Intranasal pyrrolidine dithiocarbamate decreases brain inflammatory mediators and provides neuroprotection after brain hypoxia–ischemia in neonatal rats

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ABSTRACT

Brain injury due to birth asphyxia is the major cause of death and long-term disabilities in newborns. We determined whether intranasal pyrrolidine dithiocarbamate (PDTC) could provide neuroprotection in neonatal rats after brain hypoxia–ischemia (HI). Seven-day old male and female Sprague–Dawley rats were subjected to brain HI. They were then treated with intranasal PDTC. Neurological outcomes were evaluated 7 or 30 days after the brain HI. Brain tissues were harvested 6 or 24 h after the brain HI for biochemical analysis. Here, PDTC dose-dependently reduced brain HI-induced brain tissue loss with an effective dose $(ED)_{50}$ at 27 mg/kg. PDTC needed to be applied within 45 min after the brain HI for this neuroprotection. This treatment reduced brain tissue loss and improved neurological and cognitive functions assessed 30 days after the HI. PDTC attenuated brain HI-induced lipid oxidative stress, nuclear translocation of nuclear factor κ-light-chain-enhancer of activated B cells, and various inflammatory mediators in the brain tissues. Inhibition of inducible nitric oxide synthase after brain HI reduced brain tissue loss. Our results suggest that intranasal PDTC provides neuroprotection possibly via reducing inflammation and oxidative stress. Intranasal PDTC may have a potential to provide neuroprotection to human neonates after birth asphyxia.

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Introduction

The World Health Organization estimates that 4 to 9 million neonates suffer from birth asphyxia each year in the world (World-Health, 2003). This leads to about 1.2 million deaths and the same number of infants with severe disability (Bang et al., 2005; Minino et al., 2007). These deaths and disabilities are mostly due to hypoxic–ischemic (HI) brain injury. The long-term neurological or cognitive disabilities include cerebral palsy, epilepsy and mental retardation (Lynch and Nelson, 2001; Sran and Baumann, 1988; Sreenan et al., 2000). Thus, it is very important to identify methods to reduce HI brain injury in neonates. However, clinically practical methods to reduce this brain injury, especially in low-income countries, have not been established yet.

There are at least two types of insults that contribute to HI brain injury. Hypoxia and ischemia, the primary insults, interrupt energy supply to cells and cause cell injury. Hypoxia–ischemia and the subsequent reoxygenation/reperfusion induce production of a broad range of "toxic chemicals", such as free oxygen species and inflammatory

cytokines. These "toxic chemicals" can cause additional cell injury (Allen and Bayraktutan, 2009; Lakhan et al., 2009; Lipton, 1999). Studies have shown that anti-oxidants and anti-inflammatory reagents are neuroprotective (Allen and Bayraktutan, 2009; Lakhan et al., 2009; Lampl et al., 2007; Lipton, 1999; Yamaguchi et al., 1998).

Pyrrolidine dithiocarbamate (PDTC) is an anti-oxidant and anti-inflammatory agent (Liu et al., 1999). It is a small molecule and relatively cheap. PDTC has been evaluated as an antiviral agent for humans (Si et al., 2005). A pre-clinical toxicity study has shown that PDTC is a safe drug (Chabicovsky et al., 2010). It can reduce focal brain ischemic injury in young adult rats (Nurmi et al., 2004a,b). This protective effect occurs even if the application of PDTC is at 6 h after the onset of transient focal brain ischemia in those rats (Nurmi et al., 2004b). PDTC also reduces brain injury after HI in neonatal rats (Nurmi et al., 2006). However, the previous studies only assessed the neuroprotective effect within 7 days after brain ischemia and the drug was given intraperitoneally. Our recent data showed that oral PDTC started after transient focal brain ischemia improved neurological outcome assessed 1 or 2 months later in young adult rats (Li et al., 2012). However, the PDTC application routes used in the previous studies are difficult to apply in neonates immediately after birth asphyxia.

Intranasal application (in the form of nasal drop or spray) of a drug can be performed immediately and easily by virtually any care provider

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without the need of any special equipment in the setting of birth asphyxia. The absorption is quick. Intranasal application of PDTC reaches its maximal concentration in the blood within 5 min (Chabicovsky et al., 2010). The amount of drugs that get into the brain after nasal application is similar to that of the drugs to be given intravenously (Bagger and Bechgaard, 2004). A significant amount of PDTC is found in the brain after nasal application (Chabicovsky et al., 2010). Thus, we hypothesize that intranasal PDTC reduces brain injury after neonatal brain HI. To test this hypothesis, we used a well-established rat model of neonatal brain HI. Inflammatory mediators and oxidative stress indices in the brain were measured to determine the possible mechanisms for the neuroprotection induced by intranasal PDTC.

Materials and methods

All experimental protocols were approved by the institutional Animal Care and Use Committee of the University of Virginia (Charlottesville, VA). All surgical and experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications number 80-23) revised in 1996. Efforts were made to minimize the number of animals used and their suffering. Our manuscript was written up in accordance with the Animal Research: Reporting in vivo Experiments. A total of 290 animals completed the intended study in this project.

Neonatal cerebral hypoxia/ischemia model

Cerebral HI was performed as we described before (Zhao et al., 2007). Briefly, 7-day-old male and female Sprague–Dawley rats were anesthetized by isoflurane in 30% O_2–70% N_2. Their right common carotid arteries were permanently ligated with a double 7-0 surgical silk. The neonates were returned to their cages with their mothers for 3 h and then placed in a chamber containing humidified 8% O_2–92% N_2 for 2 h at 37 °C.

Drug treatment

Sprague–Dawley rats were randomly assigned to receive intranasal PDTC (Sigma-Aldrich, St. Louis, MO, USA) or normal saline at a preset time-point. PDTC was dissolved in normal saline just before the application. The volume applied was 400 μl/kg body weight and was applied as drops to nostrils.

Some rats received intraperitoneal 1 mg/kg N-(3-(aminomethyl) benzyl)acetamidine (1400W, Enzo Life Sciences Inc., Farmingdale, NY, USA), a specific inducible nitric oxide synthase (iNOS) inhibitor, or saline at 30 min before the brain HI, 30 min before the hypoxia or 15 min after the brain HI. 1400W was dissolved in normal saline. The injected volume was from 0.15 to 0.2 ml per rat.

Brain injury/loss quantification

As we described before (Zhao and Zuo, 2004), rat brains were harvested 7 days after the brain HI. The hindbrain was removed from the cerebral hemispheres and the two hemispheres were separated and weighed. The weight ratio of right to left hemispheres was calculated.

Brain histopathology

This was performed as we described previously (Zhao et al., 2007). One month after the brain HI, rats were sacrificed under deep isoflurane anesthesia and transcardially perfused with cold normal saline. Their brains were fixed with 10% neutral buffered formalin overnight and then paraffin embedded. Five-micrometer-thick coronal sections at approximately 3.3 mm caudal to bregma were obtained and subjected to Nissl staining. These sections were examined by an observer blinded to the group assignment of the sections. The cerebral areas in each of the hemispheres were quantified by using National Institutes of Health

Image 1.60 (NIH, Bethesda, MD, USA). The cerebral area ratio of the right hemisphere to the left hemisphere was calculated and used to reflect brain tissue loss in the right hemisphere after brain HI. The number of Nissl staining positive cells (neurons) in a high magnification field ($\times 400$, ~0.2 mm^2) in the CA1 and CA3 regions was counted. Three determinations, each on different locations in these two brain regions, were performed and averaged to yield a single number (neuronal density) for each brain region of individual rats. The ratio of neuronal density in the right hippocampus to that in the left hippocampus of the same animal was then calculated.

Barnes maze

Barnes maze was used to test animals' spatial learning and memory. Eight days before the rats were euthanized for brain histopathology, they started to be tested in a Barnes maze equipped with ANY-Maze video tracking system (San Diego Instruments, San Diego, CA) as we described previously (Li et al., 2012) with minor modifications. The test was administered and evaluated by a person blinded to the group assignment of rats. Rats were placed in the middle of a circular platform with 20 equally spaced holes. One of the holes was connected to a dark chamber that was called target box. Rats were encouraged to find this box by aversive noise (85 dB) and bright light (200 W) shed on the platform. The protocol involved training sessions on 4 consecutive days that consisted of four training sessions on each day with a 15-min inter-session interval. Each session ended when the rat entered the target hole or after 3 min had elapsed. On fifth day, one trial was performed to test the animal's retention. All trials were recorded and analyzed by using the ANY-Maze tracking system to calculate the latency for the rat to enter the target hole.

Motor coordination evaluation

Evaluation of motor coordination was started at 4 days before the rats were euthanized for brain histopathology as we described before (Li and Zuo, 2009). Rats were placed on a rotarod apparatus whose speed increased from 4 to 40 rpm in 5 min. All rats were trained for two consecutive days, three times per day, before the formal tests. The latency and speed of rat's falling off the rotarod apparatus were recorded. The speed–latency index (latency in seconds × speed in rpm) of each test was calculated. Each rat was tested for three times in the formal test. The mean index value of the three trials was used to reflect the motor coordination functions of each rat.

Fear conditioning

Fear conditioning was used to determine hippocampus-dependent and hippocampus-independent learning and memory. After motor coordination evaluation was completed, fear conditioning test was performed as we described before (Lin and Zuo, 2011) and administered and evaluated by a person blinded to the group assignment of rats. Briefly, each rat was placed in a Plexiglas conditioning training chamber wiped with 70% alcohol. After a 3-min baseline exploratory period in the chamber, rats received 3 tone (2000 Hz, 90 dB)–shock (1 mA, 2 s) pairings separated by 1 min between each pairing in a relatively dark room. Twenty-four hours after the training session, each rat was placed again in the training chamber for a period of 8 min in the absence of tone and foot shock to test its contextual fear conditioning. The amount of time with freezing behavior was recorded in an 8-s interval. Two hours later, the rat was placed in a test chamber that had different context and smell from the first test chamber (this second chamber was wiped with 1% acetic acid) in a relatively light room. After a 3-min exploratory period in this new chamber, a 30-s tone (2000 Hz, 90 dB) was applied. Freezing behavior was also scored during this tone-related test period.