Respiratory Physiology & Neurobiology 229 (2016) 1–4

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

journal homepage: www.elsevier.com/locate/resphysiol

Short communication

Vagal nerve stimulation attenuates IL-6 and TNFα expression in respiratory regions of the developing rat brainstem

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ARTICLE INFO

Article history:
Received 15 February 2016
Received in revised form 19 March 2016
Accepted 22 March 2016
Available online 2 April 2016

Keywords:
Inflammation
Respiratory control
Vagus nerve

ABSTRACT

Pre-term infants are at greater risk for systemic infection due to an underdeveloped immune system. Airway infection results in immune up-regulation of early pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) in the brainstem. Current treatment for neonatal infection involves antibiotic administration. We previously showed that LPS injected into the trachea of neonatal rats causes changes in breathing and in IL-1β expression in the *nucleus tractus solitarii* (NTS) and hypoglossal motor nucleus (XII). We hypothesize that lipopolysaccharide (LPS) instilled in the trachea also causes the up-regulation of IL-6 and TNFα in the brainstem autonomic control regions. To test this hypothesis we injected LPS into the trachea of rat pups (postnatal ages 10–12 days) and then assessed changes in IL-6 and TNFα. Vagal nerve stimulation has been used in the treatment of many inflammatory disorders, including sepsis. Our experiments show that VNS attenuates the upregulation of IL-6 and TNFα caused by LPS and may be a viable alternative to antibiotics.

1. Introduction

Systemic infection is a multi-billion dollar healthcare burden in North America (Torio and Andrews, 2006). Preterm infants, especially, are at high risk for infection because exposure to pathogens occurs before innate immunity is fully developed (Simon et al., 2015). The lungs and respiratory tract are likely targets of infection, either due to chorioamnionitis, an infection caused by bacterial exposure in utero, or during normal vaginal birth. Chorioamnionitis leads to a significant proinflammatory cascade that can cause neonatal morbidity through neurodevelopmental and respiratory impairment (Kallapur et al., 2009) and, if left uncontrolled, can lead to sepsis, causing further lung and brain injury in preterm infants (Balan et al., 2011). This inflammatory cascade causes the release of early pro-inflammatory cytokines including interleukin-1-beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNFα).

We have previously shown that endotoxin (LPS) injected into the trachea of neonatal rats causes changes in breathing. We found increases in IL-1β expression in the autonomic brainstem regions responsible for the control of breathing including the *nucleus tractus solitarii* (nTS) and hypoglossal motonucleus (XII) within 2–4 h of LPS instillation (Balan et al., 2011). The *nucleus tractus solitarius* is a first order termination site for vagal afferents, including mechanosensors in the lung and chemoreceptors in the carotid bodies. The hypoglossal motor nucleus provides neural drive to the tongue and upper airway muscles (Balan et al., 2011) and is closely apposed to the nTS. The early pro-inflammatory response depends upon transcription and protein expression changes, implying longer-term effects, however, the changes we see in expression and respiration occur within an hour—suggesting that fast neural inputs to the brainstem via the vagus can cause local up-regulation of inflammatory cytokines.

Cytokines are classified as both pro- and anti-inflammatory, evoking multidimensional changes in neural activity. IL-6 and TNFα are early pro-inflammatory cytokines that participate in the early inflammatory cascade and are typically produced after the initial increase in IL-1β. TNFα is key to local inflammatory responses and serves as a co-factor for modulation of presynaptic release in the central nervous system (Santello et al., 2011), but its role in modulation of brainstem autonomic circuits is not yet known. IL-6 has been implicated in regeneration of neurons, is highly expressed early in development, and may serve a neuroprotective role (Nakashima and Taga, 2002). In addition to LPS injection, systemic IL-1β injection induces autonomic changes and increased message for IL-1β,

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http://dx.doi.org/10.1016/j.resp.2016.03.014
1569-9048/

IL-6, and TNFα in the nTS (Churchill et al., 2006) probably through short-term changes in vagal afferent input (Ek et al., 1998).

Vagal nerve stimulation, which is FDA approved to treat epilepsy and depression, is being used increasingly in the treatment of inflammatory disorders, due to its putative ability to regulate pro-inflammatory cytokine release and increase activation of descending cholinergic efferent flow to reduce inflammation. Tracey and collegues have shown that the vagus nerve and the cholinergic anti-inflammatory pathway (CAP) are implicated in the immune response and that electrical stimulation of the CAP through the vagus nerve can decrease serum levels of TNF during endotoxemia and limit synthesis of TNF in the liver, spleen and heart (Tracey, 2002).

In these experiments, we test the hypotheses that LPS induces quantifiable changes in cellular level expression of IL-6 and TNFα in brainstem nuclei and that vagal nerve stimulation can attenuate the IL-6 and TNFα upregulation caused by low doses of LPS in the trachea. To test these hypotheses we used LPS to induce IL-6 and TNFα upregulation with and without vagal nerve stimulation in neonatal rats.

2. Materials and methods

2.1. Animal preparation

Experiments were performed using Sprague-Dawley rat pups aged postnatal days (P) 10–12 (Charles-River, San Diego). All procedures were approved by the Loma Linda University Institutional Animal Care and Use committee (IACUC). For surgical procedures, pups were deeply anesthetized using 3% isofluorane and LPS (0.5 mg/kg in 10 μl saline vehicle) was injected intratracheally as previously described (Balan et al., 2011). During vagal nerve stimulation procedures, animals were anesthetized (3% isoflurane) and the vagus nerves were isolated bilaterally at the neck, silver hook electrodes were attached, and the nerves were covered with a mixture of petroleum jelly and mineral oil. The nerves were stimulated for 30 min using 1.75 mA at 25 kHz (pulse duration = 40 microseconds symmetrical duty-cycle). Injections of LPS were given at the start of VNS as described above. Animals were allowed to recover two hours before re-anesthetizing (4% isofluorane) and transcardially perfusing with 0.9% saline and 4% paraformaldehyde (PFA). The brainstems were removed and stored in 4% PFA for 24 h before transfer to 30% sucrose for cryoprotection 48 h before freezing. The brains were then frozen in *Tissue-Tek* O.C.T. and sectioned at 25 μm on a CM 3050S cryostat (Leica). Fig. 1 summarizes the animal preparation, protocol, and putative cytokine pathway.

2.2. Immunohistochemistry

Sections were stained using immunohistochemistry for IL-6 (Santa Cruz Biotechnology, Dallas TX, sc-1265-r antibody) and TNFα (Abcam, Cambridge MA, ab6671 antibody) protein. Sections were rinsed in ice cold 95% ethanol then washed in 1x PBS. Sections were incubated in dilution buffer containing bovine serum albumin, Triton-X and sodium phosphate buffer. Sections were blocked in 5% goat and 5% donkey serum for 1 h. The sections were incubated in 5% goat serum with primary antibody (IL-6 or TNFα) overnight at 4 °C. Sections were incubated for two hours at 25 °C with biotinylated secondary antibody (Vector Labs, Burlingame, CA). After a 30 min incubation with VECTASTAIN Elite ABC Kit (Vector Labs, Burlingame, CA), reactions were visualized using diaminobenzidine (DAB). Sections were dipped in ethanol to dehydrate, xylene to clean and were coverslipped using permount mounting media. Staining intensity was evaluated on an upright brightfield microscope.

2.3. Unbiased stereology

We used unbiased stereology (*Stereologer2000*, Stereology Resource Center, Tampa, FL) to reproducibly quantify the number of cells expressing IL-6 and TNFα protein. We surveyed the extent of the nTS from Bregma −11.4 to −13.92 (solitary commissure appears). This encompasses the majority of the cardiorespiratory regions of the nTS. We counted labeled cells within the XII nucleus in this extent of rostro-caudal brainstem as well. All images for Fig. 2 are acquired from within *Stereologer* and the post-processing and compositing were done using *GIMP* (http://gimp.org) and *Adobe Illustrator CS6*.

2.4. Statistics and analysis

Due to the small sample sizes and relative skew of the data distribution, we used non-parametric statistics to assess cytokine expression (Mann-Whitney) in sham, LPS, and LPS + VNS groups. All data are presented as mean ± SEM and $p < 0.05$ was considered significant.

3. Results

IL-6 was significantly upregulated ($p < 0.01$) with LPS injection over sham injection. Fig. 2 panel A shows staining in the nTS and hypoglossal (XII) regions of the brainstem through the rostro-caudal extent of the cardiorespiratory nTS. We show sections from sham, LPS-injected, and LPS + VNS treated animals. Summary data showed a significant increase in IL-6 in LPS-treated animals with an attenuation of IL-6 expression in the LPS + VNS treated animals (N = 4 for LPS + VNS and N = 3 for the LPS groups). Thus, vagal nerve stimulation immediately after LPS injection significantly decreased ($p < 0.01$) IL-6 expression (sham animals, N = 3).

TNFα was significantly upregulated ($p < 0.01$) with LPS injection when compared to sham injections. LPS injection with Vagal nerve stimulation significantly decreased ($p < 0.01$) IL-6 expression. Fig. 2, panel B shows staining in the nTS and hypoglossal (XII) regions of the through the rostro-caudal extent of the cardiorespiratory nTS in sections from sham, LPS-injected, and LPS + VNS treated animals. Summary data showed a significant increase in TNFα in LPS-treated animals with an attenuation of TNFα expression in the LPS + VNS treated animals (N = 4 for LPS + VNS and N = 3 for the LPS groups, sham N = 3).

4. Discussion

In this short communication, we present data that for the first time, shows that two other early onset pro-inflammatory cytokines, IL-6 and TNFα, are upregulated in the *nucleus tractus solitarii* and hypoglossal motor nucleus after intratracheal LPS. This builds upon prior work showing that LPS can induce local increases in the cytokine IL-1β in respiratory control regions of the brainstem (Balan et al., 2011; Jafri et al., 2013). We have also presented preliminary data that shows that vagal nerve stimulation provides a potent pathway to reduce brainstem expression of cytokines after an acute inflammatory challenge using LPS.

During the perinatal time period, when infants immune systems are immature, the airways are a frequent source of entry for pathogens. Alteration of cytokine levels by infection or clinical intervention can result in pronounced changes in neurodevelopmental outcome. LPS injections cause an increase in pro-inflammatory cytokine expression which can affect breathing control dysregulation in neonatal rats. This can cause accompanying attenuation of the ventilatory response to hypoxia, (Balan et al., 2011) which may be an underlying substrate for breathing