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

Seung Cheol Ha[1,#], A Reum Han[1,#], Dae Won Kim[2], Eun-A Kim[1], Duk-Soo Kim[3], Soo Young Choi[2] & Sung-Woo Cho[1,]*

[1]Department of Biochemistry and Molecular Biology, University of Ulsan College of Medicine, Seoul 138-736, [2]Department of Biomedical Science, Research Institute for Bioscience and Biotechnology, and Medical & Bio-material Research Center, Hallym University, Chuncheon 200-702, [3]Department 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 damage are incompletely characterized, a number of complex biochemical events appear to be involved. Ischemia can be characterized by oxidative stress and changes in the antioxidant defense system. It has been reported that oxidative stress contributes to delayed neuronal death after global cerebral ischemia (1). One of the main factors in the initiation of the pathological response to oxidative stress is the generation of reactive oxygen species (ROS). ROS participate in a wide variety of cellular functions and contribute significantly to the pathological processes underlying various human diseases (2). Reperfusion after interruption of blood flow initially induces an enormous increase in ROS in the hippocampal CA1 region that cannot be controlled by endogenous antioxidant systems (3-5). Sudden bursts of ROS during ischemia/reperfusion cause free radical production and initiate damage to lipids, proteins, and DNA (6-8). Consequences of this cellular damage include mitochondrial dysfunction, deregulation of ionic balance, and loss of membrane integrity (9, 10).

The brain is especially vulnerable to oxidative stress because of its high consumption of oxygen and low levels of endogenous antioxidants (11, 12). Therefore, antioxidative agents may offer an effective therapeutic strategy against ischemic insult. However, neuroprotective strategies that are fully effective against oxidative stress have not yet been developed, despite the abundance of data on the subject. Accordingly, the systematic search for new neuroprotective compounds remains a matter of intense research (8). We have been interested in the development of effective neuroprotective drugs, and have synthesized and examined various thiazole derivatives in cultured neuronal cells (13-16). Our recent *in vitro* study showed that 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride protects cortical astrocytes against oxidative stress (16). Thiazole derivatives have been shown to be useful for treating various diseases (13, 17-22). For instance, the benzothiazole derivative AS601245 has shown beneficial effects in models of global and focal brain ischemia (23). However, its mechanisms of action have not yet been completely elucidated. In addition, testing structurally distinct classes will be necessary to strengthen our therapeutic concept. In the cur-

*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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rent 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.

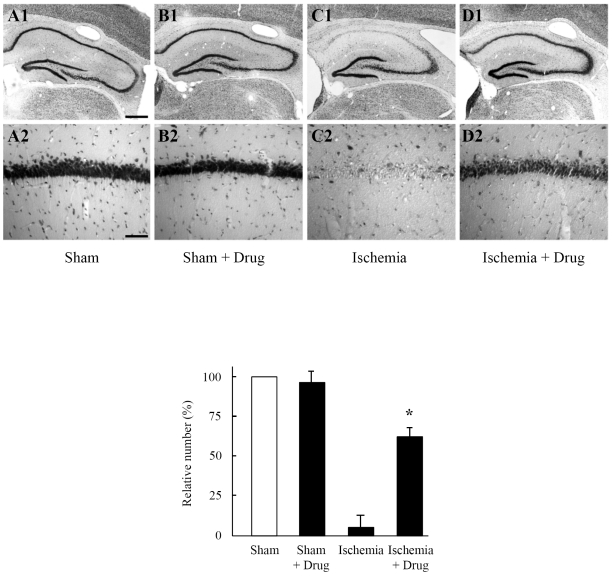

Fig. 1. Effects of 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride on neuronal cell viability after ischemic insult. Representative photomicrographs of cresyl violet-stained hippocampi of gerbil brains 4 days after ischemic insult show effects of 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride. (A1 and A2), sham-operated group; (B1 and B2), sham-operated group treated with 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride (0.3 mg); (C1 and C2), ischemia-induced group; (D1 and D2), ischemia-induced group treated with 2-cyclopropylimino-3-methyl-1, 3-thiazoline hydrochloride (0.3 mg). Scale bars: 280 μm (A1, B1, C1 and D1) and 50 μm (A2, B2, C2 and D2). Relative histological analysis for the cell number of cresyl violet-positive neurons is expressed as percentage of the sham group. Values are means ± SEM, *P < 0.01, versus ischemia-induced group.

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).

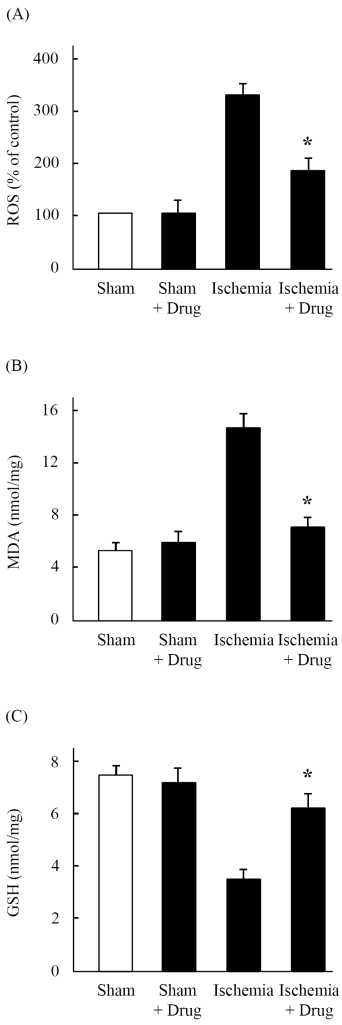

Fig. 2. Effects of 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride on oxidative parameters in the hippocampus. 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride (0.3 mg) was administered 30 min before ischemia. Hippocampi were dissected and ROS (A), MDA (B), and GSH (C) levels in hippocampal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

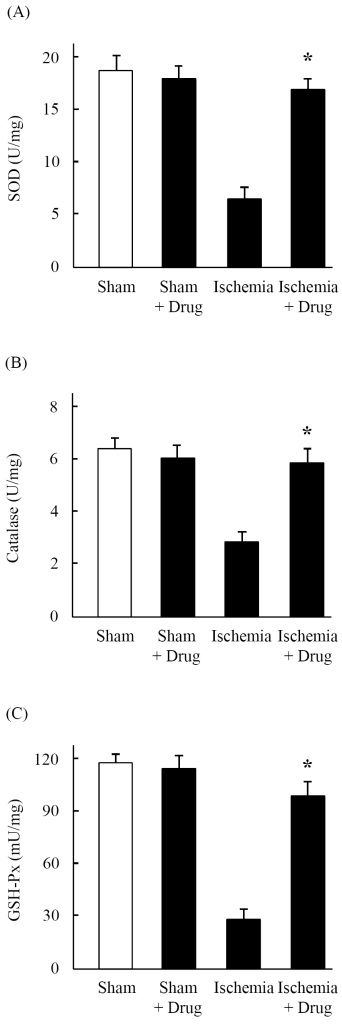

Fig. 3. Effects of 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride on antioxidative enzymes in the hippocampus. 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride (0.3 mg) was administered and hippocampi were dissected as described in Fig. 2. SOD (A), catalase (B), and GSH-Px (C) levels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

In conclusion, the results of the present study suggest that 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride protects against ischemia through its antioxidant effects, supporting the results of previous studies that have suggested that oxidative stress is an important underlying factor in the delayed neuronal death induced by ischemic insult. With the increase in oxidative stress in aging as well as in many age-related neurodegenerative diseases, our study suggests the possibility that 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride may be useful in preventing the development of diseases associated with oxidative stress. Since most of our studies were performed at one time point after ischemia/reperfusion, it is not possible to conclude whether the protective effects of 2-cyclopropylimino-3-methyl-1,3-thiazoline hydrochloride are not simply delaying the cell death process. Consequently, more studies are needed to examine the effects of 2-cyclopropylimino-3- methyl-1,3-thiazoline hydrochloride at prolonged times after ischemia.

MATERIALS AND METHODS

Materials

Cresyl violet acetate, Hank's balanced salt solution, 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), xanthine, glutathione peroxidase (GSH-px), 2',7'-dichlorofluorescein diacetate (DCF-DA), malondialdehyde (MDA), sodium dodecyl sulfate, cytochrome c, sufficient xanthine oxidase, 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 gerbils (*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 aneurysm clips. Complete interruption of blood flow was confirmed by observing the central artery in the eyeball using an ophthalmoscope. After a 5-minute occlusion, the aneurysm clips were removed from both common carotid arteries. The restoration of blood flow (reperfusion) was observed directly under the ophthalmoscope. Sham-operated control animals were subjected to the same surgical procedures except that the common carotid arteries were not occluded. Body temperature was monitored by measuring rectal temperature and was maintained 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 ischemic damage. Four days after ischemia-reperfusion, all animals (n = 6/group) were anesthetized with ketamine and perfused transcardially with phosphate-buffered saline (PBS) followed 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 sectioned with a cryostat at a thickness of 30 μm; consecutive sections were collected in six-well plates containing PBS. Neuronal damage was assessed by mounting sections on gelatin-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 Laboratory 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 as 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 as 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 μl of 0.8% thiobarbituric acid, and 300 μl of distilled water. Samples were then boiled for 1 hour at 95°C and centrifuged at 4,000 g for 10 minute. The absorbance of the supernatant 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 standard curve generated using known amounts of GSH.

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

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 H_2O_2 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 H_2O_2 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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