0031-3998/11/7001-0010
PEDIATRIC RESEARCH

Vol. 70, No. 1, 2011
Printed in U.S.A.

ARTICLES

Lipopolysaccharide-Induced Preconditioning Against Ischemic Injury Is Associated With Changes in Toll-Like Receptor 4 Expression in the Rat Developing Brain

EDWARD HICKEY, HUI SHI, GLEN VAN ARSDELL, AND RAND ASKALAN

Department of Surgery [E.H., G.V.A.], Program of Neuroscience and Mental Health, Research Institute [H.S., R.A.], Department of Pediatrics [R.A.], The Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada

ABSTRACT: Lipopolysaccharide (LPS) preconditioning reduces ischemic injury in adult brain by activating Toll-like receptor 4 (TLR-4). We sought to investigate the effect of brain maturity on the efficacy of LPS preconditioning against hypoxic-ischemic (HI) injury in the developing rat brain. Rat pups at the specified age were randomly assigned to LPS-treated (0.1 mg/kg) or saline-treated groups. HI injury was induced 48 h later by occluding the right common carotid artery followed by transient hypoxia. Brains were removed 1 wk after HI injury, and infarct volumes were compared between the two groups. TLR-4 expression was also compared among different ages. We found that LPS treated P7, P9, and P14 rat pups had significantly smaller infarct volume compared with saline-treated pups ($p = 0.006$, 0.03, and 0.01, respectively). This significant reduction in infarct volume was not observed in P3 and P5 rats. TLR-4 expression was significantly higher in older rats compared with P3 and P5 rats ($p < 0.01$). These findings indicate that LPS-induced preconditioning is a robust neuroprotective phenomenon in the ischemic developing brain that is age dependent. Pattern of TLR-4 expression is also affected by brain maturity and likely to be responsible for differences in the efficacy of LPS preconditioning. **(*Pediatr Res* 70: 10–14, 2011)**

Delayed preconditioning is a potent protective phenomenon in which a tissue like the brain develops resistance to ischemic injury after exposure to a variety of subinjurious stimuli such as brief ischemia, hypoxia, or low dose of lipopolysaccharide (LPS) (1–5). This neuroprotective effect is prolonged and can last for several days after the exposure to the subinjurious stimulus. LPS neuroprotection is well documented in adult stroke animal models (6–9), whereas studies on LPS preconditioning in the hypoxic-ischemic (HI) immature brain are scarce (10,11). In contrast to ischemic adult brain, LPS given to postnatal d 7 (P7) rat pups 72 h before HI injury increased brain damage but was neuroprotective when administered 24 h before the HI insult (10). Recently, we have shown that LPS preconditioning protected against cardiopulmonary bypass-related brain injury in neonatal piglets when administered 72 h before the procedure (11). These conflicting results on the effect of LPS treatment before HI injury in the developing brain may be attributed to differences in LPS doses, time interval between LPS treatment and the severity of the HI insult. Moreover, the influence of brain maturity on the pathophysiology of ischemic injury is well documented (12–14). It is conceivable, therefore, that there may be a critical developmental window at which the phenomenon of delayed preconditioning can be used to protect the developing brain against ischemic injury.

LPS is a known specific ligand for Toll-like receptor 4 (TLR-4), one of the Toll-like receptors (TLRs) that recognize foreign pathogens. LPS-mediated ischemic tolerance in the adult brain occurs by stimulation of TLR-4 signaling pathways (6,15), resulting in alteration in gene expression profiles (16) and dampening of ischemia-induced inflammatory response. It is not known, on the other hand, whether TLR-4 is expressed in the immature brain.

In this study, we investigated the effect of brain maturity on LPS-induced preconditioning in the HI rat brain. We hypothesized that the efficacy of TLR-4 mediated LPS preconditioning is influenced by brain maturity. Consequently, characterizing the effect of age on this phenomenon may be crucial for its translation as neuroprotective therapy for infants at high and predictable risk of ischemic brain injury.

METHODS

Preconditioning. Pairs of pregnant Wistar rats underwent natural delivery of their litters within our animal research laboratory. Litters were randomized to intraperitoneal injection of LPS or saline and nursed together with their dam. At the prespecified age, each litter was blindly injected with either 0.1 mg/kg of *Escherichia coli* lipopolysaccharide serotype 0111:B4 (Sigma Chemical Co.-Aldrich, St. Louis, MO) or an equivalent volume of saline placebo. After injection, animals were returned to a warmed incubator and housed with their dam. We did pilot studies to identify the optimum LPS dose to induce maximum protection in P7 rat pups. There was no significant difference in efficacy between low and high doses of LPS (Fig. 1); so, we

Received October 12, 2010; accepted February 2, 2011.

Correspondence: Rand Askalan, M.D., Ph.D., Division of Neurology, Hospital for Sick Children, 555 University Avenue, Toronto, ON M5G 1X8, Canada; e-mail: r.askalan@utoronto.ca

Supported by a grant from the Children's Heart Foundation, Chicago, IL, and by Clinician-Scientist Salary Award from Heart & Stroke Foundation of Ontario (CS6248) [R.A.].

Abbreviations: GFAP, glial fibrillary acidic protein; **HI,** hypoxic-ischemic; **LPS,** lipopolysaccharide; **TLR,** Toll-like receptor

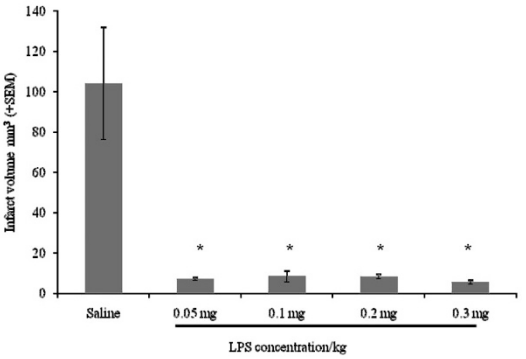

Figure 1. Low doses of LPS are adequate to achieve LPS-induced neuroprotection in P7 ischemic rat brain. Intraperitoneal injections of LPS at doses ranging from 0.05 to 0.3 mg/kg were given to P7 rats 48 h before HI injury. The animals were then killed 1 wk after the HI insult. Brains were immediately removed and processed for H&E staining. Total Infarct volume was calculated according to Cavalieri principle and compared with saline treated rats. There was no added benefit of using higher doses of LPS.

choose a low LPS dose of 0.1 mg/kg injected intraperitoneally 48 h before the HI insult. Animals were killed 1 wk later. Untreated control animals were injected with equal volume of normal saline. The study was undertaken with full approval of the Research Ethics Board and Animal Research Department at The Hospital for Sick Children.

HI insult. We used the Rice-Vannucci model (RVM), the most commonly used model to study HI brain injury in the developing brain (17). Rats (Wistar) aged 7–10 postnatal days correspond to term human newborn (14). Unilateral internal carotid artery ligation in rat pups followed by exposure to 8% hypoxia for approximately 65 min causes a reproducible unilateral infarct ipsilateral to the ligated artery involving caudate, putamen, hippocampus, and cortex (18). Body temperature was maintained at 37–37.5°C during hypoxia using an incubator. Animals were then killed at 1 wk after the HI insult using pentobarbital. Brains were removed and processed to be used in hematoxylin and eosin (H&E) and immunohistochemistry studies.

Assessment of brain damage. Treatment protocol described above was applied to pups at the specified age groups. Brains were immediately removed 1 wk after the HI insult, fixed, embedded in paraffin, and cut into 5-μm coronal sections. The total area of brain tissue loss was measured on H&E-stained sections using the Nikon NIS-Element Basic Research Image analysis software system, version 3.0. Total infarct volume in the whole effected hemisphere was calculated according to Cavalieri principle: $V = \sigma APt$, where V is the total volume expressed as mm^3, σA is the sum of the areas measured, P is the inverse of the section sampling fraction (1/200), and t is the section thickness (5 μm) (19). Adjacent sections were deparaffinized to be stained with Fluoro-Jade (Chemicon, MA) as previously described (11). Fluoro-Jade is an anionic fluorochrome that selectively stains injured neurons. Infarct volumes and number Fluoro-Jade positive cells were compared between the LPS-treated and untreated (saline) rat pups.

TLR-4 expression. Brains from rat pups aged P3, P5, P7, P9, and P14 (n = 6 rats for each age group) were removed and immediately fixed in 10% formalin. Paraffin-embedded coronal sections were cut (8 μm) at the level of the hippocampus, dewaxed with xylene, hydrated, and pretreated with heat-induced antigen retrieval technique. Sections were then stained with rabbit anti-TLR-4 (1:50; Santa Cruz) at 4°C over night. TLR-4 expression was then detected by goat anti-rabbit horseradish peroxidase (HRP; 1:100; Chemicon) for 1 h at room temperature and DAB substrate kit for Peroxidase/Vector/SK-4800. The number of TLR-4 positive cells were counted in 4–5 high power fields (40×) using Image J computer software (National Institutes of Health, Bethesda, MD) and compared among the different age groups.

Double staining. Antibody for CD68, glial fibrillary acidic protein (GFAP), and neurofilaments (NF) are well-established marker for detecting microglia, astrocytes, and neurons, respectively. Double staining with anti-CD68/anti-TLR4, anti-GFAP/anti-TLR4, and anti-NF-TLR4 was done to determine whether microglia, astrocytes, and/or neurons in P7 developing brain express TLR-4. TLR-4 staining was carried out as described above. Then, the immunohistochemical procedure for CD68, GFAP, and NF was performed. Sections were retreated with endogenous peroxidase and biotin before incubation with anti-mouse CD68 at 1/50 dilution (Santa Cruz), anti-mouse GFAP 1:1000 (Sigma Chemical Co.), or anti-chicken NF at 1:200 (Chemicon) for 60 min at room temperature. Immunodetection was performed employing the Elite ABC detection system (Vector Laboratories, Burlingame, CA), as described by the manufacturer, in conjunction with the Vector VIP substrate kit (Vector Laboratories), as the chromogen, producing a purple reaction product. The counterstain of preference was hematoxylin, for blue nuclear detail.

Statistical analysis. Comparison of continuous data between experimental groups was made using unpaired t test or one-way ANOVA for groups more than two. Normality was evaluated by the Kolmogorov-Smirnov test. Results were considered to be statistically significant if two-tailed p value was ≤ 0.05. All data were presented as mean ± SEM, and statistical analysis was done using GraphPad InStat (GraphPad Softwear Inc, San Diego).

RESULTS

LPS-induced preconditioning is modulated by brain maturity. Low dose of LPS (0.1 mg/kg), given to P7 rat pups 48 h before exposing them to HI injury, reduced brain damage as shown for example by the reduction in fluoroscopic staining of irreversibly injured hippocampal neurons in the LPS-treated pups when compared with untreated pups (Fig. 2*B* and *D*). In fact, LPS treatment resulted in 90% reduction in infarct volume compared with untreated rats of the same age (8.4 ± SEM 2.8 mm^3 *versus* 104 ± SEM 28 mm^3, respectively, $p = 0.006$; Fig. 3).

Preterm infants are known to be at high risk of HI injury (20). Therefore, we investigated the efficacy of LPS protection in P3 and P5 rat pups. Interestingly, the neuroprotective effect of LPS was abolished in very young rat pups. There was no significant difference in infarct volumes between treated and untreated pups in the two age groups. Neuroprotection could not be achieved in P3 and P5 pups even at higher LPS concentration of 0.3 mg/kg ($p = 0.06$ and $p = 0.2$, respectively, in the LPS-treated rats when compared with untreated rats; $n = 5$ pups/group/age). However, LPS continued to be neuroprotective in older rats (P9 and P14; Fig. 3).

In summary, the experimental litters in our protocol spanned the preadult mammalian developmental window—

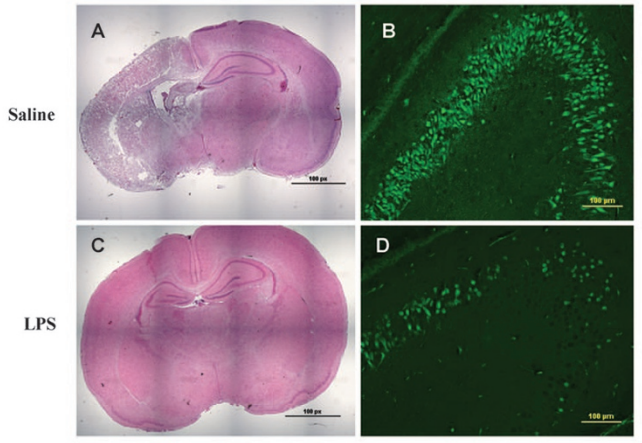

Figure 2. LPS preconditioning reduced brain damage in the rat ischemic neonatal brain. P7 rat pups were randomly assigned to LPS-treated group or untreated group (n = 5–6 rats/group). The LPS-treated group (*C* and *D*) received a single IP injection of 0.1 mg/kg LPS. The untreated group (*A* and *B*) received equal volume of normal saline. Forty-eight hours after LPS or normal saline injection, HI injury was induced by ligating and cutting the right internal carotid artery and then subjecting the animal to transient hypoxia. The animals were then killed 1 wk after the HI insult. Brains were immediately removed and processed for H&E (*A* and *C*) and Fluoro-Jade (*B* and *D*; an anionic fluorochrome that selectively stains injured neurons) staining. The area of tissue damage in the affected hemisphere and the number of injured neurons in the hippocampus are significantly less in the LPS-treated rats. Magnification ×2.

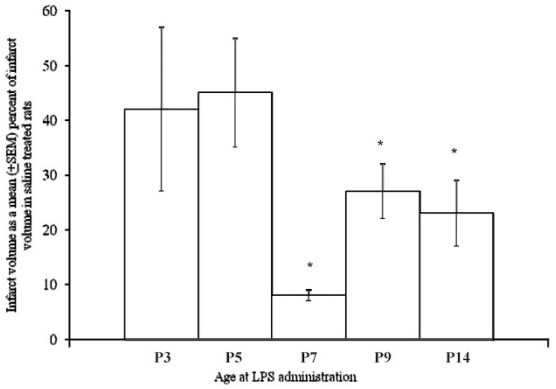

Figure 3. LPS-induced ischemic preconditioning reduced infarct volume in P7, P9, and P14 but not P3 and P5 rat pups. P3, P5, P7, P9, and P14 rat pups were randomly assigned to LPS-treated group or untreated group ($n = 5–6$ rats/group for each age). The LPS-treated group received a single ip injection of 0.1 mg/kg LPS. The untreated group received equal volume of normal saline. Forty-eight hours after LPS or normal saline injection, HI injury was induced by ligating and cutting the right internal carotid artery followed by subjecting the animal to transient hypoxia. The animals were then killed 1 wk after the HI insult. Brains were immediately removed and processed for H&E staining. Total infarct volume was calculated according to Cavalieri principle. LPS ischemic preconditioning reduced infarct size by 90%, 77%, and 73% in P7, P9, and P14 rats, respectively, but had insignificant affect on infarct volume in P3 and P5 rats. $p < 0.03$ when infarct volume compared with saline-treated rats.

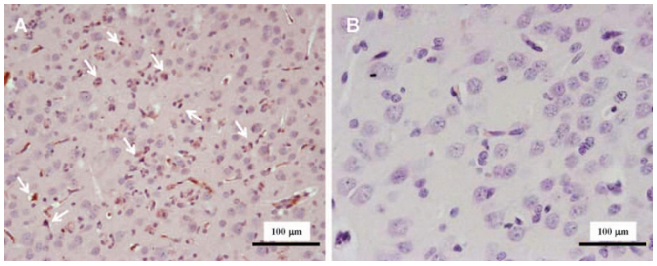

Figure 4. TLR-4 is detected in the neonatal P7 and not P5 rat brain. Representative immunohistochemical staining of brain section from animals killed at postnatal (P) d 7 (*A*) and 5 (*B*) with TLR-4 antibody. *Brown* color (*arrows*) denotes TLR-4 positive cells. Magnification ×20.

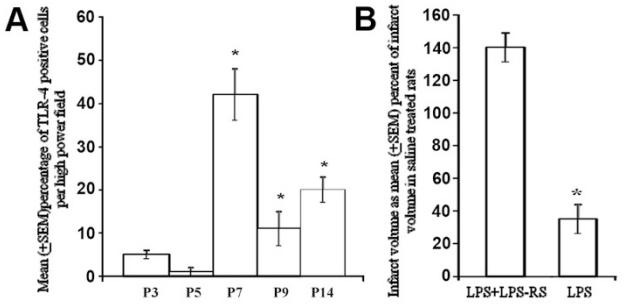

Figure 5. TLR-4 is necessary for LPS-induced preconditioning against ischemic injury. (*A*) TLR-4 is significantly more expressed in P7, P9, and P14 than P3 and P5 neonatal rat ischemic brain. Brain cortical sections from animals killed at postnatal (P) d 3, 5, 7, 9, and 14 were processed for TLR-4 immunostaining. Cumulative number of TLR-4 positive cells were counted by blinded investigator and compared between groups. *$p < 0.05$ when compared with P3 and P5. (*B*) Blocking TLR-4 abolished LPS-induced precondition. P7 rat pups were injected 0.1 mg/kg of LPS-RS followed by LPS injection 6h later as described above. Brains were immediately removed 1 wk after HI injury and processed for H&E staining. Total infarct volume was calculated according to Cavalieri principle. $p < 0.05$ when infarct volume compared with saline-treated rats.

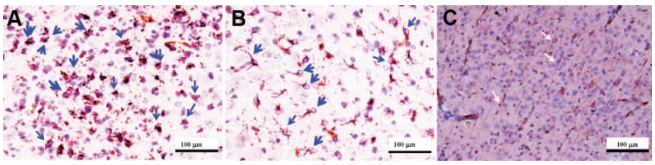

Figure 6. TLR-4 is expressed predominately in microglia and astrocytes of the P7 rat developing brain. Double staining with (*A*) anti-TLR4 and anti-CD68, a marker for detecting microglia, (*B*) anti-TLR-4 and GFAP, a marker for astrocytes, and (*C*) anti-TLR4 and anti-neurofilaments was done in P7 rat brains. Immunodetection was performed using DAB substrate kit for Peroxidase/Vector/SK-4800 to detect TLR-4 staining (brown), in conjunction with the Vector VIP substrate kit for Peroxidase/Vector/SK-4600 to detect anti-CD68, GFAP, or anti-neurofilaments (*purple*). The counterstain was hematoxylin, for blue nuclear detail. *Arrows* indicate cells expressing TLR-4 and CD68 (*A*), cells expressing TLR-4 and GFAP (*B*), and cells expressing TLR-4 and neurofilaments (*C*). Magnification ×20.

from P3 (preterm human equivalent) to P7 (human term equivalent) through to P14 (human early childhood equivalent). We were unable to effectively precondition those litters corresponding to human preterm. However, a switch in susceptibility to LPS preconditioning seemed to take place around P7—human term-equivalent. From this point onward, low-dose LPS offered marked protection against cerebral HI injury by reducing infarct volume several-fold.

Efficacy of LPS neuroprotection is associated with TLR-4 expression in the developing brain. LPS is a specific ligand for TLR-4 (15). It is plausible that LPS-mediated neuroprotection is dependent on cortical TLR-4 expression in the developing brain. Indeed, TLR-4 was highly expressed in P7 (Fig. 4). This pattern of expression was not observed in the brains of P3 and P5 rat pups. The number of TLR-4 positive cells was significantly lower in P3 and P5 rat brains compared with P7, P9, and P14 pups (Fig. 5*A*). Therefore, cerebral expression of the LPS receptor—TLR4—parallels the susceptibility to LPS preconditioning in the developing brain in a maturation-dependent fashion. These findings suggest that differences in TLR-4 expression are likely to be responsible for the age-dependent differences observed in the efficacy of LPS-preconditioning in the ischemic developing brain. To determine whether TLR-4 is necessary for LPS-mediated preconditioning, we investigated the effect of LPS-RS, which impairs the ability of LPS to activate TLR-4 signaling pathway (21), on LPS-induced neuroprotection. Neuroprotection was not achieved in P7 rat pups that were treated with LPS-RS and LPS compared with those treated with LPS alone as shown in Figure 5*B*.

Identify the cellular localization of TLR-4 in the developing brain is an important step toward understanding the mechanism of LPS-induced neuroprotection. We preformed double staining for TLR-4/CD68, TLR-4/GFAP, and TLR-4/NF markers for microglia, astrocytes, and neurons, respectively. TLR-4 was widely expressed in microglia and astrocytes, whereas its expression was limited to few neurons (Fig. 6).

DISCUSSION

The core principle in preconditioning against ischemic injury is that the dose of the preconditioning stimulus must be high enough to have an effect but at a subthreshold level that will not cause damage. In rodents, the preconditioning dose for LPS is typically in the 0.05–1.0 mg/kg range (2,9,10,22) although for other mammals this may differ (11). Another key factor influencing the efficacy of LPS-preconditioning is the timing of LPS administration before the HI insult. LPS administered 72 h before the HI injury increased brain damage in P7 rats (10). Our data have shown that low dose of LPS was neuroprotective when administered 48 h before HI injury. These studies, taken together, indicated that the time interval between exposure to the subinjurious stimulus and the injurious stimulus was very critical to elicit preconditioning. The low dose of LPS required to induce neuroprotection and the convenient interval of 24–48 h between LPS administration and ischemic injury make LPS a plausible neuroprotective agent against ischemic injury that may be used in specific clinical settings with convenient therapeutic window.

Here, we determined a third key factor in LPS preconditioning against cerebral ischemic injury, namely, the stage of brain development. We have shown that LPS preconditioning is effective in P7 rats (correspond to term human newborn), P9, and P14 but ineffective in rat pups younger than P7 (correspond to premature human newborn). Eklind *et al.* (10) previously reported that LPS administered 72 h before HI was ineffective in P4 rats. However, LPS was shown to worsen brain damage when given at this time interval to P7 rats before the HI episode. In our study, LPS administered to rat pups at all ages 48 h before HI injury reduced infarct volume by 90% in P7 rats. Nevertheless, reduction in infarct volume could not be observed in P3 and P5 pups. Our results indicate that robust neuroprotection against cerebral ischemic injury by LPS preconditioning is an age-dependent phenomenon.

The mechanism of LPS-induced preconditioning is not fully understood. LPS is a known specific ligand to TLR-4, one of the TLRs that recognize foreign pathogens. A putative mechanism suggested by Karikó *et al.* (16) proposed that activation of TLR-4 by low dose LPS causes a mild inflammatory response, which includes the production of TLR-4 inhibitors (*e.g.* phosphatidylinositol 3-kinase). These endogenous inflammatory inhibitors would remain up-regulated until the subsequent ischemic insult occurs. Presence of inflammatory inhibitors would suppress the innate immune system so that ischemia-induced inflammatory response would be reduced, resulting in reduced brain damage. TLRs were shown to be expressed in microglia, astrocytes, and oligodendrocytes of the human adult brain (23,24). In the developing brain, only TLR-8 expression was shown in neurons of embryonic and neonatal mouse brain (25). Expression of the other TLRs in the developing brain has not been previously reported. Here, we have shown that TLR-4 was highly expressed in microglia and astrocytes of P7 and P9 but in significantly less amounts in P3 and P5 rat brain. Reduced expression of TLR-4 in the younger rats may explain why LPS was not protective in this age group.

Several issues are raised by our study and will be addressed in future studies. First, do reduced TLR-4 levels simply shift the LPS dose-response preconditioning window, or is LPS preconditioning completely abrogated at all doses? Second, what are the nature and mechanisms of changing TLR-4 levels; are they restricted to TLR-4 or generic to other TLR-subtypes? Third, can preconditioning be induced in the premature brain by activating other TLR-subtypes? Activation of TLR-2 (26) and TLR-9 (27) has been shown to induce preconditioning in adult stroke models. Fourth, what is the mechanism of neuronal protection by LPS-mediated TLR-4 activation in astrocytes and microglia? Finally, do low levels of TLR-4 expression seen in P3 and P5 pups render the premature brain relatively resistant to HI injury. This suggestion is supported by the recent demonstration that adult TLR-$4^{-/-}$ mutants are resistant to injury (28).

Preconditioning is a feasible neuroprotective strategy in children with predictive high risk of cerebral ischemic injury. Newborns with congenital heart disease (10–12/1000 live births) are an example of such patient population. Acquired ischemic brain injury will occur in children before (39%) or after (35%) their cardiac procedure (29–31). Brain damage in these children could have potentially been prevented by preconditioning. However, we need to have a better understanding of the underlying molecular pathways leading to precondition in the developing brain before this neuroprotective therapy be used in the care of children with congenital heart disease.

CONCLUSIONS

We report two novel findings in this study: 1) LPS-induced preconditioning is modulated by brain maturity and 2) TLR-4 is expressed in the immature brain in a specific developmental profile. It is likely that LPS preconditioning against ischemic injury in the developing brain is mediated by TLR-4 activation. Therefore, differences in TLR-4 expression, reported here, are likely to cause the age-dependent efficacy of LPS preconditioning. Other TLRs may play a role in mediating preconditioning depending on the developmental age.

REFERENCES

1. Gidday JM 2006 Cerebral preconditioning and ischaemic tolerance. Natl Rev Neurosci 7:437–448
2. Rosenzweig HL, Lessov NS, Henshall DC, Minami M, Simon RP, Stenzel-Poore MP 2004 Endotoxin preconditioning prevents cellular inflammatory response during ischemic neuroprotection in mice. Stroke 35:2576–2581
3. Dirnagl U, Simon RP, Hallenbeck JM 2003 Ischemic tolerance and endogenous neuroprotection. Trends Neurosci 26:248–254
4. Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, Handa N, Ryuzo F, Kimura K 1990 Ischemic tolerance' phenomenon found in the brain. Brain Res 528:21–24
5. Kitagawa K, Matsumoto M, Kuwabara K, Tagay M, Ohtsuki T, Hata R, Ueda H, Handa N, Kimura K, Kamada T 1991 Ischemic tolerance phenomenon detected in various brain regions. Brain Res 561:203–211
6. Marsh BJ, Williams-Karnesky RL, Stenzel-Poore MP 2009 Toll-like receptor signaling in endogenous neuroprotection and stroke. Neuroscience 158:1007–1020
7. Bastide M, Gele P, Petrault O, Pu Q, Caliz A, Robin E, Deplanque D, Duriez P, Bordet R 2003 Delayed cerebrovascular protective effect of lipopolysaccharide in parallel to brain ischemic tolerance. J Cereb Blood Flow Metab 23:399–405
8. Bordet R, Deplanque D, Maboudou P, Puisieux F, Pu Q, Robin E, Martin A, Bastide M, Leys D, Lhermitte M, Dupuis B 2000 Increase in endogenous brain superoxide dismutase as a potential mechanism of lipopolysaccharide-induced brain ischemic tolerance. J Cereb Blood Flow Metab 20:1190–1196
9. Tasaki K, Ruetzler CA, Ohtsuki T, Martin D, Nawashiro H, Hallenbeck JM 1997 Lipopolysaccharide pre-treatment induces resistance against subsequent focal cerebral ischemic damage in spontaneously hypertensive rats. Brain Res 748:267–270

10. Eklind S, Mallard C, Arvidsson P, Hagberg H 2005 Lipopolysaccharide induces both a primary and a secondary phase of sensitization in the developing rat brain. Pediatr Res 58:112–116
11. Hickey EJ, You X, Kaimaktchiev V, Stenzel-Poore M, Ungerleider RM 2007 Lipopolysaccharide preconditioning induces robust protection against brain injury resulting from deep hypothermic circulatory arrest. J Thorac Cardiovasc Surg 133:1588–1596
12. Lesuisse C, Martin LJ 2002 Immature and mature cortical neurons engage different apoptotic mechanisms involving caspase-3 and the mitogen-activated protein kinase pathway. J Cereb Blood Flow Metab 22:935–950
13. Hu BR, Liu CL, Ouyang Y, Blomgren K, Siesjö BK 2000 Involvement of caspase-3 in cell death after hypoxia-ischemia declines during brain maturation. J Cereb Blood Flow Metab 20:1294–1300
14. Yager JY, Shuaib A, Thornhill J 1996 The effect of age on susceptibility to brain damage in a model of global hemispheric hypoxia-ischemia. Brain Res Dev Brain Res 93:143–154
15. Lu Y-C, Yeh W-C, Ohashi PS 2008 LPS/TLR4 signal transduction pathway. Cytokine 42:145–151
16. Karikó K, Weissman D, Welsh FA 2004 Inhibition of Toll-like receptor and cytokine signaling—a unifying theme in ischemic tolerance. J Cereb Blood Flow Metab 24:1288–1304
17. Yager JY, Ashwal S 2009 Animal models of perinatal hypoxic-ischemic brain damage. Pediatr Neurol 40:156–167
18. Rice JE 3rd, Vannucci RC, Brierley JB 1981 The influence of immaturity on hypoxic-ischemic brain damage in the rat. Ann Neurol 9:131–141
19. Mallard EC, Rehn A, Rees S, Tolcos M, Copolov D 1999 Ventriculomegaly and reduced hippocampal volume following intrauterine growth-restriction: implications for the aetiology of schizophrenia. Schizophr Res 40:11–21
20. Benders MJ, Groenendaal F, Uiterwaal CS, Nikkels PG, Bruinse HW, Nievelstein RA, de Vries LS 2007 Maternal and infant characteristics associated with perinatal arterial stroke in the preterm infant. Stroke 38:1759–1765
21. Coats SR, Pham TT, Bainbridge BW, Reife RA, Darveau RP 2005 MD-2 mediates the ability of tetra-acylated and penta-acylated lipopolysaccharides to antagonize *Escherichia coli* lipopolysaccharide at the TLR4 signaling complex. J Immunol 175:4490–4498
22. Ikeda T, Yang L, Ikenoue T, Mallard C, Hagberg H 2006 Endotoxin-induced hypoxic-ischemic tolerance is mediated by up-regulation of corticosterone in neonatal rat. Pediatr Res 59:56–60
23. Esen N, Tanga FY, DeLeo JA, Kielian T 2004 Toll-like receptor 2 (TLR2) mediates astrocyte activation in response to the gram-positive bacterium *Staphylococcus aureus*. J Neurochem 88:746–758
24. Bsibsi M, Ravid R, Gveric D, Van Noort JM 2002 Broad expression of Toll-like receptors in the human central nervous system. J Neuropathol Exp Neurol 61:1013–1021
25. Ma Y, Li J, Chiu I, Wang Y, Sloane JA, Lu J, Kosaras B, Sidman RL, Volpe JJ, Vartanian T 2006 Toll-like receptor 8 functions as a negative regulator of neurite outgrowth and inducer of neuronal apoptosis. J Cell Biol 175:209–215
26. Hua F, Ma J, Ha T, Kelley J, Williams DL, Kao RL, Kalbfleisch JH, Browder IW, Li C 2008 Preconditioning with a TLR2 specific ligand increases resistance to cerebral ischemia/reperfusion injury. J Neuroimmunol 199:75–82
27. Stevens SL, Ciesielski TM, Marsh BJ, Yang T, Homen DS, Boule J-L, Lessov NS, Simon RP, Stenzel-Poore MP 2008 Toll-like receptor 9: a new target of ischemic preconditioning in the brain. J Cereb Blood Flow Metab 28:1040–1047
28. Cao CX, Yang QW, Lv FL, Cui J, Fu HB, Wang JZ 2007 Reduced cerebral ischemia-reperfusion injury in Toll-like receptor 4 deficient mice. Biochem Biophys Res Commun 353:509–514
29. McQuillen PS, Barkovich AJ, Hamrick SE, Perez M, Ward P, Glidden DV, Azakie A, Karl T, Miller SP 2007 Temporal and anatomic risk profile of brain injury with neonatal repair of congenital heart defects. Stroke 38:736–741
30. Miller SP, McQuillen PS, Vigneron DB, Glidden DV, Barkovich AJ, Ferriero DM, Hamrick SE, Azakie A, Karl TR 2004 Preoperative brain injury in newborns with transposition of the great arteries. Ann Thorac Surg 77:1698–1706
31. Domi T, Edgell DS, McCrindle BW, Williams WG, Chan AK, MacGregor DL, Kirton A, deVeber GA 2008 Frequency, predictors, and neurologic outcomes of vasoocclusive strokes associated with cardiac surgery in children. Pediatrics 122:1292–1298