A Cholinergic Agonist Attenuates Endotoxin-Induced Uveitis in Rats

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PURPOSE. Investigation of physiological anti-inflammatory mechanisms can contribute to the treatment of inflammatory disorders. The purpose of the present study was to investigate the effect of nicotine, a selective cholinergic agonist, on endotoxin-induced uveitis (EIU) in rats and the underlying molecular mechanism.

METHODS. Lipopolysaccharide (LPS; endotoxin) and nicotine were injected intraperitoneally. Clinical scores were evaluated by slit lamp. Intracameral protein content and the number of cells were determined. Immunohistochemical reactivity of α7 nicotinic acetylcholine receptor (α7nAChR) was examined in the iris and ciliary body (ICB). mRNA and protein levels of cytokines and chemokines were measured by real-time PCR and enzyme-linked immunosorbent assay.

RESULTS. After LPS injection, clinical scores, as well as protein content and number of cells in the aqueous humor increased during 18 to 36 hours. Nicotine inhibited the endotoxin-induced elevation of these levels. mRNA and protein of α7nAChR expression levels were significantly increased by LPS and/or nicotine injection. Nicotine showed no effects on endotoxin-induced elevation of mRNA levels in ICB. However, nicotine decreased the endotoxin-induced elevation of interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)-α, cytokine-induced neutrophil chemoattractant (CINC)-1, and monocyte chemotactic protein (MCP)-1, but did not affect IL-10 in the serum and aqueous humor.

CONCLUSIONS. Nicotine attenuated endotoxin-induced uveitis through directly decreasing the levels of multiple cytokines and chemokines in the aqueous humor, but did not affect the mRNA levels of these factors. The findings suggest that the nicotinic anti-inflammatory pathway may be involved in the pathogenesis of EIU. (Invest Ophthalmol Vis Sci. 2007;48: 2719 – 2725) DOI:10.1167/iovs.06-0644

Uveitis frequently leads to severe vision loss and blindness, with disruption of the blood–ocular barrier and neighboring tissues associated with intraocular proliferation. Uveitis is commonly idiopathic, and treatment is difficult. Lipopolysaccharide (LPS; endotoxin)-induced uveitis (EIU) in rats is an animal model of human disease.1,2 Tumor necrosis factor (TNF)-α and interleukin (IL)-6 have been reported to be involved in the pathogenesis of LPS-induced uveitis.3-5 TNF-α, IL-6, and IL-1β, which are proinflammatory cytokines, induce uveitis when injected intraocularly in animals.6 IL-8 (CXCL8) and monocyte chemotactic protein (MCP)-1 (CCL2) are members of chemotactic families (chemokines)7 and are present in the plasma of patients with active Behçet’s disease8 and in aqueous humor of patients with active uveitis.9 Rat cytokine-induced neutrophil chemoattractant (CINC)-1 is a counterpart of the human growth-regulated oncogene product and a member of the interleukin-8 family. CINC-1 plays a key role as a mediator of neutrophil infiltration in rats during inflammation.10,11 In various cell types, CINC-1 is produced in response to inflammatory mediators such as TNF-α, IL-1β, and LPS.12-13 Physiological anti-inflammatory mechanisms provide a major advantage to the design of novel pharmacologic strategies against inflammatory diseases. The central nervous system is a pivotal regulator of the immune response and controls inflammation at various levels. Recent studies indicate that stimulation of the vagus nerve can control systemic inflammation in rodents.14-19 Accordingly, surgical vagotomy increases the susceptibility of rodents to septic shock,19 suggesting that the vagus nerve can function as a physiological anti-inflammatory system that modulates the immune response. Acetylcholine, the principal neurotransmitter of the vagus nerve, and selective nicotinic agonists can regulate inflammation through a nicotinic anti-inflammatory pathway dependent on the α7 nicotinic acetylcholine receptor (α7nAChR).20 Van Dijk et al.21 reported that nicotine inhibits TNF-α and IL-1β synthesis in mouse colonic mucosa. Borovikova et al.22 reported that vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Wang et al.23 reported that α7nAChR is an essential regulator of inflammation. We therefore investigated the in vivo effect of nicotine, a selective cholinergic agonist, on EIU in rats.

MATERIALS AND METHODS

Animals

Six- to eight-week-old Wistar rats (160-180 g) were obtained from Sankyo Labo Service Co. Inc. (Tokyo, Japan). The animals were housed in 12-hour dark and 12-hour light conditions, and were given food and water ad libitum during the experiment. All studies were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved by the Institutional Committee for Ethical Animal Care and Treatment.

Injection of LPS and Nicotine

To induce uveitis, 150 µg LPS (Escherichia coli, serotype 955; B5, from Sigma-Aldrich, Inc., St. Louis, MO), in 150 µL of pyrogen-free 0.9% sodium chloride, was injected intraperitoneally. Rats treated with 0.9% sodium chloride served as the control. Nicotine (Sigma-Aldrich, Inc.) was dissolved in 0.9% sodium chloride just before use, and 0.2 to 2 mg/kg of nicotine was injected intraperitoneally at the same time as the LPS was administered. No repeated experiments of LPS injection in the same rats were performed. Multiple samples were used in a single experiment. The experiments were repeated at least twice to ensure reproducibility.
Clinical Evaluation of EIU

Clinical scoring of EIU was performed as described by Behar-Cohen et al.22 The eyes were examined by slit-lamp after LPS injection and nicotine treatment. The severity of EIU was graded from 0 to 4, by a masked investigator, as follows: 0, no inflammatory reaction; 1, discrete dilation of the iris and conjunctival vessels; 2, moderate dilation of the iris and conjunctival vessels with moderate flare in the anterior chamber; 3, intense iridal hyperemia with flare in the anterior chamber; and 4, same clinical signs as 3 plus the presence of fibrinous exudates in the pupillary area and miosis.

Measurement of Intracameral Protein and Number of Cells

Aqueous humor was sampled at 0, 6, 12, 18, 24, 30, and 36 hours after injection of LPS. Nicotine was injected at the same time as LPS administration. Mean ± SD (n = 4 pairs of eyes). *P < 0.05; **P < 0.01 compared with LPS injection.

Histopathology

Twenty-four hours after LPS administration, rats were euthanatized. The eyes were enucleated immediately and stored in a mixture of 10% formalin and 2.5% glutaraldehyde for 24 hours. Subsequently, the eyes were embedded in OCT compound. Sagittal sections (6-μm thick) were cut near the optic nerve head and stained with hematoxylin and eosin.

RNA Extraction and Real-Time Polymerase Chain Reaction

After intraperitoneal injection of pentobarbital (50 mg/kg body weight), the eye globe was enucleated and submerged in RNA stabilization reagent (RNeasy Protect Mini kit, treated with an RNase-free Dnase Set; Qiagen) to remove any residual genomic DNA. cDNA from each sample was obtained by reverse transcription with random hexamers and multiscribe reverse transcriptase (MultiScribe; Applied Biosystems [ABI], Foster City, CA). Based on the database, real-time PCR primers and probes were designed. Probes and primers are as follows:

For 7nAChR (GenBank accession no. L31619): forward primer 5'-AGCTGAGGTGAGTGGTGG-3', reverse primer 5'-CAGGCTCCTGTTGTGGG-3', probe 5'-AGGCTGAGGTGAGTGGTGAGTGCAGGTGCTGG-3'.

FIGURE 1. Effects of nicotine on LPS-induced uveitis. Clinical scores (A) and protein content in the aqueous humor (B) after injection of nicotine and LPS at each time point are shown. Clinical score evaluation and aqueous sampling were performed at 0, 6, 12, 18, 24, 30, and 36 hours after injection of LPS. Nicotine was injected at the same time as LPS administration. Mean ± SD (n = 4 pairs of eyes). *P < 0.05; **P < 0.01 compared with LPS injection.

FIGURE 2. Effects of different doses of nicotine on LPS-induced uveitis. Clinical scores (A), protein content (B), and number of cells (C) in the aqueous humor after injection of nicotine and LPS are shown. Clinical score evaluation and aqueous sampling were performed at 24 hours after injection of LPS. Nicotine was injected at the same time as LPS was administered. Means ± SD (n = 6 pairs of eyes). *P < 0.05; **P < 0.01 compared with LPS injection.
**Anterior chamber.** Severe inflammatory cell infiltration was observed in LPS-injected rats. A significant attenuation of inflammation was observed in rats treated with nicotine (2 mg/kg) and LPS simultaneously. Results are representative of those in four pairs of eyes. Hematoxylin and eosin; original magnification, ×200.

GAAGCCAA-3′, and TaqMan (ABI) probe 5′-(FAM)CCACCGAATG-GCAACCTGCT(TAMRA)-3′; for TNF-α (accession no. NM_012675); forward primer 5′-ACAGGCCATGCGCCACTAC-3′, reverse primer 5′-TCTCCTGGTATGAAATGGCACC-3′, and TaqMan probe 5′-(FAM)TGCTCTCTACCCACCGGATCGC(TAMRA)-3′; for IL-6 (accession no. NM_012509), forward primer 5′-TCAACTCCATCTGCTCTCTCAG-3′, reverse primer 5′-AAAGCAACTGCTGGAAGAATC-3′, and TaqMan probe 5′-(FAM)AAACCTGCTGGAAGATCTCT-3′, for IL-1β (accession no. NM_051512), forward primer 5′-AAACGCAATGGTGCGGACATA-3′, reverse primer 5′-CATTAGGATAGTGGAGGACTCTTG-3′, and TaqMan probe 5′-(FAM)TTCGACTTGACACCCGCTTGCTCCT(TAMRA)-3′; for TNF-α (accession no. NM_031512), forward primer 5′-ACAGTTGCGTGAACAGCAACTGTGAACAA-3′, reverse primer 5′-CATGCTATTGCAGACAACATG-3′, and TaqMan probe 5′-(FAM)TTCGACTTGACACCCGCTTGCTCCT(TAMRA)-3′, and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, accession no. AF100860); forward primer 5′-CCGGAGGCCCCTCTGAAAGG-3′, reverse primer 5′-GCTGTTGAAGTCCACAGGAGACAA-3′, and TaqMan probe 5′-(FAM)CATCTCCTGGGCTACTGAGGACCA (TAMRA)-3′.

cDNA was used to detect real-time PCR products (TaqMan Universal Master Mix and PRISM 7700 sequence detection system; ABI) with specific primers and probes. The thermal profile for each primer consisted of 2 minutes at 50°C and 10 minutes at 95°C followed by 40 cycles for 15 seconds at 95°C and 1 minute at 60°C. The expression levels of α7nAChR, IL-6, IL-1β, TNF-α, and MCP-1 mRNAs were normalized by the mRNA level of GAPDH in each sample, and the relative changes in expression were shown as an n-fold increase relative to value of rats treated with 0.9% NaCl.

**Immunohistochemistry**

At 0, 12, 18, 24, 36, and 48 hours after LPS injection, the rat was anesthetized. The blood was washed out with phosphate-buffered saline (PBS), and the animals were perfusion-fixed with 4% cold paraformaldehyde in PBS. The eyes were enucleated, cut in half, and fixed with 4% paraformaldehyde in PBS for 20 minutes at 4°C.

Specimens were OCT embedded, frozen, and cryosectioned at a thickness of 10 μm. After they were blocked with 10% normal bovine serum in PBS, the slides were incubated with rabbit anti-rat α7nAChR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour and immunolabeled with FITC-conjugated bovine anti-rabbit IgG (Santa Cruz Biotechnology) for 30 minutes. Negative control samples were incubated with normal bovine IgG or primary antibody.

**Enzyme-Linked-Immunosorbent Assay**

After intraperitoneal injection of pentobarbital (50 mg/kg body weight), aqueous humor was aspirated with a 50-gauge needle under microscopic visualization. Aspirated samples were centrifuged at 2500 rpm for 20 minutes at 4°C, to obtain the supernatant. The aqueous humor from both eyes of a rat was diluted up to 30 to 40 μL for the assay. Serum samples were collected and pooled. Protein levels of TNF-α, IL-6, IL-1β, IL-10 (Pierce Biotechnology, Inc. Rockford, IL), CINC-1, and MCP-1 (IBL Co., Ltd., Gunma, Japan) were determined by enzyme-linked immunosorbent assay.

**Statistics**

All data are expressed as the mean ± SD. Statistical analysis was performed using the Scheffe procedure after the ANOVA test for multiple comparison of means. P < 0.05 was considered as statistically significant.

**RESULTS**

**Animal Behavior**

No abnormal behavior, including diarrhea and vomiting, was noted in rats treated with LPS or nicotine. No deaths occurred during the experiment.

**Clinical scores, Protein Content, and Number of Cells in the Aqueous Humor after Injection of Nicotine and LPS**

After LPS injection, clinical scores, protein content, and number of cells in the aqueous humor increased at 6 hours; reached maximum levels (score, 3.8; protein, 12.6 mg/mL; cells, 18.2 × 10^3/mL) at 24 hours; maintained high level until 36 hours; and...
decreased at 48 hours (data not shown). However, injection of nicotine (2 mg/kg) improved clinical scores, decreased protein levels in the aqueous humor from 12 to 36 hours (Fig. 1). Nicotine decreased the elevation of these levels in a dose-dependent manner (Fig. 2). By intraperitoneal injection of 2 mg/kg nicotine, significant inhibition effects (score, 2.2; protein, 7.3 mg/mL; cells, 6.8 × 10^5 /mL) were observed (Fig. 2).

Meanwhile, nicotine treatment alone did not affect the scores, the protein content, or the number of cells in the aqueous humor in the rats untreated with LPS. No inflammation was observed histopathologically in rats treated with nicotine alone (Fig. 3B). However, severe inflammation was found in the anterior and posterior chamber 24 hours after LPS administration (Fig. 3C). Significant reductions of inflammation were observed in eyes of rats treated with nicotine (2 mg/kg) simultaneously with the LPS injection (Fig. 3D).

**Changes in α7nAChR mRNA and Protein Levels in the Iris and Ciliary Body**

α7nAChR mRNA was expressed in the iris and ciliary body of rats untreated with LPS. The level increased to its maximum at 9 hours after LPS injection (Fig. 4A). Therefore, we observed the effect of nicotine on α7nAChR mRNA level at this time point. The result showed that the α7nAChR mRNA level was elevated by injection of nicotine alone or of both LPS and nicotine (Fig. 4B).

Immunofluorescent staining for α7nAChR in the iris and ciliary body was negative after injection of saline (Fig. 5A). After injection of LPS alone, α7nAChR proteins were present (Fig. 5B, after 18 hours; Fig. 5C, after 24 hours). Twenty-four hours after injection of nicotine alone, α7nAChR proteins were observed (Fig. 5D). After injection of both nicotine and LPS, α7nAChR proteins increased in the iris and ciliary body (Fig. 5E, after 18 hours; 5F, after 24 hours). The negative control (normal IgG) showed no autofluorescence or non-specific staining.

**Changes in mRNA Levels of Cytokines and Chemokines in the Iris and Ciliary Body**

After injection of LPS, mRNAs of IL-6, IL-1β, TNF-α, and MCP-1 in the iris and ciliary body increased at 1.5 hours, reached maximum levels at 3 hours, and then gradually decreased (data not shown). Nicotine did not change the elevated levels of IL-6 mRNA (Fig. 6A), IL-1β mRNA (Fig. 6B), TNF-α mRNA (Fig. 6C), and MCP-1 mRNA (Fig. 6D) at 3 hours after injection of LPS.

**Changes in Cytokines and Chemokines in the Serum and Aqueous Humor**

High levels of IL-6, CINC-1, MCP-1, and IL-10 were detected in the serum at 24 hours after LPS administration. Treatment with 2 mg/kg of nicotine significantly reduced the levels of serum IL-6, CINC-1, and MCP-1, but did not suppress release of the anti-inflammatory cytokine IL-10 (Fig. 7). After LPS injection, TNF-α and IL-1β serum levels were below the detection sensitivities of the assays in rats (Fig. 7). All the cytokines and chemokines tested were below detection levels in control rats injected with saline or nicotine alone.

**FIGURE 4.** Changes in α7nAChR mRNA levels in the iris and ciliary body. α7nAChR mRNA levels at various times after LPS (150 μg) injection are shown (A). The mRNA level reached its maximum at 9 hours after injection of LPS. Therefore, the effect of nicotine (2 mg/kg) on the mRNA level of α7nAChR in LPS-injected rat was investigated at 9 hours after LPS injection (B). Mean ± SD (n = 6 pairs of eyes). **P < 0.01 compared with the value in rats treated with saline.

**FIGURE 5.** Immunohistochemistry of α7nAChR in the iris and ciliary body. Rats without LPS injection (A), 18 hours (B) and 24 hours (C) after LPS injection, treated with nicotine alone (D), and 18 hours (E) and 24 hours (F) after injection of 2 mg/kg nicotine together with LPS. Fluorescein indicates localization of α7nAChR in the iris and ciliary body. Results are representative of those in four pairs of eyes. Original magnification, ×200.
After injection of LPS, IL-6, IL-1β, TNF-α, CINC-1, MCP-1, and IL-10 reached maximum levels at 24 hours in the aqueous humor. Nicotine (1–2 mg/kg) decreased the elevated levels of proinflammatory cytokines and chemokines in the aqueous humor 24 hours after LPS administration in a dose-dependent manner, but failed to prevent the constitutive release of the anti-inflammatory cytokine IL-10 (Fig. 8).

**DISCUSSION**

Physiological anti-inflammatory mechanisms represent efficient systems that have been selected by evolution to control inflammation. These mechanisms may provide advantages that can be exploited for the treatment of inflammatory disorders. Previous studies have shown that nicotine, a more selective cholinergic agonist, prevents endotoxin-induced TNF-α production in macrophage cultures and microvascular endothelial cells. It is still unclear whether this pharmacological strategy can be applied in vivo to improve EIU. In the current study, nicotine improved clinical scores and decreased protein content and the number of cells in rats with EIU (Figs. 1, 2, 3).

The LD₅₀ (median lethal dose) of nicotine in mice was reported to be 3 to 9 mg/kg. In the present study, we used intraperitoneal injection of nicotine (0.2–2 mg/kg), and the effective doses (1–2 mg/kg) were similar to those described by Saeed et al., in which nicotine (2 mg/kg) inhibits endothelial cell adhesion molecule expression in mice.

To examine whether the α7nAChR is essential for the cholinergic anti-inflammatory pathway in vivo, Wang et al. measured TNF-α production in α7nAChR-deficient mice. The serum TNF-α level in α7nAChR-deficient mice after administration of endotoxin was significantly higher than that in wild-type endotoxemic mice, indicating a critical function of the α7nAChR in the regulation of systemic inflammatory responses in vivo. α7nAChR is expressed in macrophages and endothelial cells and protein in the iris and ciliary body (Figs. 4, 5) in the present study may exist in inflammatory cells and vascular endothelial cells in the tissues. Nicotine alone or both nicotine and LPS injection elevated the expression of α7nAChR in the iris and ciliary body. Nicotine inhibited LPS-induced elevation of serum IL-6, CINC-1, and MCP-1 (Fig. 7). It reduced the LPS-induced release of proinflammatory cytokines and chemokines, such as IL-6, TNF-α, IL-1β, CINC-1, and MCP-1, but not anti-inflammatory cytokine IL-10 in a dose-dependent way in the aqueous humor (Fig. 8). It is possible that nicotinic anti-inflammatory pathways are involved in the pathogenesis of EIU in rats.

Nicotine inhibited the expression of cytokines and chemokines in aqueous humor, but did not influence the mRNA levels of these cytokines and chemokines (Fig. 6), indicating that activation of the cholinergic receptor transduces intracellular signals that inhibit synthesis of cytokines and chemokines at a posttranscriptional stage, as stated by Tracey and Ulloa. Also, Wang et al. have reported that nicotine may affect macrophage activation by modulating HMGB1 acetylation or phosphorylation as a posttranslational regulation.

The NF-kB pathway is crucial for macrophage activation and the production of proinflammatory cytokines. In an earlier study, we reported that NF-kB translocation is involved in the pathogenesis of LPS-induced uveitis. Wang et al. and Saeed et al. reported that nicotine suppresses the production of proinflammatory cytokines from macrophages and microvascular endothelial cells by inhibiting the NF-kB pathway through an α7nAChR-dependent anti-inflammatory pathway.
Because α7nAChR can control the NF-κB pathway, a pharmacological target for several inflammatory disorders, nicotine and selective nicotinic agonists could provide a therapeutic potential target for the treatment of a variety of inflammatory disorders. The clinical therapeutic use of nicotine has been suggested for the treatment of a large number of human diseases, including depression, Parkinson’s disease, and inflammatory disorders such as Crohn’s disease and ulcerative colitis.29-31

Our results indicate that cholinergic agonists suppress cytokine and chemokine release through a novel nicotinic anti-inflammatory pathway depending on α7nAChR. These results support α7nAChR as a potential pharmacological target for EIU and systemic inflammation, and further investigation is warranted to determine its potentially clinical translation.

References