Neuroscience 255 (2013) 292–299

HESPERIDIN PRETREATMENT PROTECTS HYPOXIA–ISCHEMIC BRAIN INJURY IN NEONATAL RAT

Z. RONG,[a] R. PAN,[b] Y. XU,[d] C. ZHANG,[d] Y. CAO[c] AND D. LIU[d]*

[a] *Department of Pediatrics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*

[b] *Department of Pediatrics, Xiangyang Central Hospital, Xiangyang 441021, China*

[c] *Department of Orthopedic, Xiangyang Central Hospital, Xiangyang 441021, China*

[d] *Department of Pharmaclogy , Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*

Abstract—Neonatal hypoxia–ischemic encephalopathy (HIE) remains a major cause of brain damage, leading to high disability and mortality rates in neonates. *In vitro* studies have shown that hesperidin, a flavanone glycoside found abundantly in citrus fruits, acts as an antioxidant. Although hesperidin has been considered as a potential treatment for HIE, its effects have not been fully evaluated. In this study, the protective effect of hesperidin pretreatment against hypoxia–ischemic (HI) brain injury and possible signal pathways were investigated using *in vivo* and *in vitro* models. *In vivo* HI model employed unilateral carotid ligation in postnatal day 7 rat with exposure to 8% hypoxia for 2.5 h, whereas *in vitro* model employed primary cortical neurons of neonatal rats subjected to oxygen and glucose deprivation for 2.5 h. Hesperidin pretreatment significantly reduced HI-induced brain tissue loss and improved neurological outcomes as shown in 2,3,5-triphenyltetrazolium chloride monohydrate staining and foot-fault results. The neuroprotective effects of hesperidin are likely the results of preventing an increase in intracellular reactive oxygen species and lipid peroxide levels. Hesperidin treatment also activated a key survival signaling kinase, Akt, and suppressed the P-FoxO3 level. Hesperidin pretreatment protected neonatal HIE by reducing free radicals and activating phosphorylated Akt. © 2013 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: hypoxia–ischemia encephalopathy, hesperidin, reactive oxygen species.

INTRODUCTION

Neonatal hypoxia–ischemic encephalopathy (HIE) is associated with high morbidity and mortality rates. The incidence of moderate and severe HIE among infants is approximately 0.1% to 0.3%, and up to 35% of the survivors had significant handicap, such as cerebral palsy and mental retardation (Finer et al., 1981; Costello and Manandhar, 1994; Rivkees and Wendler, 2011). To date, hypothermia is rapidly becoming the standard therapy for full-term neonates with moderate to severe HIE. However, hypothermia does not completely protect an injured brain, and available evidence suggests that neonates with the most severe forms of HIE may not be rescued (Azzopardi et al., 2009; Perrone et al., 2010; Cilio and Ferriero, 2010; Lin et al., 2011). Thus, an effective therapy to mitigate brain damage and improve the prognosis of HIE infants should be determined.

Reactive oxygen species (ROS) plays a role in normal physiology and accumulates rapidly after hypoxia–ischemic (HI) brain injury. A number of studies have considered oxidative stress in the mechanism of ischemia–reperfusion injury (Raza et al., 2011; Lu et al., 2012). Failing to eliminate excessive ROS leads to subsequent oxidative damage to the pathogenesis of ischemia–reperfusion (Ten and Starkov, 2012). Another important mechanism that leads to neuronal death is the inactivation of Akt, a key survival signaling kinase, and its downstream target FoxO3 (Skurk et al., 2005a,b).

Hesperidin, a flavanone glycoside found abundantly in citrus fruits, is believed to play a role in plant defense. The biological activities of hesperidin cover a broad spectrum, from anticancer and antibacterial activities to inhibition of bone reabsorption and neuroprotection effect (Amado et al., 2009). Although emerging evidence suggests that hesperidin has an important role in brain development, little is known about its mechanism. Several *in vitro* studies have proved that hesperidin has a pharmacological profile as a sedative and sleep-inducing compound. Other studies have suggested that this compound has an important neuroprotective property related to its antioxidant and anti-inflammatory effects (Nones et al., 2011). Martinez, by applying a hesperidin structural analog that exerted a minor sedative effect of hesperidin, found that hesperidin

*Corresponding author. Address: 1095 Jiefang Avenue, Tongji Hospital, Wuhan 430030, China. Tel: +86-011-27-83663643.
E-mail address: dliu428@gmail.com (D. Liu).

Abbreviations: BBB, blood–brain barrier; DCFH-DA, 2′,7′-dichlorodihydro-fluorescein diacetate; DIV, days *in vitro*; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethyl sulfoxide; FJB, fluoro-jade B; HI, hypoxia–ischemic; HIE, hypoxia–ischemic encephalopathy; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, MTT; NB, neurobasal; NS, normal saline; OGD, oxygen and glucose deprivation; PFA, paraformaldehyde; TTC, 2,3,5-triphenyltetrazolium chloride monohydrate; ROS, reactive oxygen species.

0306-4522/13 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2013.09.030

accomplishes its depressant action by decreasing brain pERK1/2 signal pathway (Martinez et al., 2009). Gaur demonstrated that hesperidin treatment significantly improved neurobehavioral alterations in adult rat ischemic reperfusion brain injury, and that this protective effect was attenuated by L-arginine but significantly potentiated by L-NAME treatment. This result suggested that L-arginine–NO signaling pathway is involved in the protective effect of hesperidin against cerebral ischemic reperfusion-induced brain damage (Gaur and Kumar, 2010). Moreover, Youdim found that hesperidin can pass the blood–brain barrier (BBB) (Youdim et al., 2003). Hesperidin possibly provides similar neuroprotection against HI injury *in vivo*.

To date, the majority of studies on hesperidin have focused on its protective effect on cultured primary cortical neurons or astrocytes, but the neuroprotective effects of hesperidin in *in vivo* model such as HI brain injury have not been fully investigated. The present study is intended to evaluate the effects of hesperidin in protecting neurons from HI injury using corresponding *in vitro* and *in vivo* models and to explore potential mechanisms of the effects of hesperidin in the same oxygen and glucose deprivation (OGD) and neonatal HI model.

EXPERIMENTAL PROCEDURES

Primary cortical neuron culture

The neurons were collected from newborn Sprague–Dawley rats using the procedures described in the previous studies (Zhong et al., 2004; Pan et al., 2012). Briefly, after removing the meninges, the cortical tissue was minced and maintained in Dulbecco's Modified Eagle Medium (DMEM) medium. An aliquot of 0.25% trypsin (Invitrogen 15050-065) and DNAse (Sigma DN25) was added and incubated for 15 min at 37 °C to produce a single cell suspension. After centrifugation, the cells were resuspended in neurobasal (NB) medium (GIBCO 10888) supplemented with 2% B-27 (Invitrogen 17504-044), 0.5 mM glutamine (GIBCO 25030-081), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were plated into poly-L-lysine-coated (Sigma P1399) dishes at an appropriate density. The culture medium was changed at 24 h and 4 d *in vitro* (DIV), and b-fibroblast growth factor (final concentration, 5 ng/mL; Sigma F0291) was added into the culture medium. The cells were ready to use at 7 DIV.

OGD

At 7 DIV, cultured primary cortical neurons were gently washed with phosphate-buffered saline (PBS) and then changed to glucose-free DMEM (GIBCO 11965) before being placed into a humidified chamber gassed with 95% N_2/5% CO_2. The control cells were changed to DMEM with glucose media (GIBCO 11966) and remained in a regular incubator (5% CO_2 and 21% O_2). After 2.5 h, the cells were removed from the hypoxic chamber to a regular incubator after changing back to NB media. The cells were pretreated with hesperidin (dissolved in dimethyl sulfoxide (DMSO), Sigma h5254) for 0.5 h to evaluate its effect.

LDH release and MTT assay

Cell injury or death was assessed by measuring the amount of lactate dehydrogenase (LDH) released into the culture medium using a cytotoxicity detection kit (Promega G1780) following the manufacturer's procedure. Briefly, 50 μL of culture medium was mixed with 50 μL substrate and incubated in a dark place at 37 °C for 30 min. Then, 50 μL stop solution was added, and the intensity was measured at 490 nm.

Cell viability was monitored by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) colorimetric assay (Sigma M2003). MTT (10 μL) was added to the cell culture medium and incubated at 37 °C for 4 h. Then, after discarding the medium, 500 μL DMSO was added, and absorbance at 570 nm was recorded.

Intracellular ROS and TBARs

For ROS measurement, 2′,7′-dichlorodihydro-fluorescein diacetate (50 μM, DCFH-DA, Sigma D6883) was added to the culture medium. After 30 min incubation, the cells were washed twice with PBS to remove excessive DCFH-DA. The DCF fluorescence was measured in a microplate reader with excitation at 485 nm and emission at 535 nm, and the results were normalized by protein concentration.

The lipid peroxidation products were measured in cultured neurons using a commercial kit (TBAR assay kit, Cayman, 10009055). The primary neurons were seeded in a 24-well plate on 7 DIV. The cells were washed with PBS to remove dead cells, and 50 μl of RIPA buffer was added to lysate cells. The lipid peroxide levels were determined and normalized by protein concentration (following the manufacturer's instructions).

Neonatal HI model and treatment

Seven-day-old Sprague–Dawley rat pups (eight per litter, weighing 13 g to 18 g) were anesthetized with a mixture of isoflurane (3–4% for induction and 2% for maintenance) and 30% oxygen/70% N_2. The left carotid artery of each pup was exposed and ligated with 6-0 surgical silk. After a 2-h recovery period, the pups were placed in 2-L airtight and watertight glass flasks submerged in a water bath at 37.0 °C and exposed to a humidified mixture of 8% oxygen and 92% nitrogen. After 2.5 h of hypoxia, the pups were returned to their dams. Hesperidin (50 mg/kg/day) (Raza et al., 2011; Kamisli et al., 2013) was dissolved in normal saline (NS), and the pups were pretreated orally for 3 d (by gavage). The vehicle group received the same volume of NS. The sham group underwent the same operative procedure, except for the left carotid artery ligation and hypoxia. The animal experiments were approved by the ethics committee of the Huazhong University of Science and Technology. The observer was blinded with the treatment condition when he was evaluating the outcome.