Neuroprotective effects of the antioxidant action of 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride against ischemic neuronal damage in the brain

Seung Cheol Ha1,*, A Reum Han1,#, Dae Won Kim2, Eun-A Kim1, Duk-Soo Kim3, Soo Young Choi2 & Sung-Woo Cho1,*

1Department of Biochemistry and Molecular Biology, University of Ulsan College of Medicine, Seoul 138-736, 2Department of Biomedical Science, Research Institute for Bioscience and Biotechnology, and Medical & Bio-material Research Center, Hallym University, Chuncheon 200-702, 3Department of Anatomy, College of Medicine, Soonchunhyang University, Cheonan 330-090, Korea

Ischemia is characterized by oxidative stress and changes in the antioxidant defense system. Our recent in vitro study showed that 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride protects cortical astrocytes against oxidative stress. In the current study, we examined the effects of 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride on ischemia-induced neuronal damage in a gerbil ischemia/reperfusion models. Extensive neuronal death in the hippocampal CA1 area was observed 4 days after ischemia/reperfusion. Intraperitoneal injection of 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride (0.3 mg/kg body weight) significantly prevented neuronal death in the CA1 region of the hippocampus in response to transient forebrain ischemia. 2-Cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride administration reduced ischemia-induced increases in reactive oxygen species levels and malondialdehyde content. It also attenuated the associated reductions in glutathione level and superoxide dismutase, catalase, and glutathione peroxidase activities. Taken together, our results suggest that 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride protects against ischemia-induced neuronal damage by reducing oxidative stress through its antioxidant actions. [BMB Reports 2013; 46(7): 370-375]

INTRODUCTION

Although the precise cellular events initiated by ischemic dam-

*Corresponding author. Tel: +82-2-3010-4278; Fax: +82-2-3010-4278; E-mail: swcho@amc.seoul.kr
#These authors contributed equally to this work.

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recent study, we report the effects of the thiazole derivative, 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride on neuronal damage following ischemic insult in the brain.

RESULTS AND DISCUSSION

Effects of 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride on neuronal cell viability after ischemic insult

Oxidative stress has been implicated as a critical factor in ischemic brain damage. Therefore, antioxidative agents may offer an effective therapeutic strategy against ischemic damage. Previous studies from our laboratory have demonstrated the ability of 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride against oxidative stress in primary cultured cortical astrocytes (16). 2-Cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride also protected against damage from glutamate, an excitotoxic neurotransmitter (14). To determine whether 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride functions similarly in vivo, we examined its effects on neuronal cell viability after transient forebrain ischemia in a gerbil model. The gerbil model of transient global cerebral ischemia is well studied for the underlying mechanisms of delayed neuronal death pertaining mainly to the pyramidal neurons in the hippocampal CA1 region and increased oxidative stress and induction of neuronal apoptosis are known to occur in this model (23, 24). 2-Cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride (0.3 mg) or saline was injected 30 min before ischemia. Four days after the ischemic insult, animals were killed and brain sections were prepared to identify the histological changes of delayed neuronal cell death induced by ischemia in the CA1 region. As shown in Fig. 1, induction of transient ischemia produced neuronal loss in the CA1 region of the hippocampus in ischemia-induced group. The average number of cresyl violet-stained neurons in these animals (Fig. 1C1, C2) was approximately 10% of that in the sham-operated group (Fig. 1A1, A2). However, in animals in groups treated with 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride (Fig. 1D1, D2), the number of stained neurons was protected up to 68% of that in animals in the sham-operated group. The drug itself in the sham-operated group had no effects on neurons (Fig. 1B1, B2). These results indicate that 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride effectively protects against neuronal cell damage following ischemic insults in the brain.

Effects of 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride on oxidative parameters in the hippocampus

Global ischemia is known to induce a rapid increase in lipid peroxidation in the hippocampus (25-27). Therefore, the protective effects of 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride in the ischemic brain were further evaluated by measuring the levels of various oxidative stress parameters in the hippocampus. A primary factor in the initiation of the pathological response to ischemia injury is the generation of ROS, and the increase in the levels of ROS produced upon ischemia/reperfusion appears to be essential for the development of astrocyte dysfunction and delayed death (28-30). Formation of ROS in the brain after various insults is respiration dependent, and mitochondria in vitro are sensitive to ROS (31). Lipid peroxidation, a hallmark of ROS-induced brain injury, commences when a free radical removes a hydrogen atom from an unsaturated fatty acid. In this study, lipid peroxidation was evaluated by measuring the level of malondialdehyde after the drug was administered and ischemic damage was induced. The level of ROS (Fig. 2A) and MDA (Fig. 2B) in the ischemia group markedly increased compared to those in the sham group. This increase in ROS and MDA levels was similar to what has been reported after ischemic insults (32-34). Notably, the administration of 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride (0.3 mg) efficiently prevented the formation of ROS and MDA (Fig. 2A, 2B). GSH is a central component in the antioxidant defense of cells that acts both by directly detoxifying ROS and by serving as a substrate for various peroxidases (35, 36). GSH content was significantly decreased in animals in the
ischemia group, whereas pretreatment with 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride significantly mitigated ischemia-induced depletion of GSH (Fig. 2C). Collectively, these results suggest that the protective effect of 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride is due, at least in part, to its antioxidative properties.

Cellular defenses against ROS include antioxidant enzymes such as SOD, catalase, and GSH-Px. Accordingly, we investigated the intracellular activities of these enzymes. We found a significant decrease in the activities of SOD (Fig. 3A), catalase (Fig. 3B), and GSH-Px (Fig. 3C) in the ischemia group. However, animals in the groups treated with 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride showed a significant recovery in SOD, catalase and GSH-Px activity compared with those in the ischemia group (Fig. 3A-C). These results suggest that 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride protected the brain against ischemia injury through regulation of the oxidation-reduction system, specifically by increasing antioxidant capacity. The antioxidant effects of 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride in vivo is consistent with the antioxidant and free-radical-scavenging properties of this compound in vitro (16).

**Fig. 2.** Effects of 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride on oxidative parameters in the hippocampus. 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride (0.3 mg) was administered 30 min before ischemia. Hippocampi were dissected and ROS (A), MDA (B), and GSH (C) levels in hippocampal extracts were determined. Each bar represents the mean ± SEM, *P < 0.01, versus ischemia-induced group.

**Fig. 3.** Effects of 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride on antioxidative enzymes in the hippocampus. 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride (0.3 mg) was administered and hippocampi were dissected as described in Fig. 2. SOD (A), catalase (B), and GSH-Px (C) levels in hippocampal extracts were determined. Each bar represents the mean ± SEM, *P < 0.01, versus ischemia-induced group.
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MATERIALS AND METHODS

Materials
Cresyl violet acetate, Hank’s balanced salt solution, 3-(4,5-di-
methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT),
xanthine, glutathione peroxidase (GSH-px), 2’,7’-dichloro-
fluorescein diacetate (DCF-DA), malondialdehyde (MDA), so-
dium dodecyl sulfate, cytochrome c, sufficient xanthine oxido-
dase, NADPH, glutathione, and dimethyl sulfoxide (DMSO)
were purchased from Sigma Chemical Co. (St. Louis, MO, USA).
2-Cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride was synthesized as described previously (14). All other
chemicals and reagents were the highest analytical grade available.

Induction of forebrain ischemia
To induce forebrain ischemia, we performed a surgery similar to
that described in a previous study (26). Male Mongolian ger-
bils (M. unguiculatus) weighing 80-88 g were placed under
general anesthesia using 2.5% isoflurane in a mixture of 33% oxygen and 67% nitrous oxide. A midline ventral incision was
made in the neck. Both common carotid arteries were isolated,
freed of nerve fibers, and occluded with nontraumatic aneur-
ysm clips. Complete interruption of blood flow was confirmed
by observing the central artery in the eyeball using an ophthal-
omoscope. After a 5-minute occlusion, the aneurysm clips were removed from both common carotid arteries. The re-
stitution of blood flow (reperfusion) was observed directly un-
der the ophthalmoscope. Sham-operated control animals were
subjected to the same surgical procedures except that the com-
mon carotid arteries were not occluded. Body temperature was
monitored by measuring rectal temperature and was main-
tained at 37°C during surgery and throughout the immediate
postoperative period until the animals had recovered fully from
anesthesia. Thirty minutes before occlusion of both common
carotid arteries, gerbils were injected intraperitoneally (i.p.)
with saline or with 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride (0.3 mg) to test for protective effects against is-
chemic damage. Four days after ischemia-reperfusion, all ani-
mal (n = 6/group) were anesthetized with ketamine and per-
fluenced transcardially with phosphate-buffered saline (PBS) fol-
lowed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains
were removed and post-fixed in the same fixative solution for 4 h and brain tissues were cryoprotected by infusing with 30% sucrose overnight. Thereafter, the tissues were frozen and sec-
tioned with a cryostat at a thickness of 30 μm; consecutive sec-
tions were collected in six-well plates containing PBS. Neu-
ronal damage was assessed by mounting sections on gela-
tin-coated slides and then staining with cresyl-violet. Procedures involving animals and their care conformed to institutional
guidelines, which are in compliance with current international
laws and policies (NIH Guide for the Care and Use of Labora-
tory Animals, NIH Publication No. 85-23, 1985, revised
1996) and were approved by the Hallym Medical Center
Institutional Animal Care and Use Committee. All efforts were
made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

Oxidative stress parameters
Oxidative parameters in hippocampal extracts were determined
described below. Intracellular ROS levels were determined as
described previously (14) using DCF-DA, which is converted by
ROS into fluorescent DCF. The cells were washed twice with
PBS and incubated with DCF-DA (10 mM) for 30 minute.
Fluorescence intensity was measured using a SpectraMax
GEMINI XS fluorescence spectrophotometer (Molecular Devices,
Sunnyvale, CA, USA) at an excitation wavelength of 485 nm and
an emission wavelength of 538 nm. Challenge with H2DCF-DA
and measurement of fluorescence intensity was performed in the
dark. Neuronal malondialdehyde (MDA) levels were determined
described previously (26) in a reaction mixture containing 100 μl of 8.1% sodium dodecyl sulfate, 750 μl of 20% acetic acid (pH
3.5), 750 ml of 0.8% thiobarbituric acid, and 300 μl of distilled
water. Samples were then boiled for 1 hour at 95°C and centri-
fuged at 4,000 g for 10 minute. The absorbance of the super-
natant was measured by spectrophotometry at 532 nm. Intracellular GSH content was measured in protein-free extracts as
described by Lombardi et al. (37) with minor modifications. To
assay GSH, 100 μl of 5’,5’-dithio-bis(2-nitrobenzoic acid) (6 mM),
25 μl of protein-free extracts, 875 μl of NADPH (0.3 mM), and 10
μl of GSH reductase (10 U/ml) were mixed, and the absorbance
changes were monitored at 412 nm with a spectrophotometer.
Intracellular GSH content was quantified by reference to a stand-
ard curve generated using known amounts of GSH.

Superoxide dismutase (SOD) activity was measured by moni-
torng inhibition of the ferricytochrome c reduction reaction by
xanthine/xanthine oxidase, as described previously (26). The re-
action mixture contained 10 μM cytochrome c, 50 μM xan-
thine, and sufficient xanthine oxidase to produce a reduction

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rate of cytochrome c of 0.025 absorbance units per min at 550 nm. The assay was performed in 3 ml of 50 mM potassium phosphate buffer (pH 7.8), containing 0.1 mM EDTA in a cuvette at 25°C. Under these defined conditions, the amount of SOD required to inhibit the reduction rate of cytochrome c by 50% (to a rate of 0.0125 absorbance units per min) was defined as 1 unit of activity. Glutathione peroxidase (GSH-px) activity in the supernatants from different groups was measured by the enzymatic reaction which was initiated by addition of H₂O₂ to a reaction mixture containing reduced glutathione, NADPH, and glutathione reductase (38). The change in the absorbance at 412 nm was monitored with a spectrophotometer. Catalase activity assay was performed according to the method described elsewhere (39), by determining H₂O₂ decomposition rate at 240 nm.

Statistical analysis
Data are expressed as means ± SEM. The data were analyzed for statistical significance using one-way ANOVAs. Bonferroni’s test was used for post hoc comparisons. All statistical analyses were performed using the SPSS 12.0 package. P values < 0.05 were considered statistically significant.

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