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Brain acetylcholinesterase activity controls systemic cytokine levels through the cholinergic anti-inflammatory pathway

Valentin A. Pavlov¹, William R. Parrish¹, Mauricio Rosas-Ballina¹, Mahendar Ochani¹, Margot Puerta¹, Kanta Ochani¹, Sangeeta Chavan¹, Yousef Al-Abed², and Kevin J. Tracey¹

¹Laboratory of Biomedical Science, The Feinstein Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030

²Laboratory of Medicinal Chemistry, The Feinstein Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030

Abstract

The excessive release of cytokines by the immune system contributes importantly to the pathogenesis of inflammatory diseases. Recent advances in understanding the biology of cytokine toxicity led to the discovery of the “cholinergic anti-inflammatory pathway,” defined as neural signals transmitted via the vagus nerve that inhibit cytokine release through a mechanism that requires the $\alpha 7$ subunit-containing nicotinic acetylcholine receptor ($\alpha 7$ nAChR). Vagus nerve regulation of peripheral functions is controlled by brain nuclei and neural networks, but despite considerable importance, little is known about the molecular basis for central regulation of the vagus nerve-based cholinergic anti-inflammatory pathway. Here we report that brain acetylcholinesterase activity controls systemic and organ specific TNF production during endotoxemia. Peripheral administration of the acetylcholinesterase inhibitor galantamine significantly reduced serum TNF levels through vagus nerve signaling, and protected against lethality during murine endotoxemia. Administration of a centrally-acting muscarinic receptor antagonist abolished the suppression of TNF by galantamine, indicating that suppressing acetylcholinesterase activity, coupled with central muscarinic receptors, controls peripheral cytokine responses. Administration of galantamine to $\alpha 7$ nAChR knockout mice failed to suppress TNF levels, indicating that the $\alpha 7$ nAChR-mediated cholinergic anti-inflammatory pathway is required for the anti-inflammatory effect of galantamine. These findings show that inhibition of brain acetylcholinesterase suppresses systemic inflammation through a central muscarinic receptor-mediated and vagal- and $\alpha 7$ nAChR-dependent mechanism. Our data also indicate that a clinically used centrally-acting acetylcholinesterase inhibitor can be utilized to suppress abnormal inflammation to therapeutic advantage.

Correspondence to: Valentin A. Pavlov, PhD, Laboratory of Biomedical Science, The Feinstein Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030, Tel: (516) 562-2316, Fax: (516) 562-2356, vpavlov@nshs.edu.

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Keywords

acetylcholinesterase; endotoxemia; galantamine; huperzine A; cytokines; brain muscarinic receptors; vagus nerve; $\alpha 7$ subunit-containing nicotinic acetylcholine receptor; the cholinergic anti-inflammatory pathway; inflammation

1. Introduction

The excessive production of TNF and other pro-inflammatory cytokines from innate immune cells and their release in the bloodstream is critically associated with the pathology of inflammatory disorders. Cytokine production is controlled by neural input via an inflammatory reflex (Tracey, 2002; Tracey, 2007). Efferent vagus nerve activity, which comprises the motor arm of the inflammatory reflex, regulates cytokine production specifically via $\alpha 7$ nAChR-dependent signaling, termed the “cholinergic anti-inflammatory pathway” (Borovikova *et al.*, 2000; Wang *et al.*, 2003; Pavlov and Tracey, 2005; Tracey, 2007). Stimulating the cholinergic anti-inflammatory pathway by electrical or pharmacological methods significantly suppresses the systemic levels of TNF and other pro-inflammatory cytokines during endotoxemia (Borovikova *et al.*, 2000; Pavlov *et al.*, 2003; Gallowitsch-Puerta and Pavlov, 2007). Clinical and experimental studies indicate that the inflammatory reflex is impaired, and vagus nerve outflow depressed, during endotoxemia and cytokine-mediated diseases (Tracey, 2002; Tracey, 2007).

The vagus nerve control of peripheral functions is modulated centrally. However, little is known about the brain regulation of the efferent vagus nerve-based cholinergic anti-inflammatory pathway. We have previously shown that muscarinic receptor ligands, administered i.c.v., suppress serum TNF levels in endotoxemic rats and the efferent vagus nerve may mediate this effect (Pavlov *et al.*, 2006). These findings have indicated that brain cholinergic muscarinic networks may regulate the cholinergic anti-inflammatory pathway. Vagus nerve outflow to peripheral organs is centrally regulated in the brainstem and in “higher” forebrain areas, including the cerebral cortex, hippocampus and hypothalamus that receive cholinergic innervations (Benarroch, 1993; Levey, 1996). Previous studies indicate that brain acetylcholinesterase activity and muscarinic receptors, which regulate cholinergic network signaling, modulate vagus nerve outflow (Gotoh *et al.*, 1989; Pavlov *et al.*, 2006). Accordingly, we reasoned that brain acetylcholinesterase activity may have a role in regulating the vagus nerve-based cholinergic anti-inflammatory pathway. Here we explored the efficacy of inhibiting brain acetylcholinesterase and thus facilitating brain cholinergic transmission in suppressing the systemic cytokine response during endotoxemia.

2. Methods

2.1. Animals

Male BALB/c mice (24–28g, Taconic) were allowed to acclimate for at least two weeks prior to the corresponding experiment. C57BL/6 mice with $\alpha 7$ nAChR deficiency and wild-type littermates were purchased from The Jackson Laboratory (B6.129S7-Chrna^{7tm1Bay}, number 003232) and a breeding program to obtain homozygous knockout mice or wild-type mice was established in the Center for Comparative Physiology at the Feinstein Institute for

Medical Research. Male and female $\alpha 7$ nAChR knockout mice 5–8 months old and age- and sex-matched wild-type controls were utilized in the study. All animals were housed in standard conditions (room temperature 22°C with a 12h light–dark cycle) with access to regular chow and water. All animal experiments were performed in accordance with the National Institutes of Health Guidelines under protocols approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee of the Feinstein Institute for Medical Research, Manhasset, NY.

2.2. Endotoxemia and drug treatment

Endotoxemia in animals was induced by administering LPS (endotoxin, *Escherichia coli* 0111:B4; Sigma, 6 mg/kg, i.p.). Groups of animals were treated with sterile saline (controls), galantamine (galantamine hydrobromide, Calbiochem, 0.1, 1.0 or 4.0 mg/kg, i.p.), or huperzine A (huperzine A $\pm$, Sigma, 0.04 or 0.4 mg/kg, i.p.) at different time points prior to endotoxin administration. Atropine sulfate or atropine methyl nitrate (Sigma, 1 mg/kg or 4 mg/kg, i.p.) was administered 15min prior to galantamine or huperzine A treatment where indicated. Animals were euthanized by CO₂ asphyxiation 1.5h or 3h following endotoxin administration for TNF or IL-6 determination respectively, and blood was collected via cardiac puncture. In another set of survival experiments, groups of mice were treated with galantamine (1.0 or 4.0 mg/kg, i.p.) or huperzine A (0.04 or 0.4mg/kg, i.p.) 1h prior to endotoxin (6 mg/kg, i.p.) administration. Where indicated, mice were pretreated with atropine sulfate or atropine methyl nitrate (4 mg/kg, i.p.) 15 min prior to galantamine or huperzine A treatments. Survival was routinely monitored for two weeks.

2.3. Vagotomy

Mice were anesthetized by isoflurane inhalation and the right cervical vagus nerve was exposed, ligated with a 4-0 silk suture, and divided. In sham-operated animals, the cervical vagus nerve was visualized, but was neither isolated from the surrounding tissues nor transected. All animals were permitted to recover for 7 days following the surgical procedure and before their inclusion in endotoxemia experiments.

2.4. Cytokine analysis

To obtain serum samples, blood was collected via cardiac puncture immediately following euthanasia, allowed to clot for 1h and then centrifuged at 5,000 rpm (1,500 $\times$ g) for 15min. Supernatants were subsequently collected for TNF or IL-6 determination via an ELISA technique (R&D Systems) according to the manufacturer's recommendations. For splenic TNF determination spleens were rapidly excised, rinsed of blood, and homogenized by polytron (Brinkman) in homogenization buffer (PBS, containing a protease inhibitor cocktail, pH 7.2). Homogenates were centrifuged at 1,000g for 10 min; the supernatants were centrifuged at 12,000g for 10 min, and the resulting supernatants were used for TNF analysis by ELISA (R&D Systems). Protein concentrations were determined by the method of Bradford.

2. 5. Corticosterone determination

Groups of mice were treated with saline or galantamine (1 mg/kg or 4 mg/kg, i.p.) 1h prior to endotoxin (6 mg/kg, i.p.) administration. Mice were euthanized 1.5h following endotoxin administration and blood was withdrawn via cardiac puncture. Serum corticosterone levels were determined using a corticosterone immunoassay kit (R&D Systems Inc, Minneapolis, MN) according to the manufacturer's recommendations.

2. 6. Statistical analysis

Values are presented as mean $\pm$ SEM. ANOVA, for multiple comparisons, and a two-tailed two-sample equal variance Student's *t*-test were performed to determine statistical significance. The statistical significance of differences between groups of animals in survival experiments was analyzed by Log-rank test. P values equal to or below 0.05 were considered significant.

Results

Galantamine is a competitive and reversible acetylcholinesterase inhibitor that crosses the blood brain barrier, significantly increases brain cholinergic network activity(Reichman, 2003; Ellis, 2005), and activates vagus nerve outflow(Waldburger *et al.*, 2008). Galantamine dose-dependently lowered serum TNF levels in endotoxemic mice (Fig. 1a). and the highest suppression was achieved with a drug dose (4 mg/kg, i.p.) that was previously shown to inhibit mouse brain acetylcholinesterase activity by 43%.(Bickel *et al.*, 1991). Surgical transection of the right cervical vagus nerve, which innervates the celiac ganglia through abdominal posterior branches(Berthoud and Powley, 1993) and predominantly controls TNF release from spleen(Huston *et al.*, 2006), reduced galantamine-induced suppression of TNF (Fig. 1b). Galantamine administration in doses that significantly inhibited serum TNF levels (Fig. 1b) failed to significantly alter serum corticosterone levels, which were elevated during endotoxemia (Fig. 1c). This observation indicated that the suppressive effect of galantamine on serum TNF could not be attributed to elevated anti-inflammatory corticosteroids. In addition to reducing serum TNF levels, administration of galantamine conferred significant protection against endotoxin lethality (Fig. 1d). We next studied whether central muscarinic acetylcholine receptors, which mediate the activity of brain cholinergic pathways, are required for the anti-inflammatory effect of acetylcholinesterase inhibition. Pre-treatment with atropine sulfate, a muscarinic receptor antagonist that crosses the blood brain barrier, reversed the effect of galantamine on serum TNF levels (Fig. 1e). Pre-treatment with atropine methyl nitrate, a muscarinic receptor antagonist that does not penetrate the blood brain barrier, failed to alter galantamine suppression of TNF levels (Fig. 1e). Atropine sulfate, but not atropine methyl nitrate, eliminated the survival improvement conferred by galantamine (Fig. 1f). Pre-treatment with atropine sulfate or atropine methyl nitrate alone did not significantly alter serum TNF and survival during endotoxemia (data not shown). Together these data indicate that galantamine suppresses TNF production and endotoxin lethality through a centrally controlled, muscarinic receptor-, vagus nerve-dependent mechanism.

The molecular mechanism of the cholinergic anti-inflammatory pathway requires the $\alpha 7$ nAChR (Wang *et al.*, 2003). To determine whether the $\alpha 7$ nAChR is required for galantamine suppression of TNF, we next measured TNF levels in wild type mice and $\alpha 7$ nAChR knockout mice treated with galantamine and subjected to endotoxemia. Galantamine reduced serum TNF levels in wild type mice, but failed to significantly alter cytokine levels in $\alpha 7$ nAChR knockout mice (Fig. 2a), indicating that the $\alpha 7$ nAChR was required for galantamine suppression of serum TNF levels. We had previously shown that the spleen is the principal organ that integrates the cholinergic anti-inflammatory pathway control of TNF release during endotoxemia (Huston *et al.*, 2006). Galatamine administration significantly inhibited TNF in the spleen of wild type mice, but not in the $\alpha 7$ nAChR knockout mice (Fig. 2b). These results indicate that the suppression of TNF production by galantamine requires cholinergic anti-inflammatory pathway signaling to spleen via the $\alpha 7$ nAChR.

To determine the specificity of acetylcholinesterase inhibition in preventing cytokine release, we utilized huperzine A, a structurally distinct, highly selective, centrally-acting acetylcholinesterase inhibitor (Zangara, 2003). Huperzine A (0.4 mg/kg, i.p.) administration significantly reduced serum TNF levels (Fig. 3a) and improved survival (Fig. 3b) during endotoxemia. Administration of atropine sulfate significantly decreased the survival-improving efficacy of huperzine A (Fig. 3c), whereas administration of atropine methyl nitrate failed to significantly alter huperzine A-induced survival benefit (Fig. 3c). Huperzine A or galantamine also reduced serum IL-6 levels (Fig. 3d), as expected from activation of the cholinergic anti-inflammatory pathway.

Discussion

Here we show that galantamine, a clinically used acetylcholinesterase inhibitor suppresses systemic cytokine levels during endotoxemia. The cholinergic anti-inflammatory pathway, through $\alpha 7$ nAChR-mediated signaling, conveys the primary central, muscarinic receptor-dependent galantamine effect to inhibition of systemic TNF release. These findings have major implications for understanding how the brain regulates the innate immune response during infection or injury, and for the possible clinical use of clinically approved centrally-acting cholinergic drugs as anti-inflammatory agents to suppress peripheral inflammation. Galantamine doses in the range reported here significantly inhibit brain acetylcholinesterase activity in levels that can be achieved in patients (Barnes *et al.*, 2000; Geerts, 2005). The brain cholinergic system modulates attention, learning, memory, REM sleep, the control of movements, and other peripheral functions (Gotoh *et al.*, 1989; Steriade, 1992; Sarter and Bruno, 1997; Perry *et al.*, 1999; Li *et al.*, 2003). Now it appears that this neuromodulatory system regulates the peripheral cytokine response to lethal endotoxemia. through brain mechanisms that may also involve other neurotransmitter systems. Our data indicate that brain cholinergic muscarinic networks communicate with the cholinergic anti-inflammatory pathway to suppress peripheral inflammation. These findings do not exclude contribution by other immunoregulatory mechanisms, including catecholaminergic pathways (Pavlov and Tracey, 2004; Paton *et al.*, 2005; Flierl *et al.*, 2007). Decreased activity in central cholinergic networks, a decline of vagus nerve outflow, and increases in serum cytokine levels are associated with inflammatory disease and aging (Bartus *et al.*, 1982; Luine and

Hearns, 1990; Muir, 1997; Csiszar *et al.*, 2003; Krabbe *et al.*, 2004; De Meersman and Stein, 2007; Marsland *et al.*, 2007). It is interesting to consider whether dysfunction in central cholinergic signaling underlies the risk of developing cytokine-mediated tissue injury. Galantamine is widely used in the treatment of cholinergic insufficiency and memory loss during Alzheimer's disease, but the potential use of this agent for suppressing peripheral inflammation had not been considered previously. It is now plausible that pharmacologically targeting brain acetylcholinesterase to increase activity of the cholinergic anti-inflammatory pathway will suppress inflammation in diseases mediated by cytokines (e.g. rheumatoid arthritis, septic shock, and inflammatory bowel disease).

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Figure 1. Galantamine exerts anti-inflammatory activity through vagus nerve- and central muscarinic receptor-dependent mechanisms

(a) Galantamine administered 1h prior to endotoxin (6 mg/kg, i.p.) dose-dependently suppressed serum TNF in endotoxemic mice (n=8 to 9 animals per group, * $P<0.002$, ** $P<0.0001$ as compared with saline (S)-treated controls). (b) Galantamine (Gal, 4 mg/kg, i.p.) administered 1h prior to endotoxin (6 mg/kg, i.p.) in mice with intact vagus nerves (Sham) suppressed serum TNF levels and right cervical vagotomy (Vg) reduced the magnitude of this effect (n=7–10 per group, * $P<0.03$, ** $P<0.003$, $P<0.0003$ ***). (c) Galantamine (1 mg/kg (Gal1), 4 mg/kg (Gal4), i.p.) administered 1h prior to endotoxin (LPS, 6 mg/kg, i.p.) does not alter serum corticosteroid levels, as compared to endotoxin alone (n=5 animals per group, * $P<0.002$, ** $P<0.0001$, *** $P<0.00005$ as compared with saline (no LPS)-administered animals). (d) Galantamine administered 1h prior to endotoxin (6 mg/kg, i.p.) improved survival i endotoxemia (n=20 animals per group, * $P<0.0001$ as compared with saline-administered controls). (e) Pre-treatment with 4 mg/kg atropine sulfate (AS), but not 4 mg/kg atropine methyl nitrate (AMN) abolished the

inhibition of serum TNF by 4 mg/kg galantamine (Gal) in endotoxemic mice, (n=7 to 11 per group, $*P<0.0001$, $**P<0.0009$) as compared with (Sal)-administered mice). (f) Pretreatment with atropine sulfate (AS, 4 mg/kg, i.p.), but not atropine methyl nitrate (AMN, 4 mg/kg, i.p.), abrogated the survival-improving effects of galantamine (4 mg/kg, i.p.) in endotoxemic mice (n=19 to 20 per group, $*P<0.0005$ as compared with AS-pretreated mice).

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Figure 2. The anti-inflammatory function of galantamine is dependent on the $\alpha 7$ nAChR
(a) Galantamine (4 mg/kg, i.p.), injected 1h prior to endotoxin (6 mg/kg, i.p.) suppressed significantly serum TNF levels in wild type mice, but did not alter serum TNF levels in $\alpha 7$ nAChR knockout mice (n=7 to 8 per group, * $P < 0.002$ as compared with saline (Sal)-treated controls). **(b)** Galantamine (4 mg/kg, i.p.), administered 1h prior to endotoxin (6 mg/kg, i.p.) inhibited significantly splenic TNF levels in in wild type mice, but failed to suppress splenic TNF levels in $\alpha 7$ nAChR knockout mice (n=7 to 8 per group, * $P < 0.002$ as compared with saline (Sal)-treated controls).

Figure 3. Huperzine A suppresses cytokine responses and improves survival through a central muscarinic receptor-dependent mechanism

(a) Huperzine A administered i.p. 1h prior to endotoxin (6 mg/kg, i.p.) reduced serum TNF levels in endotoxemic mice (n=5 per group, $*P<0.0001$ as compared with saline (0)-treated controls). (b) Huperzine A administered 1h prior to endotoxin (6 mg/kg, i.p.) in mice improved survival in endotoxemia (n=20 per group, $*P<0.0001$ as compared with saline-treated controls). (c) Pre-treatment with atropine sulfate (AS, 4 mg/kg, i.p.), but not atropine methyl nitrate (AMN, 4 mg/kg, i.p.) abrogated the survival-improving effects of huperzine A (0.4 mg/kg, i.p.) (n=20 per group, $*P<0.0001$ compared with AS). (d) Huperzine A (HupA, 0.4 mg/kg, i.p.) or galantamine (Gal, 4 mg/kg, i.p.) administered i.p. 1h prior to endotoxin (6 mg/kg, i.p.) reduced serum IL-6 levels analyzed in blood obtained 3h after endotoxin administration (n=5 per group, $**P<0.0005$, $*P<0.0001$ as compared with saline (Sal)-administered controls).