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Full-length Article

Vesicular acetylcholine transporter knock down-mice are more susceptible to inflammation, c-*Fos* expression and sickness behavior induced by lipopolysaccharide

Hércules Ribeiro Leite [a,b,*], Onésia Cristina de Oliveira-Lima [b], Luciana de Melo Pereira [b], Vinícius Elias de Moura Oliveira [b], Vania Ferreira Prado [c], Marco Antônio Máximo Prado [c], Grace Schenatto Pereira [b], André Ricardo Massensini [b,*]

[a] *Laboratório de Inflamação e Metabolismo (LIM), Programa de Pós-graduação em Ciências Fisiológicas, Centro Integrado de Pesquisa e Pós-Graduação em Saúde – CIPq-Saúde, Universidade Federal dos Vales do Jequitinhonha e Mucuri (UFVJM), Campus JK, Alto da Jacuba, Minas Gerais 39100 000, Brazil*

[b] *Núcleo de Neurociências (NNC), Departamento de Fisiologia e Biofísica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (UFMG), Avenida Antônio Carlos, Pampulha, n° 6627, Belo Horizonte, Minas Gerais 31270 901, Brazil*

[c] *Molecular Medicine, Robarts Research Institute, Department of Physiology and Pharmacology, Department of Anatomy & Cell Biology, Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario N6A 5K8, Canada*

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ABSTRACT

In addition to the well-known functions as a neurotransmitter, acetylcholine (ACh) can modulate of the immune system. Nonetheless, how endogenous ACh release inflammatory responses is still not clear. To address this question, we took advantage of an animal model with a decreased ACh release due a reduction (knockdown) in vesicular acetylcholine transporter (VAChT) expression (VAChT-KD^{HOM}). These animals were challenged with lipopolysaccharide (LPS). Afterwards, we evaluated sickness behavior and quantified systemic and cerebral inflammation as well as neuronal activation in the dorsal vagal complex (DVC). VAChT-KD^{HOM} mice that were injected with LPS (10 mg/kg) showed increased mortality rate as compared to control mice. In line with this result, a low dose of LPS (0.1 mg/kg) increased the levels of pro-inflammatory (TNF-α, IL-1β, and IL-6) and anti-inflammatory (IL-10) cytokines in the spleen and brain of VAChT-KD^{HOM} mice in comparison with controls. Similarly, serum levels of TNF-α and IL-6 were increased in VAChT-KD^{HOM} mice. This excessive cytokine production was completely prevented by administration of a nicotinic receptor agonist (0.4 mg/kg) prior to the LPS injection. Three hours after the LPS injection, c-Fos expression increased in the DVC region of VAChT-KD^{HOM} mice compared to controls. In addition, VAChT-KD^{HOM} mice showed behavioral changes such as lowered locomotor and exploratory activity and reduced social interaction after the LPS challenge, when compared to control mice. Taken together, our results show that the decreased ability to release ACh exacerbates systemic and cerebral inflammation and promotes neural activation and behavioral changes induced by LPS. In conclusion, our findings support the notion that activity of cholinergic pathways, which can be modulated by VAChT expression, controls inflammatory and neural responses to LPS challenge.

1. Introduction

Lipopolysaccharide (LPS) is a component of the outer cell wall of gram-negative bacteria (Raetz and Whitfield, 2002). LPS stimulates the immune system and induces a release of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin (IL)-1β, and IL-6 (Raetz and Whitfield, 2002; Rosas-Ballina et al., 2008; Wang et al., 2003); this in turn initiates an acute inflammatory phase response comprising immune, endocrine, behavioral, and metabolic components (Doğan et al., 2002; Konsman et al., 2008).

The dual interaction between the immune system and central nervous system (CNS) is important for regulating the immunological, physiological, and behavioral responses to immune stimulation (Steinman, 2004). In fact, an inflammatory response that is generated peripherally can affect the brain because cytokines can reach the CNS via neural and humoral pathways (Benarroch, 2011; Duvernoy and Risold, 2007; Quan and Banks, 2007). This

* Corresponding authors at: Laboratório de Inflamação e Metabolismo (LIM), Programa de Pós-Graduação em Ciências Fisiológicas, Centro Integrado de Pesquisa e Pós-Graduação em Saúde – CIPq-Saúde, Universidade Federal dos Vales do Jequitinhonha e Mucuri, Rodovia MGT 367, Km 583, Alto da Jacuba, n° 5000, CEP 39100-000 Diamantina/MG, Brazil (H.R. Leite).

E-mail addresses: hercules.leite@ufvjm.edu.br (H.R. Leite), massen@icb.ufmg.br (A.R. Massensini).

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inflammatory input causes CNS cells, such as microglia and macrophages, to produce pro-inflammatory mediators, which activate specific forebrain areas and elicit sickness behavior syndrome, which is an attempt by the body to conserve energy and redirect the resources to fight off inflammation (Dantzer, 2004; Dantzer and Kelley, 2007; Marvel et al., 2004b; McCusker and Kelley, 2013; Pittman, 2011). In general, these symptoms include impaired cognitive function, loss of interest in food, anhedonia, alterations in sleep, low locomotor activity, and reduced social interaction (Bluthé et al., 2000, 1994; Harden et al., 2015; Henry et al., 2008; Konsman et al., 2008).

Sub diaphragmatic vagotomy has been shown to worsen sickness behavior symptoms and cerebral production of cytokines after LPS exposure, thus pointing to an important role of the vagal nerve in modulation of inflammation and sickness behavior (Borovikova et al., 2000; Goldbach et al., 1997; Kapcala et al., 1996; Marvel et al., 2004a); this is now known as the cholinergic anti-inflammatory reflex (Borovikova et al., 2000; Rosas-Ballina et al., 2008; Tracey, 2002). Vagal sensory nerve endings are located in the dorsal vagal complex (DVC) situated in the brainstem. The DVC encompasses a sensory component, the area postrema (AP), and the nucleus of the solitary tract (NTS) as well as a motor component: the subjacent dorsal motor nucleus (DMN) of the vagus (Rogers et al., 1995). Moreover, some studies on c-Fos expression as a marker of neuronal activation have shown that the NTS expresses substantial amounts of c-Fos in response to intraperitoneal LPS administration (Elmquist et al., 1996; Konsman et al., 1999; Laye et al., 1994). This finding supports the notion that the DVC may function as a "portal" for transmission of peripheral immune stimuli from the vagus nerve (neural), or the general circulation (humoral), to higher brain regions (Goehler et al., 1998; Marvel et al., 2004a).

Central and peripheral cholinergic activities are thought to contribute to regulation of the immune system by the CNS via the anti-inflammatory reflex (Rosas-Ballina and Tracey, 2009). Nonetheless, whether presynaptic regulation of the ACh release contributes to such modulation is not fully understood because of the contribution of non-neuronal and nonsynaptic ACh secretion to this pathway (Rosas-Ballina et al., 2011). To study the possibility of regulation of immune responses by endogenous ACh secretion, we used mice with a targeted mutation in vesicular acetylcholine transporter (VAChT), a protein crucial for ACh storage and release from synaptic vesicles in neurons (de Castro et al., 2009; Prado et al., 2006; Rodrigues et al., 2013). Although VAChT may be involved in secretion of non-neuronal ACh from cardiomyocytes (Roy et al., 2013), it is apparently not involved in secretion of ACh from immune T cells (Fujii et al., 2012; Kawashima and Fujii, 2003). In this context, a long-term VAChT deficiency induces airway hyper-responsiveness, inflammation, and remodeling in a murine model of allergic airway inflammation (Pinheiro et al., 2015) as well as upregulation of pro-inflammatory cytokines 15 days after cecal ligation and puncture (CLP) (Jeremias et al., 2015). Taken together, these results indicate that cholinergic tone is necessary for inflammatory homeostasis. Here, we used this unique mouse model to test whether long-term VAChT downregulation affects systemic and cerebral inflammation, neuronal c-Fos expression in the DVC, and the concomitant sickness behavior after systemic LPS administration.

2. Materials and methods

2.1. Animal care

Male wild-type (WT) and homozygous VAChT knockdown (VAChT-KD^{HOM}) mice aged 2–3 months and weighing 18–23 g were used in the study (n = 124). The VAChT knockdown mice were generated as previously described (Prado et al., 2006). Heterozygous mice were intercrossed to generate VAChT-KD^{HOM} mice and WT littermate controls used in these experiments. These mice have a knockdown of the *VAChT* mRNA and show a ~65% reduction in VAChT protein, compromising the synaptic ACh release (Lima et al., 2010; Prado et al., 2006). The animals were maintained in a 12 h/12 h light–dark cycle, and food and water were available *ad libitum*. Every effort was made to not cause any unnecessary distress to the animals, and all the experiments were conducted in accordance with NIH guidelines for the care and use of laboratory animals. Our animal protocols were approved by the Institutional Animal Care and Use Committee at Federal University of Minas Gerais Universidade Federal de Minas Gerais (protocol No. 280/2012).

2.2. Drug preparations

LPS (0.1 or 10 mg/kg, serotype 0111:B4, derived from *Escherichia coli*; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sterile pyrogen-free 0.9% NaCl (saline). The mice were injected intraperitoneally (i.p.) with either LPS or saline (SAL); the volume of the injection was 10 μL/mg. Activation of nicotinic receptors was accomplished using nicotine (0.4 mg/kg, Sigma-Aldrich) (Amaral et al., 2015) 30 min prior to LPS administration. Nicotine is an exogenous ligand for nicotinic acetylcholine receptors (nAChRs) and simulates cholinergic anti-inflammatory responses (Cloëz-Tayarani and Changeux, 2007).

2.3. The experimental protocol

Experiment 1 was designed to test whether VAChT-KD^{HOM} mice are more susceptible to endotoxic shock caused by administration of a high dose of LPS (10 mg/kg).

Experiment 2 was designed to determine the effects of a long-term reduction in cholinergic neurotransmission on cytokine production induced by LPS. The cytokine profile was evaluated in the spleen, brain, and serum after injection of a low dose of LPS (0.1 mg/kg) (Borovikova et al., 2000).

Experiment 3 was designed to determine the effects of a nicotinic receptor agonist, nicotine (0.4 mg/kg, administered 30 min before LPS injection) on cytokine levels in the spleen, brain, and serum.

Experiment 4 was designed to evaluate LPS-induced activation of the DCV (AP, NTS, and DMN) in mice with the long-term reduction in cholinergic neurotransmission.

Experiment 5 was intended to evaluate LPS-induced sickness behavior (exploratory behavior, locomotor activity, and social interaction) in mice with the long-term reduction in cholinergic neurotransmission.

2.4. The survival rate

To evaluate the response to endotoxic shock (such as that observed in sepsis), the mice were injected with 10 mg/kg LPS. The animals were examined every 2 h for 24 h after the LPS injection.

2.5. Quantification of cytokines in the spleen, brain, and serum

Levels of cytokines in the brain, spleen, and serum were measured by ELISA. Blood and spleen were collected 1 h after LPS administration to measure TNF-α levels or 3 h after LPS administration to quantify IL-1β, IL-6, and IL-10 expression. The brain was excised 3 h after the LPS injection to quantify IL-1β, TNF-α, IL-6, and IL-10. Blood samples were obtained by decapitation and