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Research report

Combined L-citrulline and glutathione administration prevents neuronal cell death following transient brain ischemia

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ABSTRACT

We previously reported that oral L-citrulline (L-Cit) administration antagonizes neuronal cell death in hippocampus following transient brain ischemia and that oral glutathione (GSH) administration prevents neuronal death through antioxidant activity. Here, we tested potential synergy of combined L-Cit and GSH administration in protection against neuronal death following cerebral ischemia. One day after a 20-min bilateral common carotid artery occlusion (BCCAO), mice were orally administered L-Cit or GSH alone (at 40 or 100 mg/kg p.o.) or both (at 40 mg/kg p.o. each) daily for 10 days. The combination, but not L-Cit or GSH alone at 40 mg/kg p.o., significantly prevented neuronal death in the hippocampal CA1 region in BCCAO mice. Consistently, combined L-Cit and GSH administration improved memory-related behavioral deficits observed in BCCAO mice. Combination treatment also significantly rescued reduced endothelial nitric oxide synthase (eNOS) protein levels and antagonized eNOS S-glutathionylation seen following BCCAO ischemia. Recovery of eNOS activity was confirmed by in vivo NO production in hippocampus of BCCAO mice. Taken together, combined administration of L-Cit with GSH rescues eNOS function, thereby inhibiting delayed neuronal death in hippocampus.

1. Introduction

Cerebral ischemia is the most common cause of permanent disability and leads to cerebral hypoperfusion and endothelial injury (Janigro et al., 1994; Pluta et al., 1994; Han et al., 2006), in turn causing motor and memory impairments (Das et al., 2008). Up to 23% of strokes occur due to transient ischemic attack (TIA) (Rothwell and Warlow, 2005). In Japan, treatment with the antioxidant edaravone has been approved for use in only in the acute phase of TIA, but edaravone is associated with side effects such as acute renal failure (Edaravone Acute Infarction Study Group, 2003). Thus, safer and more effective therapies are necessary for the subacute phase to prevent brain injury after TIA.

Nitric oxide (NO) levels play a key role in survival of neurons following brain ischemia. (Han and Fukunaga, 2009; Liu et al., 2015). Under ischemic conditions, abnormal NO elevation aggravates brain injury in the subacute phase (Coeroli et al., 1998; Liu et al., 2013). In fact, in mice neuronal nitric oxide synthase (NOS) or inducible NOS (iNOS) deficiency decreases middle cerebral artery occlusion (MCAO)-induced infarct volume (Huang et al., 1994; Park et al., 2006). By contrast, increased infarct size is observed in endothelial NOS (eNOS) knockout relative to wild-type mice following MCAO (Huang et al., 1996). These findings suggest that NOS activities have diverse effects on neuronal injury or survival following cerebral ischemia and that eNOS-derived NO may be neuroprotective.

L-Citrulline (L-Cit) is a reaction product of NOS-catalyzed NO synthesis from L-arginine (L-Arg) (Hayashi et al., 2005). However, argininosuccinate synthase and lyase convert L-Cit to L-Arg in vivo, facilitating L-Arg recycling for NO production (Cynober et al., 2010). Several reports indicate a protective effect of L-Cit on peripheral blood vessels. For example, L-Cit administration antagonizes injury to the thoracic aorta in rats fed high-fat and high-cholesterol diets (El-Kirsh et al., 2011). L-Cit administration

Abbreviations: ANOVA, analysis of variance; APP, amyloid precursor protein; BCCAO, bilateral common carotid artery occlusion; eNOS, endothelial nitric oxide synthase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; GSH, glutathione; GSSG, glutathione-disulfide; iNOS, inducible nitric oxide synthase; -Arg, -arginine; -Cit, -citrulline; MCAO, middle cerebral artery occlusion; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PI, propidium iodide; TIA, transient ischemic attack.

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also antagonizes endothelial damage and microcirculatory dysfunction in rats following cardiac ischemia/reperfusion injury (Ikeda et al., 2000). Furthermore, Marquet-de Rougé et al. (2012) report that L-Cit supplementation antagonizes increases in levels of amyloid precursor protein (APP), the C99-APP-C terminal peptide, caveolin-1 and cholesterol seen in hippocampus of aging rats. Recently, we reported that oral L-Cit administration protects against cerebrovascular injury and neuronal cell death in bilateral common carotid artery occlusion (BCCAO) mice, improving memory deficits (Yabuki et al., 2013). L-Cit activity is closely associated with restoration of reduced eNOS protein expression and function in hippocampus following BCCAO (Yabuki et al., 2013). These observations indicate that L-Cit may be beneficial in attenuating eNOS dysfunction in both the periphery and in cerebral vascular diseases.

Glutathione (GSH) serves as an antioxidant by scavenging ROS directly (Marí et al., 2009). When GSH detoxifies ROS, oxidized GSH, called glutathione-disulfide (GSSG), is generated by glutathione peroxidase (Marí et al., 2009). Several reports support the importance of the glutathione antioxidant system in neurodegenerative diseases, including cerebral ischemia (Anderson and Sims, 2002; Jung et al., 2011; Schulz et al., 2000). Recently, we reported that oral GSH administration rescues reduced GSH levels and decreases elevated GSSG levels caused by BCCAO ischemia, preventing neuronal cell death in the hippocampal CA1 region (Yabuki and Fukunaga, 2013).

GSH also plays a key role in NO synthesis. L-Cit levels as an indicator of NO synthesis are dependent on intracellular GSH levels in human and murine endothelial cells (Ghigo et al., 1993, 1996). GSH treatment promotes L-Arg turnover and increased NOS activity (Hofmann and Schmidt, 1995). In addition, the oxidized form GSSG reportedly alters function of several proteins by promoting reversible protein S-glutathionylation consisting of formation of a disulfide bond between glutathione and susceptible cysteine thiol residues (Mieyal et al., 2008; Popov, 2014). Chen et al. (2010) reported that S-glutathionylation of eNOS causes functional uncoupling, decreasing NO biosynthesis and increasing generation of superoxide anions. Moreover, McKinley-Barnard et al. (2015) report that chronic administration of combined L-Cit and GSH increases NO bioavailability in blood during exercise in humans.

Here, we investigated benefits and potential neuroprotective mechanisms underlying effects of combined L-Cit and GSH administration on brain injury using ischemic mice. We demonstrate that following brain ischemic injury, a combined low dose of L-Cit and GSH rescues neuronal cell loss comparably or in some cases more efficiently than high doses of either drug alone, improving memory deficits in BCCAO mice. We also confirm that BCCAO ischemia-induced eNOS S-glutathionylation is inhibited by L-Cit and GSH combination treatment, improving eNOS function in brain.

2. Results

2.1. Combined L-Cit and GSH administration prevents neuronal cell death in hippocampal CA1 following BCCAO

To assess neuroprotective effects of L-Cit or GSH or their combined administration, we evaluated cell survival in hippocampal CA1 regions after BCCAO as shown in Fig. 1. We detected viable cells in the CA1 region using propidium iodide (PI) staining (Fig. 2A, B). There were significant group effects on cell viability [$F(6, 21) = 32.603$, $p < 0.001$]. L-Cit or GSH administration (at 40 mg/kg p.o.) did not block cell death in CA1 following transient brain ischemia (GSH40: 28.9 ± 10.8% of control, $p < 0.01$ vs. control, $p > 0.05$ vs. BCCAO; L-Cit40: 30.1 ± 6.9% of control, $p < 0.01$ vs. control, $p > 0.05$ vs. BCCAO; Fig. 2A, B). High doses (100 mg/kg, p.o.) of either L-Cit or GSH attenuated neuronal death relative to vehicle-treated BCCAO animals (GSH100: 59.9 ± 8.2% of control, $p < 0.01$ vs. control, $p < 0.01$ vs. BCCAO; L-Cit100: 62.9 ± 5.4% of control, $p < 0.01$ vs. control, $p < 0.01$ vs. BCCAO; Fig. 2A, B), consistent with our previous studies (Yabuki et al., 2013; Yabuki and Fukunaga, 2013). Interestingly, administration of low dose combinations of L-Cit and GSH (at 40 mg/kg, p.o. each) had a neuroprotective effect against BCCAO ischemia as potent as a high dose alone of Cit or GSH (70.2 ± 11.8% of control, $p < 0.01$ vs. control, $p < 0.01$ vs. BCCAO; Fig. 2A, B).

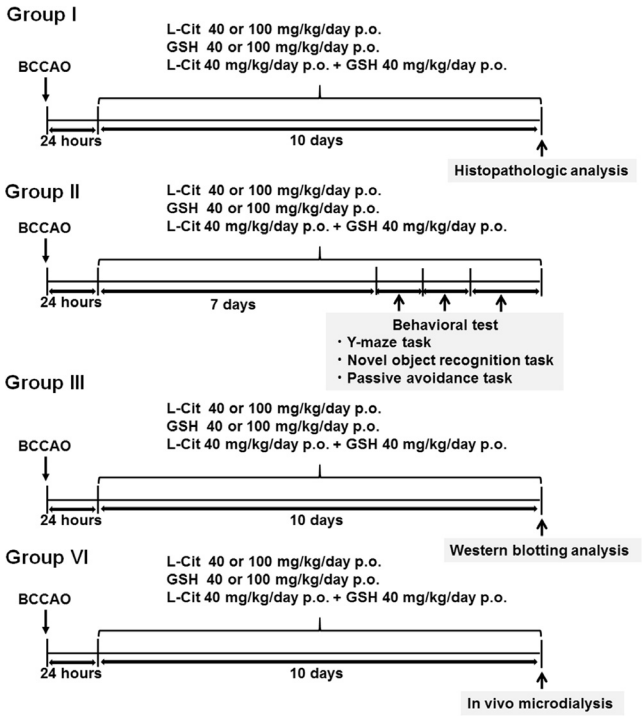

Fig. 1. Experimental protocols for groups analyzed in this study.

2.2. Combined L-Cit and GSH administration decreases memory impairment seen in BCCAO mice

Next, we asked whether combined L-Cit and GSH administration could rescue memory impairment observed in BCCAO mice. To do so, we first evaluated spontaneous alternation behavior and total number of arm entries in a Y-maze task. We observed significant group effects on spontaneous alternation behavior [$F(6, 38) = 7.579$, $p < 0.001$]. The decreased percentage of alternations seen in BCCAO mice was improved by high doses (100 mg/kg, p.o.) of L-Cit or GSH but not by low doses (40 mg/kg, p.o.) of either, without observed changes in the number of total arm entries (Fig. 3A, B). Combined administration of low doses of L-Cit and GSH significantly reversed the decrease in alternation behaviors seen in untreated BCCAO mice (69.8 ± 4.8%, $p < 0.01$ vs. control, $p < 0.01$ vs. BCCAO; Fig. 3B) without changing the total number of arm entries (Fig. 3A). The improvement of alternation behavior in animals with combined administration of low doses was comparable to that seen in animals given high doses of single drugs. To evaluate contextual fear memory, which requires hippocampal activity, we performed a step-through passive avoidance task. Whereas significant differences in latency time to enter the dark chamber were not observed in each group in trial sessions (Fig. 3C), a significant group effect was observed on retention time [$F(6, 38) = 49.935$, $p < 0.001$]. Twenty-four hours after foot shock, latency was