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Tert-butylhydroquinone post-treatment attenuates neonatal hypoxic-ischemic brain damage in rats

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ABSTRACT

Hypoxic-ischemic (HI) encephalopathy is a leading cause of dire mortality and morbidity in neonates. Unfortunately, no effective therapies have been developed as of yet. Oxidative stress plays a critical role in pathogenesis and progression of neonatal HI. Previously, as a Nrf2 activator, tert-butylhydroquinone (TBHQ) has been demonstrated to exert neuroprotection on brain trauma and ischemic stroke models, as well as oxidative stress-induced cytotoxicity in neurons. It is, however, still unknown whether TBHQ administration can protect against oxidative stress in neonatal HI brain injury. This study was undertaken to determine the neuroprotective effects and mechanisms of TBHQ post-treatment on neonatal HI brain damage. Using a neonatal HI rat model, we demonstrated that TBHQ markedly abated oxidative stress compared to the HI group, as evidenced by decreased oxidative stress indexes, enhanced Nrf2 nuclear accumulation and DNA binding activity, and up-regulated expression of Nrf2 downstream antioxidative genes. Administration of TBHQ likewise significantly suppressed reactive gliosis and release of inflammatory cytokines, and inhibited apoptosis and neuronal degeneration in the neonatal rat cerebral cortex. In addition, infarct size and neuronal damage were attenuated distinctly. These beneficial effects were accompanied by improved neurological reflex and motor coordination as well as amelioration of spatial learning and memory deficits. Overall, our results provide the first documentation of the beneficial effects of TBHQ in neonatal HI model, in part conferred by activation of Nrf2 mediated antioxidative signaling pathways.

1. Introduction

Neonatal hypoxic-ischemic encephalopathy (HIE) is the most common perinatal brain disorder, and is associated with fearful morbidity and mortality (Fatemi et al., 2009; Zhu et al., 2015). HIE often leads to severe neurological sequelae, such as cerebral palsy, epilepsy and intellectual disabilities. Treatment and care for these neurological deficits imposes considerable financial and lifelong burdens to the affected individuals, their families, and society at large (Pazos et al., 2012). As a result, HIE is a significant global public health care problem. Many studies have been aimed at investigation of feasible and effective strategies for the treatment of HIE, such as therapeutic hypothermia (TH), hyperbaric oxygen therapy, stem cell therapy, anticonvulsant and neuroprotective drugs including erythropoietin, melatonin, desferrioxamine, resveratrol and sulforaphane (Buonocore et al., 2012; Zhu et al., 2015). Despite these efforts, however, there are limited effective therapy options currently available. Herein, there is an urgent need for more effective treatments to ameliorate the damage and disability associated with neonatal HIE.

The pathogenesis of HIE is complex and multifactorial, including energy failure, glutamatergic excitotoxicity, calcium overload, inflammation, reactive oxygen species (ROS)-mediated toxicity, endothelial cell dysfunction, etc., which culminates finally in neurocyte death by a mixture of necrosis and apoptosis (Northington et al., 2011). Recently, oxidative stress has been reported as a critical causative factor in HIE (Noor et al., 2007). The rich polyunsaturated fatty acid content, high rate of oxygen consumption, increased availability of free iron, and low antioxidant capabilities in the immature brain, compared to the adult brain, all contribute to sensitize the neonatal brain to oxidative stress after HIE (Esih

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et al., 2017). In pathological conditions, excessive free radical production can effectively overwhelm production of antioxidative enzymes (Birben et al., 2012). Free iron further contributes to cytotoxicity by promoting the generation of ROS (Abdal Dayem et al., 2017). Increased ROS output rapidly damages cell membranes, proteins, lipids, DNA, and results in a cascading inflammatory response. These derivatives contribute to a complex interplay of apoptosis, autophagy and necrosis resulting in brain injury (Zhao et al., 2016b). Previous investigations have indicated that, as a new therapeutic approach, exogenous antioxidant therapy exhibits effective neuroprotection against neonatal HI brain damage. Some of these exogenous antioxidants have demonstrated neuroprotective effects in human trials, i.e., the erythropoietin and melatonin (Arteaga et al., 2017). Before vital molecules are subject to insult, antioxidants can safely interact with oxygen free radicals and terminate that chain response to protect against oxidative damage (Lobo et al., 2010).

Notably, the nuclear factor erythroid 2-related factor-2 (Nrf2) pathway has pleiotropic actions in antioxidative stress, anti-apoptotic, anti-inflammatory, anti-atherosclerotic, and anti-tumorogenic signaling, and is known to regulate glutathione (GSH) synthesis and cerebrovascular reactivity, conferring neuroprotection against oxidative insult. In this manner, Nrf2 activators have become attractive neuroprotective candidates for cerebral ischemia (Jiang et al., 2017a; Lu et al., 2016). It has been shown that mice deficient of Nrf2 are especially sensitive to oxidative stress (Ma, 2013). Under normal conditions, Nrf2 is sequestered in the cytoplasm, but is translocated to the nucleus in response to oxidative stress. Next, it can activate a range of antioxidant downstream genes, such as heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase-1 (NQO1) and superoxide dismutase2 (SOD2), to scavenge ROS and prevent damage by oxidative stress (Jiang et al., 2017b).

The Nrf2 activator, TBHQ is an effective phenolic antioxidant widely used in foods, as well as in medicines and cosmetics. Numerous previous studies reported that TBHQ has a hydroquinone-type electrophilic structure that contributes to activate Nrf2 transcription (Satoh et al., 2009), counteract oxidative damage and exhibit neuroprotective effects in different models of central nervous system injury (Jin et al., 2014; Lu et al., 2014; Shih et al., 2005). As the effects of TBHQ treatment against HIE has yet to be determined, the present study aims to examine whether TBHQ post-treatment confers neuroprotection in a neonatal HI rat model. As well, this study seeks uncover the underlying mechanisms involved to determine whether TBHQ may serve as a possible exogenous antioxidant therapeutic agent against HIE.

2. Materials and methods

2.1. Animal model of HI brain injury

In a modified version of a previously described HI model (Yuan et al., 2014), unsexed 10-day-old Sprague-Dawley rats (Charles River Laboratories) were anesthetized, and the right common carotid artery (RCCA) was isolated and ligated permanently through a midline neck incision. Animals were returned to cages for 1.5 h after wound closure. Rats were then placed in a hypoxic environment (6% oxygen/94% nitrogen) for 2 h. The temperature was maintained at 37 °C. All procedures were approved by the Animal Care and Use Committee of Augusta University and were in accordance with National Institutes of Health guidelines.

2.2. Experimental design and administration of drugs

Rats were randomly allocated into four groups: (a) Sham + vehicle control group, treated with 1% DMSO in PBS without RCCA ligation; (b) HI + vehicle group, treated with 1% DMSO in PBS; (c) HI + TBHQ group, treated with TBHQ (Acros Organics); and (d) HI + TBHQ + brusatol (Brus) group, treated with brusatol (Sigma-Aldrich), a unique inhibitor of the Nrf2 pathway. TBHQ was dissolved in 1% DMSO and injected intraperitoneally (i.p.) at a dose of 20 mg/kg after 1 h of HI and repeated once daily for seven consecutive days. Fresh TBHQ solutions were prepared for each injection. To confirm the role of Nrf2 pathway in TBHQ neuroprotection, brusatol was dissolved in 1% DMSO and administrated at a dose of 1.0 mg/kg via i.p. in the combination with TBHQ. The experimental protocol was shown in Fig. 1A. HI brain injury was induced on rat postnatal day 10 (P10). From P11 to P14, the righting reflex test was applied to investigate the short-term behavioral outcomes. From P28 to P31, the long-term behavioral tests were performed, such as the beam-walking, cylinder and Barnes maze task. Rats were sacrificed under anesthesia at P17 and P31, respectively. The brains were collected for further analysis.

2.3. Histology examination and infarct measurement

Rats were anesthetized and perfused transcardially with cold PBS followed by 4% paraformaldehyde (PFA). Brains were extracted and postfixed overnight in 4% PFA, and were then submersed in 30% sucrose until they sank. Brains were then immersed in cryoprotectant and frozen overnight at −80 °C. Coronal brain sections (25 μm) were prepared using a cryostat microtome. Histological examination was carried out using the methods as described previously by our laboratory (Lu et al., 2017a; Zhang et al., 2013). At least 3–5 representative sections in the coronal plane (>100 μm gap between each section, ~2.5–4.5 mm posterior from Bregma) of each animal were selected for analysis. Sections were stained with 0.01% (w/v) cresyl violet (CV) for 30 min, following graded ethanol dehydration as described previously by our laboratory (Ahmed et al., 2016). Stained sections were mounted and examined under light microscopy and the mean infarct area of each section was quantified with ImageJ software. Histological analysis was performed by investigators blinded to the staining groups. Infarct size was calculated according to the following formula: size = (area of contralateral hemisphere − area of intact ipsilateral hemisphere)/area of contralateral hemisphere × 100%.

2.4. Brain homogenates and subcellular fractionations

The ipsilateral damaged cerebral cortex was microdissected quickly 7 d after HI (P17) and frozen in liquid nitrogen immediately. The cytosolic and nuclear extraction were performed, as previously reported by our laboratory (Zhang et al., 2008), with a few modifications. Briefly, tissue samples were homogenized in ice cold homogenization medium buffer A. Then, the samples were sharply vortexed and sonicated 2 times for 2s, then centrifuged at 800×*g* for 10 min. Supernatants were centrifuged again at 15,000×*g* for 30 min at 4 °C, and were kept at −80 °C as cytosolic fractions. The nuclear pellets were washed three times using buffer A, resuspended in buffer B, and then were vigorously rocked at 4 °C for 30 min and sonicated 2 times for 2s. The samples were then centrifugated at 12,000×*g* for 15 min at 4 °C. The protein samples were aliquoted and frozen in liquid nitrogen for long-term storage at −80 °C. Protein concentrations were detected using a modified Lowry protein assay.

2.5. Western blotting

Western blotting was performed as described previously by our laboratory (Zhang et al., 2008). The protein from cytosolic and