Published in final edited form as:

Respir Physiol Neurobiol. 2011 September 30; 178(3): 458–464. doi:10.1016/j.resp.2011.03.003.

Vagal afferents modulate cytokine-mediated respiratory control at the neonatal medulla oblongata

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Abstract

Perinatal sepsis and inflammation trigger lung and brain injury in preterm infants, and associated apnea of prematurity. We hypothesized that endotoxin exposure in the immature lung would upregulate proinflammatory cytokine mRNA expression in the medulla oblongata and be associated with impaired respiratory control. Lipopolysaccharide (LPS, 0.1 mg/kg) or saline was administered intratracheally to rat pups and medulla oblongatas were harvested for quantifying expression of mRNA for proinflammatory cytokines. LPS-exposure significantly increased medullary mRNA for IL-1 β and IL-6, and vagotomy blunted this increase in IL-1 β , but not IL-6. Whole-body flow plethysmography revealed that LPS-exposed pups had an attenuated ventilatory response to hypoxia both before and after carotid sinus nerve transection. Immunochemical expression of IL-1 β within the nucleus of the solitary tract and area postrema was increased after LPS-exposure. In summary, intratracheal endotoxin-exposure in rat pups is associated with upregulation of proinflammatory cytokines in the medulla oblongata that is vagally-mediated for IL-1 β and associated with an impaired hypoxic ventilatory response.

1. Introduction

Respiratory control during the neonatal period is vulnerable to a diversity of factors and apnea of prematurity is a major source of morbidity in this population (Abu-Shaweesh and Martin, 2008). Sepsis in preterm infants is frequently associated with changes in breathing pattern and apneic episodes. The respiratory tract is a likely source of infection for organisms acquired by infants in the neonatal setting. The lung is also a major potential site of entry for diverse organisms during fetal life, especially in the presence of chorioamnionitis. The latter has been implicated in a significant proinflammatory cascade that may affect neonatal morbidity via both neurodevelopmental as well as respiratory outcomes (Kallapur et al., 2009; Leviton et al., 2010). These clinical observations led us to explore the interrelationship between intrapulmonary endotoxin exposure and an inflammatory response in the developing brain.

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There are two major types of sensors in the lung: mechanosensitive receptors and chemosensitive receptors (Lee and Pisarri, 2001; Yu, 2005) both of which project through vagal afferents terminating in the nucleus of the solitary tract (nTS) (Kalia and Mesulam, 1980a, b). The nTS is the first central synapse for barosensory and chemosensory fibers coursing through the carotid sinus nerve. Furthermore, second order neurons arising from the nTS project to neurons in the rostral ventrolateral medulla (RVLM). Systemic administration of interleukin-1 β (IL-1 β) has been shown to induce time- and dose-dependent expression of early immediate genes in the nTS and RVLM (Ericsson et al., 1994; Koshiya and Smith, 1999). However the cytokine, IL-1 β is a large lipophobic protein that does not readily diffuse across the blood-brain barrier (BBB) (de Boer and Breimer, 1998). It is unclear whether activation of lung afferents in response to intratracheal administration of LPS in the neonatal rat model might signal the medulla oblongata to increase transcripts for medullary proinflammatory cytokines.

The role of neonatal sepsis in affecting peripheral and central respiratory control mechanisms and resultant changes in breathing pattern is not clearly understood. Prior studies in immature rodent models have explored the role of systemic intraperitoneal endotoxin exposure on respiratory control. Attenuation of the ventilatory responses to hypoxia after systemic *Escherichia coli* endotoxin administration has been attributed to the inhibitory effect of endothelial NO on peripheral chemoreceptors (Ladino et al., 2007). However, central ventilatory depression during hypoxia has been associated with increased synthesis of IL-1 β mediated prostaglandin at the level of the CNS (Olsson et al., 2003). Moreover, IL-1 receptor mRNA was observed in trigeminal and hypoglossal motor nuclei, as well as in the area postrema, although it was not detected in the nTS (Ericsson et al., 1995; Yabuuchi et al., 1994). In the present study, we hypothesized that intrapulmonary instillation of LPS in rat pups would elicit a proinflammatory cytokine response in the medulla oblongata, and that vagal afferents would play a role in this response at the medullary cardiorespiratory centers. We further hypothesized that this would be associated with a perturbation of ventilatory responses to hypoxia or hypercapnia at the level of centrally-mediated medullary mechanisms.

2. Materials and Methods

2.1. Animals

Male and female wild-type specific-pathogen free Fischer 344 (F344) rats at postnatal age 10 – 12 days (n = 125, Harlan Laboratories, Indianapolis, Indiana, USA) were employed. Body weight of these pups was 19.2 ± 0.37 g. Based on comparative interspecies data on brain growth (Davis and Dobbing, 1974), it is estimated that this postnatal age corresponds to full term human birth. All pups were reared by their mothers, fed rat chow and water *ad libitum* and housed under standardized conditions with a light-dark cycle of 12:12 hr. The procedures used in the care and handling of the animals, were in accordance with the guidelines of the National Institutes of Health. The study protocol was reviewed and approved by the Case Western Reserve University, School of Medicine, Institutional Animal Care and Use Committee.

2.2. Surgical Procedures

2.2.1. Intratracheal instillation of *E.coli* lipopolysaccharide (LPS)—On the day of the experiment, the rat pups were intraperitoneally anesthetized with a short-acting anesthetic cocktail of xylazine (0.4 mg/100 g) and ketamine (3 mg/100 g). The rats were then instilled intratracheally via a modified tracheal cannula with either sterile pyrogen-free saline (1.5 ml/kg) or with 0.1 mg/kg of LPS (*E. coli* endotoxin O55:B5, Sigma-Aldrich, St. Louis, MO, USA) in the same volume of saline, and allowed to recover. Placement of the

cannula was confirmed randomly by gently opening the sternohyoid muscle and visualizing entry of the cannula into the trachea. The pups recovered from anesthesia within 30 min and were returned to the mothers for feeding. Deeply sedated pups were cervically dislocated at varying intervals (2, 4, 8 and 24 hr; $n = 6$ per treatment group) and the medulla oblongata (rostral to the spinal cord and caudal to the ponto-medullary border) was excised to assess the mRNA expression profile for proinflammatory cytokines (IL-1 β , IL-6 and TNF- α) by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR).

Prior to tissue collection, pups were deeply sedated and blood sample was obtained via cardiac puncture. Blood gas values were determined (Model ABL5, Radiometer, Westlake, OH) in LPS-instilled pups (pH 7.37 ± 0.03 , PaCO₂ 30 ± 1 mmHg, PaO₂ 94 ± 2 mmHg and SaO₂ $97 \pm 1\%$) and these were comparable to saline-treated pups ($n = 6$ per group).

2.2.2. Vagotomy—These pups ($n = 10$ per group) were anesthetized (section 2.2.1.) and the vagus nerves were isolated bilaterally via a midline cervical incision. Both nerves were transected and the animals were allowed to recover with the mother for at least an hour following which intratracheal saline (1.5 ml/kg) or LPS (0.1 mg/kg) was instilled as described in 2.2.1. Following vagotomy, effectiveness of the transection was confirmed by diaphragm electromyographic recording (D_{EMG}). Briefly, electrodes made from teflon-coated stainless steel wires were inserted into the right side of the costal region of the diaphragm. D_{EMG} signals amplified, filtered, and both the raw and full-wave rectified signals were recorded. The integrated data were analyzed breath-by-breath for frequency and peak amplitude. A significant reduction in respiratory frequency (before vagotomy 73 ± 3 bpm, after vagotomy 15 ± 3 bpm) and increase in breath amplitude (before vagotomy 0.31 ± 0.01 arbitrary units, after vagotomy 0.55 ± 0.06 arbitrary units) was observed ($n = 6$ per group). The pups were sacrificed 2 – 3 hr post instillation and RT-PCR quantification was performed for IL-1 β and IL-6 mRNA expression in the medulla oblongata in intact and vagotomized rat pups.

Prior to tissue collection, blood gas values via cardiac puncture were determined in vagotomized LPS-instilled pups and these were not different from those in vagotomized saline-instilled pups ($n = 6$ per treatment group). However, vagotomy did induce a mild respiratory acidosis (pH 7.31 ± 0.01 , PaCO₂ 55 ± 2 mmHg, PaO₂ 81 ± 3 mmHg and SaO₂ $93 \pm 2\%$).

2.3. RNA isolation and RT-PCR for Pro-inflammatory cytokines

mRNA for IL-1 β , IL-6 and TNF- α was measured using RT-PCR analysis with protocols described previously (Wilson et al., 2007). Briefly, total cellular RNA was extracted from the medulla oblongata by using the standard Trizol RNA extraction reagent (Invitrogen, Carlsbad, CA, USA). The contaminating genomic DNA in the RNA samples was digested using DNaseI (Ambion, Austin, TX, USA), and 1 μ g total cellular RNA was reverse transcribed using random hexamers as primers in a 20- μ l reaction volume (Retroscript kit; Ambion). Finally, 1.5- μ l cDNA was amplified with Accuprime DNA polymerase in a 25- μ l reaction volume (Invitrogen). PCR primers were designed using the Primer3Plus software program (Untergasser et al., 2007). The primers for IL-1 β , IL-6 and TNF- α are the following: IL-1 β , oligonucleotide transcript of 57-bp size [forward: 5'-gcaactgcctgaactc-3'; reverse: 5'-ggctattctcctcactgtc-3'], IL-6 transcript of 50 bp [forward: 5'-tccgcaagagacttcag-3'; reverse: 5'-tgtcaacaacatcagtc-3'] and TNF- α transcript of 99 bp [forward: 5'-ctcctctcatcagttcc-3'; reverse: 5'-cttggtggtttgtacga-3']. The PCR cycling profile, using a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA, USA), was as follows: one cycle at 94°C for 5 min, 32 cycles at 94°C for 30 s, 58°C for 30 s, 68°C for 30 s followed with a final extension cycle at 68°C for 2 min.

As an internal control for RNA input and RT efficiency, the housekeeping gene, hypoxanthine phosphoribosyltransferase (HPRT) was also quantified for each sample. The primers for HPRT are the following: oligonucleotide transcript size of 123 bp [forward: 5'-ctcatggactgattatggacaggac-3'; reverse: 5'-gcaggtcagcaaagaacttatagcc-3']. An amplification of 32 cycles was chosen based on a preliminary study determining the linear range of amplification for each cytokine and housekeeping gene (data not shown) and furthermore PCR for each cDNA was performed in duplicate. A 10- μ l aliquot of each amplified sample was electrophoresed on a 2.0% agarose gel. As a negative control, 0.5 μ g of RNA extract was added directly to the PCR cocktail as a template for amplification. Contamination from genomic DNA would be detected as amplified DNA, and none was detected, ruling out contamination of the samples. The average densities were calculated using the gel analyzer options of *ImageJ* software (<http://rsb.info.nih.gov/ij/>) and the results are expressed as the ratio of mRNA of interest and HPRT.

2.4. Measurement of hypoxic and hypercapnic ventilatory responses

Two to three hours after the intratracheal instillation of saline or LPS, pups were placed in a thermoregulated (30°C), flow-through barometric plethysmograph (BUXCO Research Systems, Wilmington, NC, USA). The time point for investigation corresponded to the maximal induction of pro-inflammatory cytokines in the medulla oblongata. Briefly, pressure differences across two-screen mesh pneumotachographs located on top of the plethysmograph were measured using a flow transducer. With appropriate software, the recorded flow signal was then integrated on a breath-by-breath basis to obtain relative ventilation (VE). Tracheal-instilled LPS pups ($n = 10$) were compared with saline-instilled pups ($n = 10$). The pups were allowed to adapt to the plethysmograph until they were in quiet sleep (eyes closed, recumbent with limbs adducted, regular and stable respiratory rhythm with minimal sighs or periodic breathing and absence of movements except for intermittent brief startles). Then, after baseline respiration was measured for 10 minutes, the respiratory responses to hypoxia and hypercapnia were tested. To evaluate the response of the rat pup to hypoxia, the inflow to the plethysmograph was switched to the test gas: 10% inspired oxygen, balance N₂ for 5 to 10 minutes. To evaluate the response of breathing to an increase in inhaled CO₂ (hypercapnia), pups were exposed to 5% CO₂, 21% O₂, balance N₂ for 5 minutes, after which the pups were allowed to return to their baseline minute ventilation in room air. Minute ventilation (the product of respiratory rate and tidal volume) was calculated. Minute ventilation was averaged over 20 sec intervals before and during the test gas exposure, and expressed as ml/min/100g weight of the rat.

To determine whether the decreased ventilatory response to hypoxia in LPS exposed pups is a consequence of central versus peripheral chemoreceptor mediated mechanisms, in a subgroup of 10 – 12 day old pups ($n = 6$) the carotid sinus nerve (CSN) was transected using an anatomic approach described by McDonald (1980). Briefly, the rat pups were anesthetized and the CSN was identified, freed from the closely adjacent pharyngeal branch of the vagus, and transected as it joined the glossopharyngeal nerve. The pups were then sutured, hydrated and left to recover with the mother for 48 hr. The hypoxic ventilatory response was then recorded by whole body flow plethysmography before and after LPS instillation (0.1 mg/kg) in the same pups.

2.5. Immunohistochemistry for IL-1 β

Paraformaldehyde-fixed medulla oblongata sections (20 μ m) were washed for 1 hr in tris-buffered saline (TBS; pH 7.4) to remove traces of fixative and then the tissues were placed in 0.5% hydrogen peroxide for 30 minutes to quench endogenous peroxidase. The tissues were washed in TBS 3 times, 10 minutes each and then immersed for 1hr in a solution of 10% normal donkey serum to block nonspecific binding of antibodies. The tissues were then

incubated overnight at 4°C in goat polyclonal antibody to IL-1 β (4 μ g/ml, R&D Systems, Inc., Minneapolis, MN, USA). The sections were then washed in three changes of TBS and incubated for 2 hr at room temperature in a 1:200 dilution of biotinylated donkey anti-goat. This was followed by three changes of PBS to remove unreacted secondary antibody. The sections were then reacted for 1 hr at room temperature in Avidin-Biotin Complex (Vector Laboratories, Burlingame, CA, USA). The reaction was visualized by treatment for 2 minutes in 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) solution in Tris-buffered saline (TBS; pH 7.6) containing 0.05% hydrogen peroxide. The color reaction was stopped with several washes of TBS. Some sections were mounted on gelatin-coated glass slides, and lightly counterstained with neutral red, dehydrated in alcohol series, then cleared in xylene before coverslipping. Control sections were incubated in TBS containing normal donkey serum instead of primary antibody.

2.6. Statistical Analysis

Each value is represented as a mean $\pm$ SEM. Changes in tissue proinflammatory cytokines and ventilatory responses between groups were analyzed by ANOVA, followed by post hoc Bonferroni correction. For ventilatory responses, changes in V_E are shown as percentage change from the normalized baseline values.

3. Results

3.1. Time course for medulla oblongata proinflammatory cytokine mRNA response to intratracheal LPS instillation

We initially explored the ability of intratracheal LPS instillation to elicit a medullary cytokine response and the resultant time course. Figure 1 shows the effect of intratracheal LPS on medulla oblongata tissue mRNA levels for proinflammatory cytokines IL-1 β , IL-6 and TNF- α in 10 – 12 d neonatal rats. The expression levels for IL-1 β and IL-6 peaked at two hours after LPS exposure, decreasing to baseline levels at 8 hr and 24 hr respectively. IL-1 β mRNA levels in the medulla oblongata were significantly higher at 2-hr (2 fold, $p < 0.001$) and 4-hr (2 fold, $p < 0.001$) compared to baseline levels. IL-6 mRNA levels in the medulla oblongata were significantly higher at 2-hr (3 fold, $p < 0.001$), 4-hr (2 fold, $p < 0.001$) and 8-hr (1 fold, $p < 0.01$) compared to baseline levels. However, there was no quantifiable expression for TNF- α at our dose of LPS and over the time-course of this experimental model (Figure 1).

3.2. Effects of vagal transection on medulla oblongata cytokines after intratracheal LPS instillation

We then sought to determine whether vagal transmission would alter medullary cytokine mRNA expression two hours following intratracheal LPS. Figure 2 shows the effect of cervical vagotomy on medullary transcript levels for IL-1 β and IL-6 two hours post LPS administration. Vagotomy clearly blunted the increase of LPS-mediated medullary mRNA for IL-1 β but not IL-6. Compared with non-vagotomized rat pups in which LPS induced a 2 fold increase in IL-1 β ($p < 0.001$), prior vagotomy resulted in a marginal non-significant change in medulla IL-1 β transcript response 2-hr post LPS administration. The IL-1 β transcript levels prior to LPS instillation did not differ significantly between vagus intact versus vagotomized saline-instilled control pups suggesting no influence of vagotomy on the level of baseline medullary IL-1 β mRNA expression.

In contrast, both vagus intact and vagotomized pups elicited a significant LPS-mediated medullary IL-6 transcript response (Figure 2). Non-vagotomized rat pups showed a 3 fold increase in IL-6 transcript ($p < 0.001$) as compared to a 2-fold increase ($p < 0.01$) after vagotomy. In saline-instilled rat pups, vagotomy resulted in a 2-fold increase ($p < 0.05$) in

baseline IL-6 mRNA compared to vagus intact controls, suggesting an effect of vagotomy on baseline IL-6 mRNA expression.

3.3. Ventilatory response to hypoxia and hypercapnic challenge

We next sought to correlate the LPS induced increase in medullary cytokine expression with any alteration in chemical control of breathing. Baseline values for minute ventilation in rat pups did not significantly differ between saline- (114 ± 6 ml/min/100g) and LPS-instillation (137 ± 11 ml/min/100g). As seen in Figure 3, LPS-instillation resulted in a significant attenuation of ventilatory response to moderate hypoxia (10% O₂). The percent increase in ventilation over baseline was reduced at 2, 5, 8 and 10 minutes after hypoxic exposure in LPS exposed versus control pups (Figure 3). This attenuated change in $\dot{V}_E$ after hypoxic exposure was due to decreases in both tidal volume and respiratory rate (data not shown). No significant differences were observed in the post-hypoxic recovery phase. In contrast, as seen in Figure 4, the change in $\dot{V}_E$ over baseline in response to moderate hypercapnia (5% CO₂) did not differ between LPS-instilled pups compared to saline-instilled controls (Control $128 \pm 11\%$ versus LPS $133 \pm 11\%$).

3.4. Effect of LPS exposure on hypoxic ventilatory responses in carotid sinus nerve transected rat pups

We sought to determine whether the decreased ventilatory response to hypoxia in LPS exposed pups is a consequence of central effects versus peripheral chemoreceptor-mediated mechanisms. The effect of carotid sinus nerve transection in mediating the blunted hypoxic ventilatory response in LPS-instilled rat pups is shown in Figure 5. As expected, there is no significant increase in minute ventilation after CSN transection in either group of rat pups. However, this ventilatory response at 2 min and 5 min of 10% O₂ exposure differs significantly between pre and post LPS exposure suggestive of a centrally-mediated respiratory inhibition induced by LPS. No significant differences were observed in the post-hypoxic exposure.

3.5. Immunohistochemical localization of IL-1 β

Finally we performed an immunohistochemical evaluation for the presence of IL-1 β protein in the medulla oblongata within which lie neuronal networks involved in cardiorespiratory control. In this protocol, we have focused exclusively on IL-1 β and not IL-6 expression as IL-1 β was selectively influenced by vagal transmission. As seen in Figure 6, IL-1 β was localized within the area postrema and caudal nTS of the medulla oblongata. Qualitative assessment of the photomicrographs shows increased expression of IL-1 β within cells of the nTS in LPS-instilled rat pups. Also, IL-1 β was expressed in the cells of the area postrema in LPS-instilled but not in control animals.

4. Discussion

In the current study, we demonstrate for the first time in neonatal rats that vagal innervation of the airways and lung parenchyma plays an important and selective role in regulation of proinflammatory cytokine expression in the medulla oblongata following intratracheal instillation of *E.coli* endotoxin. Increase in mRNA transcripts for IL-1 β and IL-6 in the medulla oblongata appeared to be associated with central effects on respiratory-associated control mechanisms, particularly attenuation of the ventilatory response to hypoxia but not hypercapnia. Furthermore, immunohistochemical assessment shows a qualitative increase in IL-1 β protein in the area postrema and nTS of the rat pup medulla oblongata.

4.1. Medullary proinflammatory cytokine responses to intratracheal LPS instillation

In this model we observed a significant upregulation of mRNA transcripts for IL-1 β and IL-6 cytokines in the medulla oblongata at 2 – 4 hr post LPS exposure. These molecules represent acute-phase response mechanisms which modulate centrally-controlled facets of an acute inflammatory response, including the initiation of fever, sleep, and neuropeptide release (Dinarello, 1991). Moreover, Hansen et al., (1998) showed that intraperitoneal administration of IL-1 β resulted in a 2-fold increase in IL-1 β mRNA expression in the medulla oblongata which is congruent with our model. The cytokine IL-6 has been shown to play a role in fever regulation (Chai et al., 1996) and also stimulates hypothalamic corticotropin releasing factor expression and, as a consequence releases ACTH and glucocorticoids (Ruzek et al., 1997). Glucocorticoids can suppress multiple immune functions, including cytokine production and thus provide feedback inhibition mechanisms limiting immune responses (Brattsand and Linden, 1996). In our experimental model, there was no expression of TNF- α in the medulla oblongata suggesting that either glucocorticoids or other mechanisms may have masked a TNF- α response, or that TNF- α might not play a major role in intratracheal LPS-induced medulla oblongata cytokine expression.

4.2 Vagal regulation of medullary proinflammatory cytokines in neonatal rats

Intravenous injections of LPS or IL-1 β have been shown to activate vagal sensory lung receptors thus supporting the notion that these pulmonary receptors may function as an important afferent system for transmitting immune signals from the lung to the brain during endotoxemia (Lai et al., 2002; Yu et al., 2007). In addition, binding studies indicate that IL-1 receptors are present in the vagus (laryngeal, thoracic, hepatic and abdominal) (Goehler et al., 1997). In our experimental model we saw significant upregulation of transcripts for IL-1 β , IL-6 and TNF- α in the lungs (data not shown), however, this is not the focus of our current report. It is known that delivery of LPS to the lungs via intratracheal instillation causes neutrophil influx (Chang and Lesser, 1984), induces the release of cytokines [including interleukin-1 (Ulich et al., 1991), tumor necrosis factor (Nelson et al., 1989), and chemokine-KC (Frevert et al., 1995)] as well as release of other inflammatory mediators such as platelet-activating factor (Lantz et al., 1991) and prostaglandins (Martin et al., 1989) into bronchoalveolar lavage fluid, with a resultant increase in capillary permeability and pulmonary edema (Horgan et al., 1993; Wheeldon et al., 1992).

Our data show that transcripts for IL-1 β but not IL-6 mRNA are significantly attenuated in cervical vagotomized pups. These results complement prior study by Hansen et al., (1998) who demonstrated that subdiaphragmatic vagotomy blocked the ability of intraperitoneally administered IL-1 β to induce an increase in IL-1 β mRNA in the brainstem and hippocampus but not in the liver of an adult rat model. Moreover, other studies have demonstrated that the appearance of proinflammatory cytokines in the brain after myocardial infarction is independent of blood-borne cytokines suggesting that cardiac sympathetic afferent nerves activated by myocardial ischemia signal the brain to increase cytokine production (Francis et al., 2004). In our study, although LPS instillation resulted in a 3-fold increase of IL-6 mRNA in the medulla oblongata, we did not observe vagal control of IL-6 expression. This could be due to IL-1 α -mediated induction of IL-6 (Libert et al., 1990).

4.3. Immunohistochemical assessment of IL-1 β in the medulla oblongata

Our data show that IL-1 β synthesizing cells were localized in the area postrema and nucleus of the solitary tract regions of the caudal medulla oblongata. Differential immunoreactivity was not observed in other regions of the medulla oblongata. The brain has been long considered devoid of immune mechanisms and, proximity of circumventricular organs such as the area postrema makes it vulnerable to systemic insults. Prior studies have shown that

intraperitoneal LPS treatment induced IL-1 β -like immunoreactivity in immune cells associated with neuronal elements of the area postrema (Goehler et al., 2006). In addition, electron microscopic analysis centered on the area postrema revealed that IL-1 β -positive cells are in apposition with neuronal elements, including dendrites and terminals of vagal sensory fibers (Goehler et al., 2006). Further, neuroanatomical tracers have established neural connections of the area postrema to the nTS in rats (Shapiro and Miselis, 1985).

Lung vagal afferents terminate principally in the middle and caudal portions of the nTS (Kalia and Mesulam, 1980a, b). Thus localization of IL-1 β in the nTS could signify IL-1 β -mediated modulation of neuronal responses emanating from the nTS. In addition, intraperitoneal injection of IL-1 β results in expression of the immediate-early gene *c-fos* mRNA in the area postrema and nTS at 1-hr and 3-hr post treatment (Brady et al., 1994). In contrast *c-fos* generation in the dorsal vagus complex after systemic endotoxin is not dependent on the vagus nerve (Hermann et al., 2001).

IL-1 β receptors have been localized within brainstem regions such as the area postrema, trigeminal and hypoglossal motor nuclei; regions, which play a role in cardiorespiratory control (Ericsson et al., 1995; Yabuuchi et al., 1994). Prior studies have not demonstrated IL-1 β receptor expression in the nTS although this region represents a responsive cell population to IL-1 β -mediated infections (Chan et al., 1993). Recent studies have documented IL-1 β -mediated brainstem-specific microsomal prostaglandin E synthase-1 activation and prostaglandin E₂ binding to EP3 receptors in the brainstem. This activation resulted in increased apnea frequency and failure to autoresuscitate after a hypoxic event (Hofstetter et al., 2007; Olsson et al., 2003). Therefore we speculate that our observed localization of IL-1 β protein in the nTS along with vagal control of IL-1 β mRNA in the medulla oblongata is not just an epiphenomenon but rather IL-1 β could be a candidate molecule for altering neuronal circuitry following pulmonary inflammation.

4.4. LPS-induced inhibition of the ventilatory response to hypoxia but not hypercapnia

Brainstem regions that are hypoxia chemosensitive include the nTS, the C1 sympathoexcitatory region, and the respiratory pre-Bötzinger complex (Neubauer and Sunderram, 2004). Also, the caudal nTS is a site of central CO₂ chemoreception (Dean and Putnam, 2010). IL-1 β has been linked to altered respiration and autoresuscitation (Lindgren and Groggaard, 1996; Stoltenberg et al., 1994). In light of the convergence of IL-1 β expressing cells in the area postrema and caudal nTS, we compared hypoxic and hypercapnic ventilatory responses in intratracheal saline- and LPS-instilled neonatal pups. Our data showed that there was a significant blunting of the hypoxic but not hypercapnic response in LPS-instilled pups, while baseline ventilation remained the same in both groups. Significant blunting of the ventilatory response to hypoxia was observed throughout the ten minute duration of hypoxic exposure. This suggested either inhibition of carotid body function or centrally-mediated depression of hypoxic chemoreflex pathways.

Prior studies in 5 week old rats have shown attenuation of the ventilatory response to hypoxia during endotoxemia, in part mediated by an inhibitory effect of endothelial nitric oxide on central or peripheral respiratory control mechanisms (Ladino et al., 2007). It has also been shown that IL-1 β and IL-6 bind with their corresponding receptors on the carotid body (Wang et al., 2006; Zhang et al., 2007). Although, intratracheal exposure to LPS as performed in our study when compared to systemic administration represents a compartmentalized model, it is possible that LPS was systemically distributed resulting in a blood-borne proinflammatory stimulus affecting the carotid bodies. We therefore performed carotid sinus nerve transection in a subset of pups prior to hypoxic exposure as the carotid bodies represent the major site of hypoxic chemosensitivity. Although as expected, CSN transection suppressed the peripheral chemoreceptor response to hypoxia, the instillation of

LPS further provoked a depressant ventilatory response to hypoxia suggesting LPS-mediated cytokine effects on central respiratory neuronal pathways.

5. Conclusions and Clinical Relevance

The present investigation demonstrates that intratracheal endotoxin in the neonatal rat elicits expression of mRNA transcripts for the cytokines IL-1 β and IL-6 in the medulla oblongata; the increase in expression of IL-1 β is vagally-mediated. Our observed increase in expression of proinflammatory cytokines in the caudal nTS and area postrema of the central respiratory network was associated with blunting of the ventilatory response to hypoxia. It is widely recognized that neonatal sepsis, often presenting as pneumonia, is a common trigger for apnea in this population. Intrapulmonary endotoxin exposure may serve a trigger for the resultant alteration of respiratory control. The current study provides suggestive data that cytokine-mediated mechanisms at the medulla oblongata may be implicated in apnea of prematurity. Preterm infants are also at substantial risk for neonatal lung injury in the form of bronchopulmonary dysplasia (BPD) with its accompanying proinflammatory response. BPD has been associated with functional brainstem impairment, manifest by abnormal brainstem auditory evoked responses (Wilkinson et al., 2007). Therefore, we speculate that our findings have potentially broader translational implications for enhancing our understanding of neonatal pathophysiology.

Acknowledgments

This work is supported by the National Heart, Lung and Blood Institute Grants R21HL098628-01A1 (to R. J. Martin) and 4R00HL087620-03 (to P. Kc).

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Figure 1.

Time course for IL-1 β , IL-6 and TNF- α mRNA expression in the medulla oblongata following intratracheal instillation of LPS (0.1mg/kg). Samples were collected 2, 4, 8 and 24 hr post instillation. Note, the 2 hr time point showed optimal induction for IL-1 β and IL-6 mRNA, whereas no quantifiable expression for TNF- α at our dose of LPS was observed in this experimental model. Proinflammatory cytokine expressions were normalized to hypoxanthine phosphoribosyltransferase (HPRT), a constitutively expressed housekeeping gene. Data are expressed as mean $\pm$ S.E.M, n = 6 per group. ** = p<0.01; *** = p<0.001 compared to baseline at time zero.

Figure 2.

Relative IL-1 β and IL-6 mRNA expression in the medulla oblongata two hours after saline or LPS instillation in vagus intact or vagotomized pups. LPS instillation significantly increased IL-1 β mRNA expression in the medulla oblongata which was not apparent after vagotomy. In contrast vagotomy did not appear to diminish the increase in IL-6 mRNA expression after LPS treatment. Vagotomy per se did not alter the level of baseline medullary IL-1 β mRNA expression but did increase baseline IL-6 expression. Proinflammatory cytokine expressions were normalized to the expression of HPRT, a constitutively expressed housekeeping gene. Data are expressed as mean $\pm$ S.E.M, n = 10 per group. * = p < 0.05; ** = p < 0.01; *** = p < 0.001

Figure 3.

Effect of intratracheal LPS on minute ventilation during exposure to 10% O₂ in 10–12 day-old F344 rats (n = 10 per group). Percentage change in minute ventilation from baseline was recorded in room air (time zero), in response to 10% O₂ exposure, and during post-hypoxic response in room air using whole body plethysmography. Note, the hypoxic ventilatory response was significantly decreased in LPS as compared to saline instilled rat pups. Data are expressed as mean ± S.E.M. * = p<0.05, ** = p<0.01 and *** = p<0.001 between groups for the same time point.

Figure 4.

Effect of LPS on minute ventilation during exposure to 5% CO₂ in 10 – 12 day-old F344 rats (n = 10 per group). Percentage change in minute ventilation from baseline was recorded in room air (time zero), in response to 5% CO₂ exposure, and during post-hypercapnic response in room air using whole body plethysmography. Data are expressed as mean $\pm$ S.E.M. Note, no significant difference in hypercapnia ventilatory response was observed between saline and LPS instilled rat pups.

Figure 5.

Hypoxia-induced ventilatory changes in carotid sinus nerve (CSN)-transected pups prior to (continuous line) and 2 – 3 hr post LPS treatment (interrupted line) ($n = 6$ per group). Minute ventilation (represented as percentage change from the baseline) was recorded during 10% O_2 exposure followed by recovery in room air using whole body flow plethysmography. LPS provoked a significant ventilatory depressant response to hypoxia in CSN-denervated rat pups. Data are expressed as mean $\pm$ S.E.M. ** = $p < 0.01$ and *** = $p < 0.001$ between groups for the same time point.

Figure 6.

A: Schematic diagram showing regions of localized IL-1 β in the medulla oblongata [area postrema (AP); nucleus tractus solitarius (nTS)]. B: Photomicrographs showing localization of IL-1 β two to three hr following tracheal instillation of saline (control) or LPS in the AP (top row) and nTS (bottom row). Arrows represent IL-1 β immunostained cells and a higher magnification for IL-1 β immunoreactivity in the AP and nTS is presented for LPS-instilled pups. Note, LPS increased IL-1 β expression in both the AP and nTS. Scale bar represents 50 μ m.