Neurochemistry International 63 (2013) 741–749

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Isoquercetin protects cortical neurons from oxygen–glucose deprivation–reperfusion induced injury via suppression of TLR4–NF-κB signal pathway

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ARTICLE INFO

Article history:
Received 6 April 2013
Received in revised form 10 September 2013
Accepted 22 September 2013
Available online 5 October 2013

Keywords:
Isoquercetin
Oxygen–glucose deprivation followed by reperfusion (OGD/R)
Ischemia
Apoptosis
Inflammation

ABSTRACT

In the present study, oxygen–glucose deprivation followed by reperfusion (OGD/R), an in vitro model of ischemia, was used to evaluate the neuroprotective effect of isoquercetin in primary culture of rat cortical neuronal cells. It was found that isoquercetin administered prior to the insult could prevent OGD/R-induced intracellular calcium concentrations ($[Ca^{2+}]_i$) increase, lactate dehydrogenase (LDH) release and cell viability decrease. For the first time, isoquercetin is described as a neuroprotective agent that potentially explains the alleviation and prevention from OGD/R-induced injury in neurons. Mechanistic studies showed that the neuroprotective effect of isoquercetin was carried out by anti-inflammatory signaling pathway of inhibiting protein expression of toll-like receptor 4 (TLR4) and nuclear factor-kappa B (NF-κB), and mRNA expression of TNF-α and IL-6, accompanied by the anti-apoptotic signaling pathway of deactivation of extracellular-regulated kinase (ERK), Jun kinase (JNK) and p38, and inhibition of activity of caspase-3. Therefore, these studies highlighted the confirmation of isoquercetin, a flavonoid compound, as an anti-inflammation and anti-apoptosis factor which might be used as a therapeutic strategy for the ischemia/reperfusion (I/R) brain injury and related diseases.

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1. Introduction

About 87% of strokes are caused by ischemia, and ischemia has been the target of most drug trials (Rosamond et al., 2007). Ischemic stroke was induced by thrombosis, embolism or systemic hypoperfusion, all of which result in a restriction of blood flow to the brain, and ischemia results in insufficient oxygen and glucose delivery to support cellular homeostasis. Brain ischemia induces numerous deleterious cascades which may lead to cell death: excitotoxicity, acidotoxicity and ionic imbalance, peri-infarct depolarization, oxidative and nitrative stress, inflammation and apoptosis (Gonzalez, 2006; Juan et al., 2012). The current treatment for complete stroke is only partially successful at reversing neurodegeneration and restoring premorbid function. Therefore, there is a pressing need for new therapeutic strategies.

Ischemia/reperfusion (I/R) injury, which resulted in cerebral infarction and revascularization after thrombolysis, is the main cause for the aggravation of cerebral injury and functional impairment. Excessive formation of free radicals, inflammatory reactions, overload of calcium inside neurocytes, and the cytotoxic effect of excitatory amino acids have been proved to be the main causes contributing to cerebral I/R injury. As the main factor of aggravation of cerebral injury, acute inflammatory cascade reactions cause specific activities. There are obvious inflammatory cell aggregations, up-regulation of cytokines expression, and increased expression of intercellular adhesion molecules during cerebral I/R injury, and, what is more, oriented anti-inflammatory therapy has displayed evident neuroprotective effects on animal models.

Abbreviations: $[Ca^{2+}]_i$, intracellular calcium concentrations; DMEM, Dulbecco's Modified Eagle's Medium; ERK, extracellular-regulated kinase; FBS, fetal bovine serum; I/R, ischemia/reperfusion; JNK, Jun kinase; LDH, lactate dehydrogenase; MAP, mitogen-activated protein; MCAO, middle cerebral artery occlusion; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-kappa B; OGD/R, oxygen–glucose deprivation followed by reperfusion; PI, propidium iodide; RT-PCR, Reverse Transcription-Polymerase Chain Reaction; SD, Sprague–Dawley; TLR4, toll-like receptor 4; TLRs, toll-like receptors.

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0197-0186/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neuint.2013.09.018

Toll-like receptors (TLRs) were recently shown to participate in the recognition of endogenous proteins that are released from damaged tissues after I/R injuries (Kaczorowski et al., 2008). A large body of evidence suggests that TLRs play a central role in I/R injuries. For example, it was suggested that toll-like receptor 4 (TLR4) knockout played a neuroprotective role in ischemic brain injury induced by middle cerebral artery occlusion (MCAO) in mice (Hyakkoku et al., 2010). I/R injury induces an intense inflammatory response when a tissue is temporarily deprived of blood supply and then resupply (Arumugam et al., 2004). Such pathology commonly occurs in cases of shock, tissue transplantation, myocardial infarction, stroke, certain infections, arterial disease, and trauma. Inflammatory responses play a crucial role in cerebral ischemic injuries and neuronal cell death (Arumugam et al., 2005, 2007; Ishikawa et al., 2005). TLR4 is a leucine-rich transmembrane protein, which activates a variety of signal pathways in the cytosol of immune cells. TLR4 stimulates mitogen-activated protein (MAP) and stress kinases, such as extracellular-regulated kinase (ERK) 1/2, p38 and Jun kinase (JNK) 1/2, which in turn activate transcription regulators of inflammation in the nucleus (Schroder et al., 2001). TLR4 signaling modulates the severity of ischemia-induced inflammatory response and neuronal damage and has been demonstrated as a target of stroke therapy (Arumugam et al., 2009). Activation of TLRs generally leads to nuclear translocation of the nuclear factor-kappa B (NF-κB), a key transcription factor involved regulating the inducible expression of inflammatory mediators that lead to an inflammatory response (Kaisho and Akira, 2006). Inhibition of cerebral NF-κB activation is associated with neuroprotective effects (Bi et al., 2009; Valerio et al., 2009). TLR4 and NF-κB signaling pathway has become a research focus of cerebral ischemic injury, which involved in the inflammatory response, neurological deficits and neuronal apoptosis (Yang et al., 2011; Zhang et al., 2012). Therefore, targeting TLR4 and NF-κB signaling provides a promising intervention strategy to reduce damage induced by these pathologies (Khan et al., 2012; Qin et al., 2013; Zhang et al., 2012).

There are numerous studies on the beneficial effects of polyphenols from plants and fruits in humans and animals. Quercetin is present in various plants and particularly abundant in onions and tea, and exists predominantly in glucoside forms including isoquercetin (Lakhanpal and Rai, 2007; Scalbert and Williamson, 2000). Isoquercetin possesses many biological properties including antiviral (Kim et al., 2010), antidiabetic (Zhang et al., 2011a) and antioxidant effects (Nguyen et al., 2009). Recently, it was reported that isoquercetin protects neuroblastoma (SH-SY5Y) cells in vitro against oxidative damage (Soundararajan et al., 2008). Besides, its analogue hyperoside, has been demonstrated to exhibit neuroprotective effects against OGD/R-induced injury (Chen et al., 2006; Liu et al., 2012). Based on these research, we hypothesize that isoquercetin possess neuroprotective effects against cerebral I/R injury. Therefore, the present study was decided to investigate the neuroprotective effect of isoquercetin against OGD/R-induced injury in primary culture of rat cortical neurons. Furthermore, the possible molecular signaling mechanisms underlying the neuroprotective effect of isoquercetin was also investigated.

2. Experimental procedures

2.1. Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), trypsin, Neurobasal medium, and B27 were purchased from Gibco (Grand Island, NY). Poly-L-lysine, Hoechst 33342, nimodipine (purity above 98%), mouse monoclonal neurofilament 200 (NF200) antibody, and FITC goat anti-mouse IgG were purchased from Sigma (St. Louis, MO). Primary antibodies used for the Western Blot analyses in the present study included mouse anti-TLR4 monoclonal antibody (76B357.1, Abcam), rabbit anti-NF-κB (p65) polyclonal antibody (10745-1-AP, Proteintech), rabbit anti-CASP3 polyclonal antibody (19677-1-AP, Proteintech), rabbit anti-ERK1/2 polyclonal antibody (16443-1-AP, Proteintech), p38 MAP Kinase antibody (9212, Cell Signaling Technology), mouse anti-GAPDH monoclonal antibody (60004-1-Ig, Proteintech), JNK (FL) antibody (sc-572, Santa Crutz, CA), phospho-SAPK/JNK (Thr183/Tyr185) antibody (9251S, Cell Signaling Technology), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (D13.14.4E) XP® rabbit mAb (4370S, Cell Signaling Technology) and phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP® Rabbit mAb (4511, Cell Signaling Technology). HRP-goat anti-rabbit IgG (H + L) (00001-1, Proteintech) and HRP-goat anti-mouse IgG (H + L) (00001-2, Proteintech) were used as the secondary antibody. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Genmed (Westbury, NY). Isoquercetin (purity above 98%) was provided by the Dalian Institute of Chemical Physics, Chinese Academy of Sciences. Lactate dehydrogenase (LDH)-Cytotoxic test kit and intracellular calcium concentration assay kit were purchased from Nanjing Jiancheng Biotech, China. Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit (V13241, Invitrogen) was used for Flow Cytometry.

2.2. Primary cortical neuronal cultures and experimental treatments

All procedures on animals followed guidelines established by the Institutional Animal Care Committee and China Council on Animal Care. Primary culture of rat cortical neurons was prepared from the brain of Sprague–Dawley (SD) rat embryos at 17–19 days of gestation (obtained from the Experimental Animal Center of Nantong University, China). The dissociation and culture method was followed as described by Zhang et al. (2011b), with some modifications. Briefly, pregnant females were anesthetized with 10% chloral hydrate (0.4 ml/100 g body weight) and subjected to cesarean section in order to dissect the fetal brains. The dissected tissues were minced separately into small pieces and digested with trypsin (0.125%) for 5 min, before culture medium containing 10% FBS was added. The mixture was subjected to centrifugation at 500 rpm for 5 min. The cells were resuspended in DMEM supplemented with 10% FBS and plated onto poly-L-lysine-coated plates for 4 h incubation at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. After cells attached to the substrate, the medium was replaced with neuronal culture medium consisting of serum-free Neurobasal medium supplemented with 2% B27, 0.5 mM glutamine, followed by re-incubation for 7–8 days, the time required for maturation of cortical neurons, with half of the medium being changed every 2 days. Then, the cells were characterized by immunohistochemistry for NF200 protein, revealing that the cell cultures contained above 90% neurons.

2.3. OGD/R application

OGD was performed according to a previously described method (Liu et al., 2012; Rau et al., 2012). In brief, the culture medium was removed and cells were washed twice and incubated in DMEM without glucose. Then, the cultures were introduced into a specialized, humidified chamber filled with 95% N_2 and 5% CO_2 at 37 °C for 6 h. OGD was terminated by replacing the DMEM without glucose with Neurobasal medium supplemented with 2% B27 and the cultures were further incubated under 95% air and 5% CO_2 for 24 h at 37 °C (recovery, R). Cells in the control group were treated identically except that they were not exposed to OGD.

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