μm.
as antihypertensive agents because of their ability to block ganglionic cholinergic transmission, no effect on BP or HR was observed in the present study when the antagonists were coadministered with nicotine.

Nicotine significantly (P < 0.01) increased the uptake of [14C]sucrose into the brain (Fig. 4), as previously found (26). This effect was significantly (P < 0.05) attenuated by mecamylamine (Fig. 4), indicating that the functional effect of nicotine on the BBB is nAChR mediated. This is an important observation, as nicotine has been shown to interact directly with endothelial nitric oxide synthase (55), which could also lead to changes in BBB permeability (43, 46).

The effect of nicotine on BBB permeability was also significantly (P < 0.05) attenuated by hexamethonium (Fig. 4), further supporting the hypothesis that the effect is nAChR mediated. Furthermore, attenuation of this effect by hexamethonium strongly suggests that central nAChRs are not involved in the effects of nicotine on the BBB. Hexamethonium is a non-lipid-soluble quaternary ammonium compound that exists in a dicationic form at physiological pH. As a result, the entry of hexamethonium into the brain from the periphery is severely limited by the BBB and is highly dose dependent. Autoradiographic studies using a single intravenous bolus of [3H]hexamethonium at 2.18 mg/kg found that hexamethonium accumulated in the meninges and choroid plexus but did not penetrate into the parenchyma (24). A subsequent study found that a very small but significant fraction of [3H]hexamethonium entered the brain after intravenous injection, but only at much higher (>10 mg/kg) doses (4). Furthermore, 95–100% of a single hexamethonium dose is excreted in the urine within 18 h of injection (39). In the present study, hexamethonium was delivered via subcutaneous infusion at a constant rate of 3 mg·kg⁻¹·day⁻¹, or 0.125 mg·kg⁻¹·h⁻¹. It is therefore highly unlikely that the plasma concentration of hexamethonium would ever become sufficiently high for a pharmacologically significant amount to accumulate in the brain.

Fig. 2. Expression of nAChR subunits in hippocampal slices. Positive antibody controls for nAChR subunits α4 (A), β3 (B), and β4 (C) were performed in 20-μm coronal slices. Immunoreactivity for all subunits was observed in the hippocampus. Slices stained without primary antisera or with antisera preincubated with blocking peptides did not stain (data not shown). Magnification ×20.

Fig. 3. Multiple time uptake in situ brain perfusion. Animals were treated with 0.9% saline (sal), nicotine (4.5 mg·kg⁻¹·day⁻¹; nic), nicotine and mecamylamine (4.5 mg·kg⁻¹·day⁻¹; nic + mec), or nicotine and hexamethonium (3 mg·kg⁻¹·day⁻¹; nic + hex) via subcutaneous infusion for 7 days. Rats were perfused with [14C]sucrose for 5, 10, or 20 min. Data are mean ± SE brain-to-perfusate radioactivity ratio (Rbr) values; n = 4–6 per time point.
It is possible that as nicotine increases BBB permeability to sucrose, passage of hexamethonium into the brain may be increased as well. However, hexamethonium injected directly into the cerebral ventricles is rapidly taken up from the cerebrospinal fluid by the choroid plexus and effluxed into the blood (52), meaning that any hexamethonium gaining access to the brain would not be expected to accumulate. Malin and colleagues (44) found that an acute withdrawal syndrome could be precipitated in animals chronically treated with nicotine by peripheral administration of mecamylamine. In a follow-up study, it was found that hexamethonium administered peripherally did not precipitate a withdrawal syndrome, but hexamethonium given intracerebroventriculantly at a very low dose (≈18 ng) did (45). In both of these studies, nicotine was administered by the same route used in our experiments (subcutaneous osmotic pump) for the same length of time (7 days), but at double the dose (9 mg·kg⁻¹·day⁻¹) (44, 45). The pharmacologically effective concentration of hexamethonium in the brain when it was administered centrally was estimated by the authors to be 195,000 times smaller than the concentration in the periphery after peripheral administration. It is therefore unlikely that nicotine increased brain entry of hexamethonium to any relevant extent in the present study.

These data support the hypothesis that nicotine increases the permeability of the BBB by acting on nAChRs outside the central nervous system. It is possible that this could be a ganglionic effect of nicotine; however, this is also unlikely, as hexamethonium blocks the BBB effect well below the ganglionic blocking intravenous dose of ≈20 mg/kg in rats (51), and no changes in blood pressure were observed after treatment with hexamethonium or mecamylamine in this study. Thus the most likely mediators of the effect of nicotine on the BBB are nAChRs expressed on brain microvessels. Although the imaging techniques used in this study are inadequate to distinguish between luminal and abluminal expression of these proteins, on the basis of the pharmacological data described above, some—if not all—of these receptors are expressed on the luminal endothelial cell membrane.

This begs the question of what the physiological role of endothelial nAChRs might be in the brain. ACh has long been known to act on endothelial cells via muscarinic receptors to induce endothelium-dependent vasodilatation (20). Choline acetyltransferase, the enzyme responsible for ACh synthesis, is expressed in brain microvessels (49), and cerebral endothelial cells have been shown to synthesize ACh (3, 23). Coupled with the ubiquity of cholinesterases in the blood (41) that rapidly degrade any circulating ACh, it is possible that the endothelium itself may release ACh that acts as an autocrine factor (13) via nicotinic (as well as muscarinic) mechanisms.

It is not immediately obvious what this pathway may have to do with regulation of cerebral microvascular permeability. However, endothelial nAChRs in the periphery have been implicated in the regulation of angiogenesis (27, 28). VEGF, a potent angiogenic factor, also increases the permeability of the BBB (17). Furthermore, src-suppressed C-kinase substrate blocks cerebral angiogenesis in vivo and in vitro via suppressed expression of VEGF, increases tight junction proteins in endothelial cells, and decreases sucrose permeability (37). nAChR-mediated increases in microvascular permeability therefore might reflect an involvement of these receptors in some aspects of cerebral angiogenesis, in which nicotine acts as an agonist of an angiogenic pathway (28). Alternatively, nicotine-induced downregulation of endothelial nicotinic receptors, which has been observed in vitro (2), might impair normal cholinergic signaling necessary for the maintenance of barrier integrity (15). Another possibility is that nAChRs may be involved in regulating vesicle formation and transcytosis, the upregulation of which can be involved in BBB disruption (12).

This study demonstrates that nAChRs are expressed in the cerebral microvasculature, that increased BBB permeability after nicotine treatment is a nAChR-mediated phenomenon, and that peripheral antagonism of nAChRs is sufficient to block the effect of nicotine on the BBB. Together, these data strongly suggest that nicotine increases the permeability of the BBB via interaction with endothelial nAChRs expressed at the lumen of the cerebral microvasculature. This implies a novel physiological role for endothelial nAChRs in the modulation of cerebral microvascular permeability. These findings present a potential mechanism by which chronic nicotine use could lead to a compromised neurovascular unit, and thus increase the risk and/or severity of certain neurological diseases. Another implication of these findings is that endothelial nAChRs may be potential targets for therapeutic modulation of the neurovascular unit. Nicotine or other nicotinic agonists might be useful for facilitating the entry of low-molecular-weight drugs into the brain. Alternatively, nicotinic antagonists may be able to attenuate pathological increases in BBB permeability following an acute ischemic attack or traumatic brain injury. This work represents a novel contribution to the understanding of how the neurovascular unit might be regulated and possibly manipulated under both physiological and pathophysiological conditions.
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GRANTS

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