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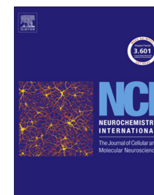

Lose dose genistein inhibits glucocorticoid receptor and ischemic brain injury in female rats

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ABSTRACT

Although acute bolus of genistein treatment has been shown to protect against neuronal damage in experimental brain injury animal models, chronic continuous low dose treatment of genistein on ischemic brain injury has not been well elucidated. In the present study, female rats were received either pure genistein (0.1 mg/kg/day via osmotic minipumps) or placebo at the time of ovariectomy, and transient forebrain ischemia was induced 7 days later. Results demonstrated that genistein treatment for 14 days significantly improved ischemic neuronal survival in hippocampal CA1 region of ovariectomized rats. Glucocorticoid receptor (GR) is a member of the adrenal steroid hormone receptor, which is highly expressed in the rat hippocampus. Activation of the GR plays a critical role in the neuronal stress responses, including ischemic brain damage. This study therefore examined the potential mechanisms by which genistein regulates GR signaling, including the protein distribution and receptor activation in hippocampus following ischemic reperfusion (*I*/*R*). Results showed that GR expression in the ovariectomized rats was excessively increased both in neurons (*I*/*R* 6 h) and activated microglial cells (*I*/*R* 7 d) in hippocampal CA1 region. Genistein treatment significantly attenuated GR induction and the enhanced GR nuclear translocation and DNA-binding capacity. The effects of genistein on the GR levels was accompanied with decreased blood plasma levels of corticosterone (primary glucocorticoid in rodents) and coupled to an E3 ubiquitin ligase Mdm2 targeted proteasomal degradation of GR, because genistein treatment could enhance the GR–Mdm2 interaction and the ubiquitination level of GR protein. In addition, our results indicated that genistein markedly prevented the excessive activation of microglia in CA1 sector. These results demonstrate the neuroprotective action of chronic low dose genistein replacement against ischemic brain damage, and a potential mechanism associated with the inhibition of both neuronal and microglial GR signaling following ischemic stress.

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1. Introduction

In experimental animals, pyramidal neurons in the hippocampal cornus ammon's 1 (CA1) region are selectively vulnerable to transient forebrain ischemia, which often resulting from cardiac arrest (Satou and Ueda, 1978; Pulsinelli and Brierley, 1979; Wolman et al., 1999). This type of delayed neuronal death in the CA1 cell layer several days after brain ischemic stress is known as apoptotic cell death (Nitatori et al., 1995; Choi, 1996; Lipton, 1999; Harukuni and Bhardwaj, 2006). Unfortunately, there is currently no effective drug available that protect the brain from cardiac arrest-induced neuronal impairment. Despite the controversies about safety and benefits of soy-based supplements, emerging data suggest that one potent and putative neuroprotective agent in cardiovascular and ischemic brain diseases is the soy isoflavonoid, genistein. Genistein has been shown to mimic the pharmacological actions of the ovarian steroid hormone, 17β-estradiol, with which it has structural similarities and weak estrogenic activity (Vincent and Fitzpatrick, 2000; Brooks and Skafar, 2004; Schreihofer and Redmond, 2009). Genistein activates anti-apoptotic pathways, inhibits the activation of mitochondria-dependent apoptosis pathways, reduces ischemia-induced reactive oxygen species (ROS) production and oxidative stress, and interacts with nitric oxide system and downstream antioxidant/detoxification Nrf2 signaling cascades to increase neuronal survival in experimental cerebral ischemia (Altavilla et al., 2004; Lovekamp-Swan et al., 2007; Schreihofer and Redmond, 2009; Qian et al., 2012; Wang et al., 2013). However, before the therapeutic potential of genistein can be translated to clinic and for drug development, detailed preclinical studies are in urgent need for understanding the precise beneficial properties and possible pharmacological targets of genistein in ischemic brain damage.

Extensive studies demonstrate that adrenal steroids, secreted during stress conditions, have potential deleterious effects to the

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brain (Sapolsky, 1996; Hibberd et al., 2000; Rose et al., 2010). Since forebrain ischemic stress is a fatal insult to internal hippocampus CA1 neurons, the adrenocortical glucocorticoids stress hormones entering the brain may have damaging effects to the vulnerable neurons after binding to glucocorticoid receptors (GR), which are predominantly expressed the hippocampal neurons (Aronsson et al., 1988; De Kloet et al., 1998). Interestingly, adrenalectomy performed after transient forebrain ischemia protects hippocampal CA1 from ischemic damage, while glucocorticoid administration results in an increase in ischemic injury (Sapolsky and Pulsinelli, 1985; Morse and Davis, 1990). While the underlying molecular mechanisms are poorly understood, it has been shown that GR activation in the rat hippocampus induces neuronal cell death by increasing the ratio of the proapoptotic protein Bax relative to the antiapoptotic proteins Bcl-2 or Bcl-xL (Almeida et al., 2000). Furthermore, studies demonstrate that prevent glucocorticoids release or block GR activation leads to protection from ischemic neuronal death induced by transient forebrain ischemia or seizures (Smith-Swintosky et al., 1996; Antonawich et al., 1999; Cheng et al., 2009). Taken together, these evidences indicate that GR activation resulting from cerebral ischemia contributes to brain neuronal damage and suggest that inhibition of GR activation plays an important role in neuroprotection. However, the protein distribution and activation patterns of GR in different hippocampal regions following forebrain ischemia have not been well studied in the ovariectomized rats. It is not entirely clear whether genistein replacement could regulate GR stress signaling and the potential mechanisms by which genistein reduces selective hippocampal damage.

Although the actions of genistein replacement may be important in ischemic brain damage, the *in vivo* neurotoxicity and other side effects at high concentrations have also been suggested (Linford et al., 2001; Lavigne et al., 2008; Kim et al., 2009; Hilakivi-Clarke et al., 2010). Therefore, caution should be noted about the potential side effects and safety in the nervous system with genistein treatment at high doses. The purpose of the present work was to determine the effectiveness of a chronic low dose genistein replacement at the time of ovariectomy on the protein distribution and activation of GR following ischemic reperfusion, and the potential protective benefits against ischemic brain injury in the ovariectomized rats. The inhibitory effects of genistein on GR induction and the underlying mechanisms in hippocampal CA1 region following ischemic insult were also investigated.

2. Materials and methods

2.1. Induction of transient forebrain ischemia

Adult (3-month old) female Sprague–Dawley rats were used for the study. The animals were housed in a temperature-controlled facility on a 12-h light/dark cycle with free access to a non-soy (phytoestrogen free) chow diet. To exclude the effect of endogenous ovarian steroids, all the rats were subjected to bilateral ovariectomy (OVX) and 10 min of transient forebrain ischemia or sham ischemia was performed on day 7 after OVX. Placebo (Pla, 50% DMSO in distilled water) and genistein (0.1 mg/kg/day, Fisher Scientific, USA) were administered by means of Alzet osmotic minipumps (14 day release, Model 1002, Alzet, Durect Corp., USA) implanted subcutaneously in the upper mid-back region under the skin at the time of OVX. Compared with previous studies (Trieu et al., 1999; Donzelli et al., 2010; Castello-Ruiz et al., 2011; Qian et al., 2012), in the present study, a relatively low dose genistein was adopted to treat the animals in a chronic manner. The four vessel occlusion model of transient forebrain ischemia was induced essentially as described previously (Pulsinelli and Brierley, 1979; Zhang et al., 2009). Briefly, under anesthesia with chloral hydrate (350 mg/kg, i.p.) on day 6 after OVX, the first and second cervical vertebral bodies were exposed through a midline incision and the vertebral arteries were permanently electrocauterized with bipolar cauterization. Twenty-four hours later, the common carotid arteries were exposed under light anesthesia with isoflurane and occlusion was employed by using carotid clips. Carotid blood flow through the arteries was inspected and confirmed before the wound was sutured after 10 min ischemia. Successful forebrain ischemia was ensured by monitoring the bilateral pupil dilation and loss of righting reflex of each subject after the occlusion of both carotid arteries. Rectal temperature was monitored using a rectal thermometer probe and maintained using a heat lamp and thermal blanket during surgical procedures. Sham-operated animals had all their common carotid arteries exposed, vertebral arteries coagulated and underwent all other identical procedures except for carotid artery occlusion. All animal research protocols were approved by the animal use committee in the institution and conformed to the local and international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and reduce their suffering and distress.

2.1.1. Histologic analysis

Seven days after transient forebrain ischemia or sham ischemia, animals were deeply anesthetized with isoflurane and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Brains were removed and postfixed in the same fixative overnight, and cryoprotected with 30% sucrose in 0.1 M PB (pH 7.4) at 4 °C until they sank. Thereafter, the tissues were embedded in optimal cutting temperature (OCT) compound and frozen coronal sections were cut through the dorsal hippocampus in twenty micron thick. For histological analysis of neuronal cell survival, sections were incubated with 10% normal horse serum in PBS containing 0.1% Triton X-100 and 0.3% hydrogen peroxide (H_2O_2) for 1 h at room temperature to block nonspecific protein-surface binding. The sections were next incubated overnight at 4 °C with mouse anti-NeuN antibody (MAB377, 1:500, Millipore Corporation, MA, USA) in PBS containing 0.1% Triton X-100. Thereafter, sections were washed for 4×10 min by 0.1% PBS-Triton X-100 followed by incubation with secondary biotinylated horse anti-mouse antibodies (Vector Laboratories) at a dilution of 1:200 for 1 h at room temperature. Sections were then washed, followed by incubation with ABC reagents for another 1 h and visualized with 3,3′-Diaminobenzidine (DAB) according to the manufacturer's instructions. Sections were washed briefly with distilled water after DAB incubation and dehydrated through graded ethanols, cleared in xylene, and coverslipped using xylene-based mounting medium. Images were finally acquired on an Olympus microscopy system. For histological assessment, surviving neurons were counted bilaterally per 250 micron length in the pyramidal cell layer at the center of the CA1 region. NeuN-positive cells with intact and round nuclei as appear in sham CA1 pyramidal cells were counted as surviving neurons. Hippocampal sections of each animal at three levels (200 micron apart, approximately 1.5–3.3 mm posterior to bregma) were selected for staining. Cell counts per animal were expressed as the mean value of surviving neurons averaged across the left and right hippocampus on the three sections. Data were presented as mean ± SE and statistically analyzed as described below.

2.1.2. Immunofluorescence staining

Single and dual immunofluorescence staining was performed as described previously (Zhang et al., 2009). Briefly, coronal brain sections were blocked for 1 h with 10% normal donkey serum at room temperature followed by incubation with appropriate primary antibodies against NeuN (1:500; MAB377, Millipore, Billerica, MA), CD11b (1:200, ab1211, Abcam, Cambridge, MA), and GR

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