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ORAL ADMINISTRATION OF GLUTATHIONE IMPROVES MEMORY DEFICITS FOLLOWING TRANSIENT BRAIN ISCHEMIA BY REDUCING BRAIN OXIDATIVE STRESS

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Abstract—Oxidative stress aggravates brain injury following ischemia. The glutathione (GSH) system plays a pivotal role in combating oxidative stress in various cell types. To determine whether oral GSH administration elicits anti-oxidative effects, we assessed its potential neuroprotective effects in transient bilateral common carotid artery occlusion (BCCAO) mice. In naïve mice, acute oral administration of GSH significantly increased GSH levels by 1 h in the cortex and hippocampus. Eleven days after BCCAO, untreated mice showed significantly decreased GSH levels and an inverse elevation of glutathione-disulfide (GSSG) levels in both the cortex and hippocampus. Oral administration of GSH (100 and 500 mg/kg p.o.) for 10 consecutive days after ischemia restored reduced GSH levels and inhibited GSSG elevation. Notably, post-administration of GSH (100 and 500 mg/kg p.o.) significantly prevented neuronal cell death in the hippocampal CA1 region in BCCAO mice, an effect closely correlated with decreased levels of oxidative markers such as 4-hydroxy-2-nonenal (4-HNE), 8-hydroxy-2-deoxyguanosine (8-OHdG) and nitrotyrosine in that region. Finally, GSH administration for 10 days improved memory deficits observed in BCCAO mice. Taken together, our findings indicate that the anti-oxidative effect of oral GSH administration ameliorates post-ischemia neuronal cell death and, in turn, may improve memory.

Key words: Ca^{2+}/calmodulin-dependent protein kinase II, bilateral common carotid arteries occlusion, glutathione, neuroprotection, oxidative stress.

INTRODUCTION

Cerebral ischemia is the most common cause of permanent disability globally and is associated with a high incidence of sensory, motor, and cognitive deficits (Das et al., 2008). Most notably, up to 23% of strokes are caused by transient ischemic attack (TIA) (Rothwell and Warlow, 2005). Anti-oxidative Radicut treatment is now the standard clinical therapy for only the acute phase of TIA, particularly in Japan (Edaravone Acute Infarction Study Group, 2003). Since Radicut has side effects including acute renal failure, safe and prolonged anti-oxidative therapy in the subacute phase is required to prevent progressive brain damage after TIA.

Oxidative stress resulting from generation of reactive oxygen species (ROS), including superoxide, hydrogen peroxide and peroxynitrite, is a major factor exacerbating neuronal damage following brain ischemia (Oliver et al., 1990). Glutathione (GSH), a natural tripeptide of glutamate, cysteine, and glycine, mediates antioxidant effects in cells to directly detoxify ROS and can serve as a substrate for various peroxidases (Marí et al., 2009). For example, superoxide rapidly changes to hydrogen peroxide by superoxide dismutase. Hydrogen peroxide is then reduced by GSH, thereby generating glutathione-disulfide (GSSG) and water by glutathione peroxidase (Marí et al., 2009). Impairment in the glutathione system is reportedly a factor in several neurodegenerative diseases, including cerebral ischemia (Schulz et al., 2000; Anderson and Sims, 2002). In rats, middle cerebral artery occlusion (MCAO) causes reduction in GSH levels and inverse increases in GSSG levels (Jung et al., 2011). Injection of glutathione monoethylester directly into the striatum reportedly reduces infarct volume in these experimental animals (Anderson et al., 2004a,b) and restores cellular glutathione levels in various tissues (Anderson et al., 1985). Likewise, oral administration of herbal antioxidants such as those derived from *Wedelia calendulacea* or *Ocimum sactum* or the plant extract Joongpoongtang 05 restores GSH levels in MCAO or bilateral common carotid artery occlusion (BCCAO) rats (Jung et al., 2011; Prakash et al., 2011; Ahmad et al., 2012). In addition, green tea extracts also restore reduced GSH levels in both the cortex and hippocampus, thereby ameliorating learning and memory deficits following MCAO ischemia (Wu et al., 2012). Although antioxidant GSH system has a key role in cerebral ischemia, neuroprotective effects against

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Abbreviations: 4-HNE, 4-hydroxy-2-nonenal; 8-OHdG, 8-hydroxy-2-deoxyguanosine; ANOVA, analysis of variance; BCCAO, bilateral common carotid arteries occlusion; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione; GSSG, glutathione-disulfide; HSP70, heat-shock protein 70; LTP, long-term potentiation; MCAO, middle cerebral artery occlusion; NEM, N-ethylmaleimide; NO, nitric oxide; OPT, o-phthalaldehyde; PBS, phosphate-buffered saline; PI, propidium iodide; ROS, reactive oxygen species; SEM, standard error of the mean; SUMO1, small ubiquitin-like modifier 1; TIA, transient ischemic attack; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-nick-end-labeling.

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brain ischemia following GSH oral administration have not been evaluated.

Here, we first asked whether oral GSH penetrates the brain in order to define its potential neuroprotective effect in the subacute phase of transient BCCAO mice. We found that oral administration of GSH to BCCAO mice restored reduced GSH levels in the brain. Oral GSH administration also rescued neurons from damage due to oxidative stress following ischemia, thereby improving higher brain functions such as cognition and memory.

EXPERIMENTAL PROCEDURES

Preparation of the ischemic model

Adult male C57/BL6N mice (10 weeks old) were obtained from Clea Japan, Inc. (Tokyo, Japan), housed under conditions of constant temperature (23 ± 1 °C) and humidity (55 ± 5%), kept on a 12-h light–dark cycle (light; 9–21 h), and fed *ad libitum*. All experimental animal procedures were approved by the Committee on Animal Experiments at Tohoku University. Efforts were made to minimize suffering and to reduce the number of animals used.

BCCAO mice were prepared as described (Yamamoto et al., 2009). Briefly, mice were anesthetized with 4% halothane and anesthesia was maintained with 2% halothane (Takeda Chemical Industries Ltd., Osaka, Japan). After a lateral neck incision, bilateral common carotid arteries were occluded for 20 min. Body temperature was maintained at 37–38 °C until the operation using a feedback-regulated heating blanket. Sham-operated mice receiving the same experimental procedures without artery occlusion were used as control animals.

GSH administration

GSH was obtained from (Kyowa Hakko Bio Ltd., Tokyo, Japan) and suspended in distilled water. One day after surgery, BCCAO mice were administered drug orally once a day at 50, 100 or 500 mg/kg of GSH in a volume of 10 mL/kg or the same volume of suspended solution for 10 days. Sham-operated mice were treated with the same volume of suspended solution or 500 mg/kg of GSH.

For behavioral analyses, animals were subjected to behavioral tests from the least to the most stressful behavioral tests such as Y-maze, novel object recognition and step-through passive avoidance tasks in this order (McIlwain et al., 2001). To minimize stress effects, five sets of experiments were conducted using different animals. (1) Group I consisting of naïve mice was subjected to analyses of brain GSH levels after oral administration of GSH or L-Cysteine (Wako Chemical Ltd., Osaka, Japan) (n = 3 per group). When the temporal change was analyzed, mice were treated with GSH or L-Cysteine orally at 0, 0.25, 0.50, 1.0 or 3.0 h prior to sacrifice. (2) Group II was subjected to analyses of brain GSH and GSSG levels at 11 days after transient BCCAO ischemia (n = 4 per group). (3) Group III was subjected to immunohistochemical (n = 4–5 per group), histopathologic (n = 6 per group), and terminal deoxynucleotidyl transferase-mediated dUTP-nick-end-labeling (TUNEL) staining (n = 3 per group) at 11 days after the Y-maze task at 8 days. (4) Group IV underwent the Y-maze task at 8 days and then was subjected to western blotting analysis at 11 days (n = 3–4 per group). (5) Group V was subjected to the Y-maze task at 8 days, novel object recognition task at 9 days, and the step-through passive avoidance task at 10 and 11 days (n = 4–8 per group). In groups II, III and IV, the Y-maze task was carried out to confirm memory impairment after BCCAO ischemia. At 24 h after final GSH oral administration, animals in groups II, III and IV were dissected or perfusion-fixed with 4% paraformaldehyde.

Y-maze task

Spontaneous alternation behavior in a Y-maze was assessed as a spatial reference memory task as described (Moriguchi et al., 2012). The apparatus consisted of three identical arms (50 × 16 × 32 cm) made from black plexiglas. Mice were placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The sequence of arm entries was recorded manually. An alternation was defined as entries into all three arms on consecutive choices. The maximum number of alternations was defined as the total number of arms entered minus two, and the percentage of alternations was calculated as actual alternations/maximum alternations × 100. The total number of arms entered during the session was also determined.

Novel object recognition task

For the novel object recognition task, mice were individually habituated to an open-field box (35 × 25 × 35 cm) for two consecutive days. The experimenter scoring the behavior was blinded to the treatment. During the acquisition phase, two objects of the same material were placed symmetrically in the center of the chamber for 10 min. One hour later, one object was replaced by a novel object, and exploratory behavior was again analyzed for 5 min. After each session, objects were thoroughly cleaned with 70% ethanol to prevent odor recognition. Exploration of an object was defined as rearing on the object or sniffing it at a distance of less than 1 cm, touching it with the nose, or both. Successful recognition was reflected by preferential exploration of the novel object. Discrimination of spatial novelty was assessed by comparing the difference between exploratory contacts of novel and familiar objects and the total number of contacts with both, making it possible to adjust for differences in total exploration contacts.

Step-through passive avoidance task

Training and retention trials of the passive avoidance task were conducted in a box consisting of dark (25 × 25 × 25 cm) and light (14 × 10 × 25 cm)