Bacterial endotoxin sensitizes the immature brain to hypoxic–ischaemic injury

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Abstract

Epidemiological studies show a markedly increased risk of cerebral palsy following the combined exposure of infection and birth asphyxia. However, the underlying mechanisms of this increased vulnerability remain unclear. We have examined the effects of a low dose of bacterial endotoxin on hypoxic–ischaemic injury in the immature brain of rats. Bacterial endotoxin (lipopolysaccharide 0.3 mg/kg) was administered to 7-day-old rats 4 h prior to unilateral hypoxia–ischaemia and the neurological outcome was determined 3 days later. Rectal temperature and cerebral blood flow was measured during the study and the expression of CD14 and toll-like receptor-4 mRNA in the brain was examined. We found that a low dose of endotoxin dramatically sensitizes the immature brain to injury and induces cerebral infarction in response to short periods of hypoxia–ischaemia that by themselves caused no or little injury. This effect could not be explained by a reduction in cerebral blood flow or hyperthermia. In association with the sensitization of injury we found an altered expression of CD14 mRNA and toll-like receptor-4 mRNA in the brain. These results suggest that the innate immune system may be involved in the vulnerability of the immature brain following the combination of infection and hypoxia–ischaemia.

Introduction

Birth asphyxia was previously considered the major cause of perinatal brain injury and cerebral palsy, however, over the past decades other aetiologies have been recognized, including infections, metabolic disease, intraventricular growth retardation and coagulopathies, acting alone or in combination (Badawi et al., 1998). Birth asphyxia is commonly preceded by antenatal infections (Grether & Nelson, 1997) and antenatal infection is intrinsically associated with the development of cerebral palsy. Furthermore, the combined exposure of infection and birth asphyxia dramatically increases the risk of spastic cerebral palsy (OR: 78, 95% CI: 4.8–406) and spastic quadriplegic cerebral palsy (OR: 367, 95% CI: 19–1974) (Nelson & Grether, 1998) fail to respond to LPS. The cytoplasmic tail of TLRs has a similar sequence homology to the interleukin-1 receptor I and TLR stimulation mediate synthesis of pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) (Kopp & Medzhitov, 1999). Increased levels of IL-1β and TNF-α are also expressed in the immature central nervous system in response to hypoxia–ischaemia (HI) and treatment with interleukin-1 receptor antagonist (IL-1ra) attenuates the injury (Hagberg et al., 1996). These studies suggest that activation of the innate immune system could exert toxic effects on the brain.

The purpose of this study was to investigate the combined effects of bacterial endotoxin and HI on the vulnerability of the immature brain by examining neuropathology, cerebral blood flow, temperature and expression of TLR-4 and CD14 mRNA in the 7-day-old rat.

Materials and methods

Lipopolysaccharide in combination with hypoxia–ischaemia

Wistar rat pups (Mollegaard Breeding and Research Centre A/S, Skensved, Denmark) were housed in accordance with the guidelines of the Animal Ethics Committee of the University of Göteborg. Animals received either a single dose of LPS (Sigma LPS O55:BS phenol extracted; n = 62, 0.3 mg/kg, i.p.) or vehicle (NaCl, 0.9%, n = 55). The dose of 0.3 mg/kg of LPS alone had no effect on postnatal day 10 body weight (NaCl: 21.8 ± 0.4 g, n = 8; LPS: 19.9 ± 0.7 g, n = 12, P > 0.05) did not result in mortality and there was no indication of brain damage or glial activation. Based on these...
results the dose of 0.3 mg/kg was used in further experiments to simulate a subclinical infection without other specific adverse physiological effects on the animals.

To induce the combination of endotoxin and HI exposure, LPS or vehicle was administered to 7-day-old rat pups. Four hours after the injections rat pups were subjected to unilateral HI as previously described (Rice et al., 1981). Briefly, the left common carotid artery was cut between ligatures after which they were exposed to 7.7% oxygen in nitrogen in a humidified chamber at 36 °C. Pups were submitted to varying duration of hypoxia; 50 (n = 30), 40 (n = 25), 30 (n = 22), 20 (n = 22) or 10 (n = 18) min.

**Tissue preparation, histological and immunohistochemical procedures**

On postnatal day 10, pups were deeply anaesthetized (Pentothal® Natrium, 50 mg/mL) and perfused intracardially with 0.9% NaCl followed by 4% buffered formaldehyde. Coronal paraffin sections of the forebrain (5 μm) were prepared and stained with thionin/acid fuchsin (Mallard et al., 1993) for morphological analysis. Adjacent sections were stained for microtubule-associated protein-2 (mouse-anti-rat-MAP-2, 1 : 2000, gift from Lars Rosengren) and immunoreactivity was visualized using 3,3-diaminobenzidine (DAB) as previously described (Gilland et al., 1998). Microglia were detected using lectin histochemistry. Sections were boiled in citric acid buffer (0.01 M, pH 6) and incubated with 10 μg/mL *Griffonia simplicifolia* isoelectin-B4-horseradish peroxidase conjugate (Sigma L5391) overnight (4°C) and visualized using DAB.

**Neuropathological analysis**

Qualitative morphological analysis of the injured hemisphere was performed on sections stained with thionin/acid fuchsin, and the expression of microglia and GFAP-positive cells were examined in adjacent sections.

The area of infarction was measured on sections stained with thionin/acid fuchsin (Mallard et al., 1993). The proportion of infarction was calculated by subtracting the MAP-2-positive area of the ipsilateral hemisphere from the contralateral hemisphere and expressed as percentage of the contralateral hemisphere.

**Reverse transcription-polymerase chain reaction**

The mRNA expression of TLR-4, CD14 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was examined in the brains using reverse transcription-polymerase chain reaction (RT-PCR) in: vehicle (n = 8), LPS 6 h after injection (n = 8), vehicle/20 min HI (n = 8) at 2 h after HI and LPS/20 min HI (n = 8) at 2 h after HI. Animals were perfused in situ with saline and the brains immediately removed and frozen in isopentane on dry ice. Total RNA was extracted from each cerebral hemisphere as previously described (Blomgren et al., 1999). Each PCR (25 μL) contained 1/25 of the cDNA synthesis reaction, 0.2 mM dNTP, 0.5 μM upstream (U) and downstream (D) primers (TLR-4 U: 5’AAAGAGCCTGGAAATACCTGGAC; TLR-4 D: 5’GAATTGCTACAGTGGTACC; GenBank accession number AF057025; CD14U: 5’GACCGAGCGAGGAAAGTTG; CD14D: 5’ACGCATACCCGCTAGAAT; GenBank accession number AF087943; GAPDH U: 5’ACCACTTGGAAGGCCTGG; GAPDH D: 5’CTCAGTGATGCCCCAGATGC; GenBank accession number M17701, primers were from Cyber Gene AB, Huddinge, Sweden), 2.5 μL 10X PCR Buffer (Sigma) and 1 U of Taq DNA polymerase (Sigma). The PCR cycling included: denaturation 20 s (5 min before the first cycle) at 94°C, annealing 20 s at 58 °C (TLR-4) or 60 °C (CD14and GAPDH) and elongation for 30 s at 72 °C and a final elongation of 5 min at 72 °C. The cycle numbers (38 cycles for TLR-4, 32 cycles for CD14and 20 cycles for GAPDH) were chosen such that the PCR product would be in the linear phase of amplification. The PCR products were separated on 1.5% agarose gels containing ethidium bromide. The gels were exposed in a LAS 1000 cooled CCD camera and the intensity of the PCR product was measured (Fujifilm, Tokyo, Japan).

**Temperature measurements**

The rectal temperature was measured in vehicle/20 min HI (n = 6) and LPS/20 min HI (n = 7) animals using a thermistor probe (BAT-12, Physiotemp Instruments, Clifton, NJ, USA). Measurements were made prior to injections and prior to, during and 1 h post-hypoxia in the humidified chamber at 32°C. Temperatures were registered every 5 min.

**Cerebral blood flow measurements**

Cerebral blood flow (CBF) was measured by the autoradiographic iodio-(14C)antipyrine method adapted to the immature rat (Gilland et al., 1998). A total of 35 animals from four different litters was assigned to either the vehicle/20 min HI (n = 16) or the LPS (0.3 mg/kg)/20 min HI group (n = 19). Immediately after the hypoxic episode 20 μCi 4-iodo-(N-methyl-14C)-antipyrine (Amersham Pharmacia Biotech, UK) in 0.2 mL saline was injected s.c. in the neck of the rat. After 60 s the animals were decapitated and blood was collected. The brains were immediately dissected out and divided into right and left hemispheres. Each hemisphere was weighed and counted in a liquid scintillation counter.

**Statistics**

All data are expressed as means ± SEM. Comparisons between groups were performed using the Mann–Whitney U-test for unpaired groups.

**Results**

**The effects of combined lipopolysaccharide and hypoxia–ischaemia on mortality and neuropathology**

There was no mortality in vehicle-treated pups at any duration of HI. LPS-treated animals showed increased mortality with increasing length of hypoxia, from 0% at 10 min HI to 44% at 50 min HI. In vehicle-treated animals brain damage increased with increasing duration of hypoxia, but no cortical infarctions were observed with less than 30 min of HI (Fig. 1). LPS increased the area of infarction in combination with 20 min and 30 min of HI compared with vehicle-treated animals (Figs 1 and 2, P < 0.01) and showed a similar degree of damage as vehicle-treated animals subjected to 50 min of HI (Fig. 1). In animals with severe injury, the damage was localized to the ipsilateral hemisphere and involved primarily the cerebral cortex, hippocampus, striatum and thalamus (Fig. 2). GFAP-positive cells were found in the area of damage except for the most severe areas of infarction where GFAP-positive cells were absent. Microglia expression was seen in all areas of damage and was also present in the area of infarction 3 days after the insult. No histological damage was detected in vehicle-treated animals subjected to 10 min HI, while selective neuronal loss or small infarctions were noted in 3/10 LPS/10 min HI animals in the area of stria terminalis, internal capsule,
The groups subjected to 10 or 50 min of HI.

There was a tendency towards an increased size of infarction in the endotoxin group compared with vehicle-treated animals. At 40 min of HI, there was a tendency towards an increased size of infarction in the endotoxin group \((P < 0.05)\), while there were no differences in the groups subjected to 10 or 50 min of HI.

There was no injury in the cerebral cortex or the hippocampus in these animals.

**RT-PCR, TLR-4 and CD14 mRNA**

No differences in the constitutive expression of the housekeeping gene GAPDH was found during the different experimental paradigms (Fig. 3). The mRNA expression of CD14 was present in the brains of control animals and increased at 6 h following LPS injection \((P < 0.01)\) and 2 h following LPS/20 min HI compared with controls (Figs 3 and 4, \(P < 0.05\)). There was no change in the CD14 mRNA expression 2 h following vehicle/20 min HI compared with control animals \((P > 0.05)\). The mRNA for TLR-4 was similar in control animals and 6 h following LPS-treatment (Figs 3 and 4, \(P > 0.05\)). Two hours following the combination of LPS/20 min HI, the expression of TLR-4 mRNA was decreased (Figs 3 and 4, \(P < 0.05\)). There was no change in the expression of TLR-4 mRNA in animals subjected to vehicle/20 min HI compared with control pups \((P > 0.05)\). In all groups, the expression was similar in ipsilateral and contralateral hemispheres.

**Temperature measurements**

There was no difference in rectal temperature at any time-point between NaCl/20 min HI and LPS/20 min HI before (NaCl: 33.8 ± 0.7 °C; LPS: 32.8 ± 0.8 °C, \(P > 0.05\)), during HI (NaCl: 35.7 ± 0.2 °C; LPS 35.5 ± 0.3 °C, \(P > 0.05\)) or during 1 h following HI (NaCl: 34.4 ± 0.5 °C; LPS: 33.8 ± 0.5 °C, \(P > 0.05\)).

**Cerebral blood flow measurements**

There was no difference in blood flow in either the contralateral hemisphere (NaCl/20 min HI: 93.5 ± 14.7 mL/100 g/min; LPS/20 min HI: 87.7 ± 24.8 mL/100 g/min, \(P > 0.05\)) or the ipsilateral hemisphere (NaCl/20 min HI: 43.9 ± 8.1 mL/100 g/min; LPS/20 min HI: 40.5 ± 6.4 mL/100 g/min, \(P > 0.05\)) between the two groups. In both groups, cerebral blood flow was reduced in the ipsilateral hemisphere compared with the contralateral hemisphere \((P < 0.05)\).

**Discussion**

The major finding in this study was that bacterial endotoxin dramatically increased the vulnerability of the immature brain to HI. The combination of 20 min of HI and endotoxin induced as extensive brain injury as observed following 40–50 min of HI without LPS. These results were obtained in spite of the fact that endotoxin alone did not affect the general health of the animals, i.e. there was no mortality and the weight gain was similar between animals injected with endotoxin and vehicle. Sensitization of brain injury by LPS has not previously been shown. On the contrary other studies have demonstrated preconditioning effects with reduced brain injury when LPS was administered several days prior to middle cerebral artery occlusion in adult rats (Dawson et al., 1999). However, sensitization of the immature brain to injury has been demonstrated following repeated brief episodes of HI and appears to be dependent on the interval between insults, with more frequent insults resulting in increased injury (Mallard et al., 1993). A similar temporal relationship could exist between LPS and HI.

Temperature during and after HI is critical for the development of brain injury. Indeed, a recent study in adult rats demonstrated that LPS only affected brain injury in febrile animals suggesting that hyperthermia was the critical factor (Thornhill & Asselin, 1998). In the present study, we found no significant effect of LPS on rectal temperature. There was a tendency, however, towards slightly lower temperatures (nonsignificant) before and after HI in LPS-treated animals, which cannot explain the dramatic increase in injury. In contrast, there is considerable evidence to show that hyperthermia is neuroprotective in this animal model (Bona et al., 1998). It is important to point out that brain temperature closely follows core temperature in these small rats and the rectal temperature is therefore considered to be representative of that in the brain.

Endotoxin may have systemic effects by lowering arterial blood pressure with subsequent development of cerebral ischaemia and brain injury. Administration of bacterial endotoxin to newborn dogs caused injury specifically in those regions with a substantial fall of cerebral blood flow (Young et al., 1982). We compared the hemispheric blood flow in animals subjected to NaCl/20 min HI (no brains with cerebral infarction) with animals that had received LPS prior to 20 min HI (most brains with cerebral infarction) and found no differences in cerebral blood flow between the groups. It is also important to stress that the blood flow of the contralateral hemisphere was within the normal range in both the LPS- and vehicle-treated group suggesting that the arterial pressure was not markedly affected by this dose of LPS. However, LPS could still have some effects on local oxygen supply to the brain as regional blood flow was not measured in the present study. Furthermore, LPS may
increase leukocyte plugging of microvessels that may not be detected by the iodoantipyrine technique, which is a measure of plasma flow rather than red corpuscular flow. It is, however, unlikely that such differences in flow could account for the large difference in cerebral infarction size noted in the present study.

Similar to previous reports in adult rats we found an upregulation of the mRNA expression of CD14 in the immature brain following LPS stimulation (Lacroix et al., 1998). In contrast, LPS exposure to human microglia has been reported to result in a decrease in CD14 mRNA expression (Becher et al., 1996). In the present study, the
increase in CD14 mRNA expression was similar following LPS alone (animals with no infarction) and LPS/20 min HI. The expression was also similar in both the injured and uninjured hemispheres, suggesting that the change in CD14 expression was not directly involved in the enhancement of the injury.

We also demonstrated the mRNA expression of TLR-4 in the immature central nervous system, which is interesting considering that the LPS response appears to depend on these receptors (Poltorak et al., 1998). In contrast to the expression of CD14, the TLR-4 expression was unchanged 6 h following LPS exposure. Mouse peritoneal macrophages demonstrate a downregulation of the expression of TLR-4 mRNA between 2.5 and 5 h after LPS exposure (Nomura et al., 2000), while monocyes stimulated with LPS show an increased TLR-4 expression, peaking at 15–30 min after LPS exposure (Jiang et al., 2000). In animals with brain injury we noted a decrease in TLR-4 mRNA 2 h following LPS/20 min HI. In contrast, myocardial infarction induces an increase in the expression of TLR-4 mRNA at 1 and 4 days after injury (Frantz et al., 1999). Additional studies are needed to clarify the time-course of TLR-4 expression and its relationship to the development of immature brain injury.

TLR stimulation has been associated with cell death and there appears to be a link between at least the activation of TLR-2 and apoptosis involving Fas-associated death domain and caspase-8 (Aliprantis et al., 2000). The activation of TLRs initiates inflammatory reactions involving oxygen free radicals, nitric oxide and the synthesis of pro-inflammatory cytokines such as IL-1β and TNF-α (Kopp & Medzhitov, 1999). We have observed an increased expression of IL-1β and TNF-α in the immature brain following administration of endotoxin (unpublished observation) and HI, and treatment with IL-1ra-attenuated the injury (Hagberg et al., 1996). Furthermore, the effects of LPS on the immature brain may be particularly severe since LPS appears to induce more pronounced inflammation in the immature than in the adult central nervous system (Lawson & Perry, 1995).

In summary, we have shown that endotoxin markedly sensitizes the immature brain to HI injury. This effect could not be explained by alterations in cerebral blood flow or hyperthermia. The expression of TLR-4 mRNA and CD14 mRNA in the immature brain was altered in association with the sensitization, however, their exact role in the development of the injury remains unclear. Speculatively, exposure to bacterial endotoxin affects the innate immune system, which may...
have severe consequences on the brain in case of a secondary insult. We propose that this may be one of the underlying mechanisms of the marked increased risk of cerebral palsy seen in infants that have been exposed to the combined effects of infection and birth asphyxia.

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Abbreviations

CBF, cerebral blood flow; D, downstream primer; DAB, 3,3-diaminobenzidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; HI, hypoxia-ischaemia; IL-1, interleukin-1; IL-1ra, interleukin-1 receptor antagonist; LPS, lipopolysaccharide; MAP-2, microtubule-associated protein-2; TLR, toll-like receptor; TNF-α, tumour necrosis factor-α, U, upstream primer.

References


