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MELATONIN AMELIORATES BRAIN INJURY INDUCED BY SYSTEMIC LIPOPOLYSACCHARIDE IN NEONATAL RATS

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Abstract—Our previous study showed that lipopolysaccharide (LPS)-induced brain injury in the neonatal rat is associated with nitrosative and oxidative stress. The present study was conducted to examine whether melatonin, an endogenous molecule with antioxidant properties, reduces systemic LPS-induced nitrosative and oxidative damage in the neonatal rat brain. Intraperitoneal (i.p.) injection of LPS (2 mg/kg) was administered to Sprague–Dawley rat pups on postnatal day 5 (P5), and i.p. administration of melatonin (20 mg/kg) or vehicle was performed 5 min after LPS injection. Sensorimotor behavioral tests were performed 24 h after LPS exposure, and brain injury was examined after these tests. The results show that systemic LPS exposure resulted in impaired sensorimotor behavioral performance, and acute brain injury, as indicated by the loss of oligodendrocyte immunoreactivity and a decrease in mitochondrial activity in the neonatal rat brain. Melatonin treatment significantly reduced LPS-induced neurobehavioral disturbances and brain damage in neonatal rats. The neuroprotective effect of melatonin was associated with attenuation of LPS-induced nitrosative and oxidative stress, as indicated by the decreased nitrotyrosine- and 4-hydroxynonenal-positive staining in the brain following melatonin and LPS exposure in neonatal rats. Further, melatonin significantly attenuated LPS-induced increases in the number of activated microglia in the neonatal rat brain. The protection provided by melatonin was also associated with a reduced number of inducible nitric oxide synthase (iNOS)+ cells, which were double-labeled with ED1 (microglia). Our results show that melatonin prevents the brain injury and neurobehavioral disturbances induced by systemic LPS exposure in neonatal rats, and its neuroprotective effects are associated with its impact on nitrosative and oxidative stress.

Key words: melatonin, lipopolysaccharide, nitrosative and oxidative damage, mitochondria, microglia.

INTRODUCTION

Periventricular leukomalacia (PVL) is a white matter disease of premature infants that is frequently related with the destruction of immature oligodendroglia and axons (Back et al., 2001; Pleasure et al., 2006). Increasing evidence indicates that perinatal infection or inflammation and hypoxia–ischemia are major contributors to PVL and result in the subsequent development of impaired neurological outcomes (Harden et al., 1996; Rezaie and Dean, 2002; Volpe, 2003). Our previous studies have shown that neonatal exposure (postnatal day 5, P5) to lipopolysaccharide (LPS) through an intracerebral (i.c.) injection results in brain inflammation and white matter and neuronal injury in rats, which was closely associated with the increased nitrosative and oxidative stress following LPS exposure (Fan et al., 2008b,c). The inflammatory response that ensues because of the initial occult exogenous oxidative/nitrosative stress becomes a secondary endogenous source of reactive oxygen species (ROS) and reactive nitrogen species (RNS). A previous study also showed that nitrosative and oxidative injury to premyelinating oligodendrocytes (OLs) was present in the infant brain with PVL (Haynes et al., 2003).

Melatonin (*N*-acetyl-5-methoxytryptamine) has been reported to have neuroprotective actions through its ability to detoxify species mediating oxidative and nitrosative damage (ROS and RNS) and to prevent activation of the pro-inflammatory enzymes, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) (Brzozowska et al., 2009; Esposito and Cuzzocrea, 2010). Clinically, melatonin has been used in pediatrics to treat respiratory distress syndrome, bronchopulmonary dysplasia, seizure disorders and to reduce oxidative stress in sepsis and asphyxia (Gitto et al., 2011; Sanchez-Barcelo et al., 2011; Aversa et al., 2012; Chen et al., 2012). Thus, melatonin may

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Abbreviations: 4-HNE, 4-hydroxynonenal; APP, α-amyloid precursor protein; COX-2, cyclooxygenase-2; DAPI, 4′,6-diamidino-2-phenylindole; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; Iba1, ionized calcium binding adapter molecule 1; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NADH, nicotinamide adenine dinucleotide; NT, nitrotyrosine; OLs, oligodendrocytes; PVL, periventricular leukomalacia; RNS, reactive nitrogen species; ROS, reactive oxygen species.

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potentially protect against brain injury induced by infection or inflammation, by attenuating the induction of nitrosative and oxidative stress in neonates. However, the effect of melatonin on brain damage induced by proinflammatory factors in neonates has not yet been tested.

Our previous study showed that intraperitoneal (i.p.) LPS exposure, which is more clinically relevant, causes pathological brain damage in the cortex, hippocampus, and striatum of neonatal rats; this damage is comparable to that caused by its intracerebral (i.c.) injection (Cai et al., 2013). In the present study, we further investigated whether systemic LPS exposure causes brain injury through activation of brain oxidative and/or nitrosative stress damage. Further, the effect of melatonin on LPS-induced nitrosative and oxidative damage was studied in the neonatal rat brain.

EXPERIMENTAL PROCEDURES

Chemicals

Unless otherwise stated, all chemicals used in this study were purchased from Sigma (St Louis, MO, USA).

Mouse monoclonal antibodies against late oligodendrocyte (OL) progenitor cell marker O4 (O4) or α-amyloid precursor protein (APP) and ED1 were purchased from Millipore (Billerica, MA, USA) and Serotec (Raleigh, NC, USA), respectively. Rabbit polyclonal antibodies against nitrotyrosine (NT), 4-hydroxynonenal (4-HNE), or iNOS and ionized calcium binding adapter molecule 1 (Iba1) were obtained from Chemicon, Alexis (San Diego, CA, USA) and Wako Chemicals USA (Irvine, CA, USA), respectively. The enzyme-linked immunosorbent assay (ELISA) kit for immunoassay of rat interleukin-1β (IL-1β) was purchased from R&D Systems (Minneapolis, MN, USA).

Animals

Pregnant Sprague–Dawley rats arrived in the laboratory on day 19 of gestation. Animals were maintained in a room with a 12-h light/dark cycle and at constant temperature (22 ± 2 °C). The day of birth was defined as postnatal day 0 (P0). After birth, the litter size was adjusted to 12 pups per litter to minimize the effect of litter size on body weight and brain size. All procedures for animal care were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center or Fu Jen Catholic University. Every effort was made to minimize the number of animals used and their suffering.

Animal treatment

An i.p. injection of LPS (2 mg/kg, from *Escherichia coli*, serotype 055: B5) was administered to 5-day-old (P5) Sprague–Dawley rat pups of both sexes. The control rats were injected with the same volume of sterile saline (0.1 mL). All animals survived the injection. Both LPS- and saline-injected animals were further divided into two groups: one received i.p. injections of melatonin and the other received vehicle. Melatonin (20 mg/kg) was dissolved in 5% ethanol in saline (Ozdemir et al., 2007; Olivier et al., 2009) and administered 5 min after LPS injection. A total of 72 rats from six litters were used in the present study. One pup from each litter was assigned to each of 12 groups, so there were six pups in each group (n = 6). Behavioral tests were performed on P6. After the behavioral tests, two-thirds of the rats were killed by decapitation to collect fresh brain tissues, which were used for mitochondrial complex I activity and ELISA assays. The remaining rats were killed by transcardiac perfusion with normal saline followed by 4% paraformaldehyde treatment for brain section preparation. Free-floating 40-μm-thick coronal brain sections were prepared using a freezing microtome (Leica, SM 2000R, Wetzlar, Germany) for immunohistochemistry.

Behavioral testing

Behavioral tests were performed as described previously with certain modifications (Fan et al., 2008a, 2013). The developmental test battery was based on previously well-established tests for neurobehavioral toxicity (Altman et al., 1971; Hermans et al., 1992). The righting reflex and wire-hanging maneuver were performed by all rat pups at P6.

Righting reflex

This test is believed to reflect muscle strength and subcortical maturation (Altman et al., 1971; Hermans et al., 1992). Pups were placed on their backs, and the time required to turn over onto all four feet and touch the platform was measured. The cut-off time was 60 s.

Wire-hanging maneuver

This maneuver tests neuromuscular and locomotor development (Altman and Sudarshan, 1975; Hermans et al., 1992). Pups are suspended by their forelimbs from a horizontal rod (5×5 mm^2 in cross-section, 35-cm long, between two 50-cm-high poles). A sawdust-filled box at the base served as protection for the falling pups. Suspension latencies were recorded, and the cut-off time was 120 s.

Immunohistochemistry

Brain injury was estimated based on the results of immunohistochemistry performed on consecutive brain sections of rats killed 1 day (P6) after LPS injection. For immunohistochemical staining, primary antibodies were used at the following dilutions: O4, 1 μg/mL; 4-HNE or Iba1, 1:500; ED1, iNOS, 1:200; and APP or NT, 1:100. O4 was used to detect late OL progenitor cells in the white matter. Upregulation of APP was used as a marker of axonal injury. Positive staining for NT or 4-HNE was used as a marker of products of RNS or lipid peroxidation, respectively. Microglia were detected using Iba1 immunostaining, which recognizes both resting and activated microglia, and by ED1 immunostaining, which detects activated microglia or