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Intrapulmonary lipopolysaccharide exposure upregulates cytokine expression in the neonatal brainstem

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Abstract

Perinatal inflammation and neonatal sepsis trigger lung and brain injury. We hypothesized that endotoxin exposure in the immature lung upregulates proinflammatory cytokine expression in the brainstem and impairs respiratory control. Lipopolysaccharide (LPS) or saline was administered intratracheally to vagal intact or denervated rat pups. LPS increased brainstem IL-1 β and vagotomy blunted this response. There was an attenuated ventilatory response to hypoxia and increased brainstem IL-1 β expression after LPS.

Conclusion—Intratracheal endotoxin exposure in rat pups is associated with upregulation of IL-1 β in the brainstem that is vagally mediated and associated with an impaired hypoxic ventilatory response.

Keywords

Brainstem cytokines; Hypoxic ventilatory response; Neonatal respiratory control

INTRODUCTION

Perinatal inflammation and infection are widely implicated in precipitating preterm birth and in contributing to both lung and brain morbidity in preterm infants (1). Bronchopulmonary dysplasia (BPD) is the most pervasive respiratory morbidity in this population. It is widely attributed to exposure to various combinations of hyperoxia, ventilation trauma and infection, and its development has been associated with the presence of proinflammatory cytokines in infant tracheal aspirates (2–4). This has led to considerable interest in studying the role of pulmonary inflammation and superimposed ventilatory strategies on the genesis of lung injury in preterm model animals (5,6). In preterm human infants, BPD is frequently accompanied by impairment of both neurodevelopment and brainstem function as measured by abnormal brainstem auditory evoked responses (7). The relationship between injurious inflammatory molecules in the immature lung and modulation of respiratory control at the level of the brainstem is, however, unclear.

Over the last decade, there has also been great interest in the association between cerebral palsy and both chorioamnionitis and increased concentrations of proinflammatory cytokines such as TNF- α , IL-1 β and IL-6 in amniotic fluid or cord blood (8,9). Animal models, such as lipopolysaccharide (LPS)-exposed, immature mice, demonstrate hypomyelination, suggesting a sensitivity of oligodendrocytes to the effects of intrauterine inflammation (10). Postnatal sepsis is also associated with delayed psychomotor development in preterm infants, accompanied by predominant abnormalities in white matter and, to a lesser extent, grey matter (11). It remains unclear whether a purely systemic cytokine response contributes to such sepsis-induced brain injury (12).

Although available data suggest that cytokine-mediated inflammatory responses contribute to the pathogenesis of both chronic neonatal lung injury and abnormalities in the immature brain, a direct relationship between these two organ responses to inflammation is not always apparent (13). Therefore, there is a need for greater understanding of the potential pathways and mechanisms by which lung inflammation initiates cytokine-mediated responses in the developing brain. The blood–brain barrier likely serves an important relay function between peripheral and central cytokine responses. However, it is also possible that inflammatory responses initiated in peripheral sites, such as the lung, may bypass the blood–brain barrier and provide a signal for cytokine production in the brain via afferent sensory nerves.

Apnoea is probably the most common clinical manifestation of sepsis in preterm infants, although the underlying mechanisms are poorly understood. Studies of this phenomenon in animal models are extremely limited. In newborn rodents, prostaglandin E₂ has been implicated in the respiratory depression elicited by intraperitoneal administration of the cytokine IL-1 β (14). In considering non-systemic or neural pathways that might impact on neonatal respiratory control, the nucleus tractus solitarius (nTS) is the main site of termination in the brainstem for vagal afferents and input from oxygen-sensitive peripheral chemoreceptors. In this series of studies, we, therefore, sought to characterize the relationship between inflammatory exposure in the immature lung and the cytokine response in the region of the brainstem that processes afferent input from the periphery. Our goals are to stimulate future study of downstream neurotransmitter-mediated effects of these and other cytokines and to apply these observations to other regions of the developing brain that are vulnerable to inflammatory insult and resultant pre- and postnatal brain injury.

EXPRESSION OF BRAINSTEM IL-1 β AFTER INTRAPULMONARY LPS EXPOSURE

Prior studies have focused on the ability of either systemic maternal, intra-amniotic or intraperitoneal neonatal LPS exposure to modulate proinflammatory cytokine expression and injury in newborn rodent lungs (15–17). We sought to minimize the systemic effects of these approaches by direct intratracheal instillation of LPS in rat pups (Fig. 1). This approach also acknowledged that the intrapulmonary route is a common portal for entry of infection both pre- and postnatally. We initially hypothesized that exposure of the immature respiratory tract to endotoxin would elicit a proinflammatory response in the brainstem.

Fischer 344 (F344) rats at postnatal age of 10–12 days ($n = 125$; Harlan Laboratories, Indianapolis, IN, USA) were employed. On the basis of comparative interspecies data on brain growth (18), it is estimated that this postnatal age corresponds to full-term human birth, although we fully acknowledge the challenge of correlating immature rodent data with human infants. All pups were reared by their mothers, fed rat chow and water *ad libitum*, and housed under standardized conditions approved by the Case Western Reserve University (CWRU), School of Medicine, Institutional Animal Care and Use Committee. Anaesthetized (xylazine and ketamine) pups were then instilled intratracheally via a modified tracheal cannula with either sterile pyrogen-free saline (1.5 mg/kg) or with 0.1 mg/kg of LPS (*Escherichia coli* endotoxin 055: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 anaesthesia within 30 min and were returned to the mothers for feeding. Deeply sedated pups were then killed at varying time intervals (2, 4, 8 and 24 h; $n = 6$ per treatment group) after LPS instillation. The medulla oblongata (rostral to the spinal cord and caudal to the ponto-medullary border) was excised to assess the mRNA expression profile for the proinflammatory cytokine IL-1 β by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) (19,20).

Our preliminary experiments demonstrated that the expression levels for IL-1 β were maximal at 2 h after LPS exposure with a subsequent decrease to baseline levels (Fig. 2). The maximal response at 2 h occurred in relation to the other time periods demonstrated in the figure. These data indicate that intrapulmonary endotoxin exposure elicits production of IL-1 β in the brainstem. We speculate that this may, in turn, regulate an acute inflammatory response and neuropeptide release at that site. These data are consistent with the prior observation that intraperitoneal IL-1 β administration to rats elicits an increase in IL-1 β mRNA expression in the brain (20). We next sought to test the hypothesis that a vagally mediated neural mechanism may be responsible for this response of IL-1 β production.

VAGAL REGULATION OF BRAINSTEM IL-1 β EXPRESSION

Rat pups ($n = 10$ per group) were anaesthetized, and the vagus nerves were isolated bilaterally via a midline cervical incision. Both nerves were transected, and the animals were allowed to recover with their mother for at least an hour following which intratracheal saline (1.5 mg/kg) or LPS (0.1 mg/kg) was instilled as already described. Figure 3 shows the effect of cervical vagotomy on medullary transcript levels for IL-1 β 2 h post LPS administration. Vagotomy clearly blunted the increase in LPS-mediated medullary mRNA for IL-1 β . Compared with non-vagotomized rat pups in which LPS induced a twofold 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 h post LPS administration.

Our data suggest that activation of vagal sensory lung receptors provides an important mode of transmitting immune signals from the immature lung to the brain. LPS exposure of the lung would be anticipated to elicit an intrapulmonary inflammatory response and activate various pulmonary receptor-mediated afferent pathways. Precise characterization of that afferent pathway and the resultant vagally mediated neural signal are subjects for future

study. Meanwhile, we sought to demonstrate an association between these molecular events and neonatal respiratory control focused on the hypoxic ventilatory response. The rationale for this approach is that lung vagal afferents terminate principally in the nTS, and hypoxic ventilatory responses elicited by peripheral chemoreceptors are processed first at this brainstem site.

LPS-INDUCED INHIBITION OF THE VENTILATORY RESPONSE TO HYPOXIA

Two to three hours after the intratracheal instillation of saline or LPS, pups were placed in a thermoregulated flowthrough barometric plethysmograph (BUXCO Research Systems, Wilmington, NC, USA). This time point for investigation corresponded to the maximal induction of proinflammatory cytokines in the medulla oblongata, based on the time periods measured and corresponding with our physiologic studies. 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 min, the respiratory response to hypoxia was tested (10% inspired oxygen, balance N_2 for 5–10 min).

Baseline values for minute ventilation in rat pups did not significantly differ between saline- (114 ± 6 mL/min/100 g) and LPS instillation (137 ± 11 mL/min/100 g). As seen in Figure 4, LPS instillation resulted in a significant attenuation of ventilatory response to hypoxia (10% O_2). The per cent increase in ventilation over baseline was reduced at 2, 5, 8 and 10 min after hypoxic exposure in LPS-exposed versus control pups. This attenuated hypoxic response was attributable to decreases in both tidal volume and respiratory rate. No significant differences were observed in the posthypoxic recovery phase.

The glomus cells of the rat carotid body, which function to detect change in arterial pO_2 and modulate respiratory drive, have been shown to express interleukin receptors, suggesting a potential immune sensing mechanism at this site (21). We therefore sought 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- to 12- day-old pups ($n = 6$), the carotid sinus nerve (CSN) was transected under anaesthesia. The pups were then left to recover with the mother for 48 h, and 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. The effect of CSN transection in mediating the blunted hypoxic ventilatory response in LPS-instilled rat pups is shown in Figure 5. As expected, there was no significant increase in minute ventilation in response to 10% O_2 after CSN transection in either group of rat pups. However, the ventilatory response at 2 and 5 min of 10% oxygen exposure was significantly depressed after LPS exposure, suggesting a centrally mediated respiratory inhibition induced by LPS.

These data demonstrate an association between centrally mediated hypoxic ventilatory depression and expression of cytokine message in the brainstem. In ongoing studies, we seek

to employ systemically or locally administered IL-1 β receptor blockade to document a causal relationship between these two phenomena. Existing data do provide evidence for both cytokine-mediated respiratory excitation and depression in early life. We have recently reported that under *in vivo* conditions intrapulmonary LPS can elicit an initial increase in respiratory frequency; however, IL-1 β injection into the nTS under *in vitro* conditions decreased the frequency of respiratory neural output (22). Future study should include characterization of cytokine-mediated neurotransmitter pathways implicated in aberrant respiratory control. Available data in immature animal models implicate prostaglandin and possibly nitric oxide signalling (14,23).

LOCALIZATION OF IL-1 β IN THE MEDULLA OBLONGATA

We have begun to localize the sites of IL-1 β cytokine expression with the developing brainstem. Following LPS or saline instillation, as already described, paraformaldehyde-fixed medullary sections were prepared for IL-1 β immunohistochemical analysis. Our focus was on the nTS as this is the major area of termination for afferents from both the vagus nerve and CSN, the latter being responsible for transmitting peripheral chemoreceptor responses. Data from a rat pup are shown in Figure 6 and demonstrate IL-1 β expression localized in the nTS region of the caudal medulla oblongata and enhanced after LPS exposure. Immunoreactivity for IL-1 β was also observed in the area postrema, a circumventricular organ in proximity to vascular elements. Enhancement of IL-1 β expression in the nTS was also documented after LPS exposure by a modest increase in immunoreactivity (Fig. 7). Prior data from mature rats have demonstrated IL-1 β like immunoreactivity in immune cells in apposition with neuronal elements including vagal afferent fibres (24,25). We are currently characterizing the specific neuronal and glial cell types that express IL-1 β in rat pups exposed to a proinflammatory stimulus. The current preliminary data demonstrated in Figure 7 do not support expression of IL-1 β in neurons of the nTS at this age. This is in contrast with our preliminary ongoing experiments in which LPS-exposed pups do demonstrate increased expression of IL-1 β in large neurons of the hypoglossal nucleus regulating respiratory output to muscles of the upper airway.

SPECULATION

The immature lung and brain are the predominant sites of longer-term morbidity in former preterm infants; inflammation has been implicated in the pathophysiologic process at both sites. Neonatal respiratory control, which is highly vulnerable in this population, serves as an important link between the developing respiratory and central nervous system. Proinflammatory states in preterm infants may be caused by chorioamnionitis, postnatal sepsis or even the recurrent hypoxia/reoxygenation cycle that is a consequence of immature respiratory control (26). Given the reservation that immature rodent data may not always coincide with human data, we suggest that intrapulmonary inflammation may, in turn, impair neonatal respiratory control and create a vicious cycle as proposed in Figure 8. We also speculate that physiologic and molecular studies focused on neonatal respiratory control provide a novel opportunity to characterize the role of inflammation in precipitating neonatal pathophysiology. Our findings may therefore have potentially broader translational implications for neonatal well-being.

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Key notes

- Neural pathways provide a link between proinflammatory mechanisms in the immature lung and brain. Respiratory control provides a useful tool to characterize the pathophysiologic link between intrapulmonary inflammation and responses of the immature central nervous system.

Figure 1.

A representation of our model to test the hypothesis that vagal afferents from the lipopolysaccharide (LPS)-exposed immature lung may feed back to the nTS and alter respiratory neural output (RVLM, rostral ventrolateral medulla; CSN, carotid sinus nerve; nTS, nucleus tractus solitarius).

Figure 2.

Representative gel image of RT-PCR products ($n = 2$) in brainstem for $IL-1\beta$ and HPRT (housekeeping) following lipopolysaccharide (LPS) instillation in trachea. Saline was instilled in control (CON) pup. M represents 100-bp DNA fragment. Note that at 2 h post LPS instillation, there was maximal $IL-1\beta$ induction over the time points measured. [Adapted and modified from: Balan et al. (19)].

Figure 3.

IL-1 β mRNA expression in the medulla oblongata 2 h after saline (C) or lipopolysaccharide (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. Vagotomy, *per se*, did not alter the level of baseline medullary IL-1 β mRNA expression. Proinflammatory cytokine expressions were normalized to the expression of HPRT, a constitutively expressed housekeeping gene. Data are expressed as mean $\pm$ SEM. $p < 0.05$ between groups, $n = 10$ per group. [Adapted and modified from: Balan et al. (19)].

Figure 4.

Effect of intratracheal lipopolysaccharide (LPS) on minute ventilation during exposure to 10% oxygen in 10- to 12-day-old rats ($n = 10$ per group). Percentage change in minute ventilation from baseline was recorded in room air (time 0) in response to 10% oxygen exposure and during the post-hypoxic response in room air using whole body plethysmography. Note: the hypoxic ventilatory response was significantly decreased in LPS (closed squares) as compared to saline-instilled control (open squares) rat pups. Data are expressed as mean $\pm$ SEM. * $p < 0.05$ between groups for the same time point. [Adapted and modified from: Balan et al. (19)].

Figure 5.

Hypoxia-induced ventilatory changes in carotid sinus nerve (CSN)-transected pups prior to (control) and 2–3 h post lipopolysaccharide (LPS) treatment ($n = 6$ per group). Minute ventilation represented as percentage change from the baseline was recorded during 10% oxygen exposure followed by recovery in room air using whole body flow plethysmography. Following LPS exposure (closed squares), there was a significant ventilatory depressant response to hypoxia in CSN-denervated rat pups when compared to the response prior to LPS exposure (open squares). Data are expressed as mean $\pm$ SEM. * $p < 0.01$ between groups for the same time point [Adapted and modified from: Balan et al. (19)].

Figure 6.

Photomicrographs and schematic diagram showing localization of IL-1 β in the nucleus tractus solitarius (nTS) 2–3 h following tracheal instillation of saline (control) or lipopolysaccharide (LPS). Scale bar represents 50 μ m. AP, area postrema. [Adapted and modified from: Balan et al. (19)].

Figure 7.

Immunofluorescent photomicrographs showing IL-1 β immunoreactivity at 2 h post-lipopolysaccharide (LPS) instillation into the trachea of 11-day-old rat pups. Compared to baseline expression (i.e. saline treated rat pups) in left upper panel, LPS instillation induced a modest increase in IL-1 β (red label in upper right panel) in the nucleus tractus solitarius (nTS), which appeared absent from the labelled neurons (green label) at this site (lower two panels). NeuN: neurons immunolabelled green. IL-1 β : cytokines immunostained red. Scale bar represents 50 μ m.

Figure 8.

A schematic representation of pre- and postnatal proinflammatory stimuli that may contribute to neonatal pathophysiology. These include chorioamnionitis, postnatal sepsis and the hypoxia/reoxygenation cycle resulting from impaired respiratory control. We have now demonstrated (dashed lines) that intrapulmonary inflammation may further inhibit respiratory neural output and contribute to a potentially vicious cycle as proposed in this figure.