Free Radical Research, August 2006; 40(8): 827–835

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S-Allyl-L-cysteine attenuates cerebral ischemic injury by scavenging peroxynitrite and inhibiting the activity of extracellular signal-regulated kinase

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Accepted by Professor J. Yodoi

(Received 20 December 2005; in revised form 9 March 2006)

Abstract

S-Allyl-L-cysteine (SAC) has been shown to reduce ischemic injury due to its antioxidant activity. However, the antioxidant property of SAC has been controversial. The present study investigated the neuroprotective mechanism of SAC in cerebral ischemic insults. SAC decreased the size of infarction after transient or global ischemic insults. While it did not alter the *N*-methyl-D-aspartate excitotoxicity, SAC significantly scavenged the endogenously or exogenously produced $ONOO^-$ and reduced $ONOO^-$ cytotoxicity. In contrast, SAC has much lower scavenging activity against H_2O_2, O_2^- or NO. Further, SAC inhibited the activity of extracellular signal-regulated kinase (ERK) increased in cultured neurons exposed to oxygen-glucose deprivation or in rat brain tissue after transient middle cerebral artery occlusion. The neuroprotective effect of SAC was mimicked by the ERK inhibitor U0125. The present results indicate that SAC exert its neuroprotective effect by scavenging $ONOO^-$ and inhibiting the ERK signaling pathway activated during initial hypoxic/ischemic insults.

Keywords: S-*Allyl-L-cysteine, antioxidant, ERK, ischemia, peroxynitrite ($ONOO^-$)*

Introduction

Oxidative stress has been associated with neuronal loss in brain ischemia. Thus, a variety of antioxidants has been used in attempts to protect neuronal cells from ischemic injury [1–4]. In both *in vivo* and *in vitro* studies, *S*-allyl-L-cysteine (SAC), an active organosulfur compound derived from garlic, has been shown to inhibit oxidative damage by: (i) scavenging superoxide anion (O_2^-) or hydroxyl radicals (HO^-) [5] and hydrogen peroxide (H_2O_2) [6], (ii) preventing oxidized LDL-induced endothelial cell injury [6] and low-density lipoprotein oxidation [7,8] and/or (iii) regulating nitric oxide (NO) production associated with anti-inflammatory responses [5]. SAC also reduced the formation of edema in the ischemic rat brain by inhibiting lipid peroxidation [9,10]. Many *in vitro* studies, however, indicated that SAC exerted its antioxidant effect only at millimolar concentrations [6–8,11], implying that the antioxidant property of SAC may not play a critical role in *in vivo* neuroprotection in cerebral ischemic injury. In our preliminary experiments, we also found that SAC significantly blocked H_2O_2-induced injury/death in cultured astrocytes only at millimolar concentrations.

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2006 Informa UK Ltd
DOI: 10.1080/10715760600719540

Therefore, in the present study we aimed to investigate the exact mechanism for the neuroprotective effect of SAC in cerebral ischemic insults.

Reactive oxygen species (ROS) appear to regulate various signaling pathways including the mitogen-activated protein kinases (MAPKs). Thus, oxidative stress such as H_2O_2 [12], NO [13], O_2^- [14] and peroxynitrite ($ONOO^-$) [15,16] lead to activation of MAPKs. Such activation of extracellular signal-regulated kinase (ERK), one family of MAPKs, was suggested to contribute to glutamate neurotoxicity [17], and inhibition of ERK activity protected neuronal injury/death in *in vivo* [18,19] and *in vitro* ischemic insults [19]. In the present study, therefore, we also studied whether the neuroprotective effect of SAC in ischemia was associated with the down-regulation of ERK activity.

Materials and methods

Materials

SAC was provided by TCI Chemical Co. (Tokyo, Japan). Dulbecco's modified Eagle's media (DMEM: Ham's F12 (1:1 mixed)), Minimum essential media (MEM), DMEM/F12, glucose-free DMEM, trypsin/EDTA, penicillin/streptomycin, fetal bovine serum (FBS), and interferon-γ (IFN-γ) were purchased from Gibco BRL (Grand Island, NY, USA). Dihydrorhodamine 123 was obtained from Molecular Probes (Eugene, OR, USA). Triphenyltetrazolium chloride (TTC) was from Sigma Chemical Co. (St Louis, MO, USA). Antibodies to ERK and active (phosphorylated) ERK were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-nitrotyrosine and HRP-conjugated secondary antibodies were from Upstate Biotechnology (Lake Placid, NY, USA). All other chemicals were purchased from Sigma Chemical Co.

Animals

Male Mongolian gerbils and Sprague-Dawley rats were purchased from Charles River Laboratories (Seoul, Korea) and kept on a 12 h light/dark cycle with *ad libitum* access to food and water. Animals were acclimated to their environment for at least 5 days prior to experiments. All animal experimental procedures were approved by the institutional animal care and use committee.

Global cerebral ischemia model

Male Mongolian gerbils weighing 80 ~ 88 g were placed under general anesthesia with a mixture of 2.5% isoflurane in 33% oxygen and 67% nitrous oxide. A midline ventral incision was made in the neck. Both common carotid arteries were isolated, freed of nerve fibres and occluded using nontraumatic aneurysm clips. Complete interruption of blood flow was confirmed by observing the central artery in eyeballs using an ophthalmoscope. After 5 min of occlusion, the aneurysm clips were removed from both common carotid arteries. Restoration of blood flow (reperfusion) was observed directly under the microscope.

Sham-operated controls ($n = 7$) were subjected to the same surgical procedures except that common carotid arteries were not occluded. Body temperature was monitored and maintained at 37 ± 0.3°C during surgery and during the immediate postoperative period until the animals recovered fully from anesthesia. SAC was injected intraperitoneally (300 mg/kg body weight) once (immediately after occlusion) or twice (immediately after occlusion and reperfusion) in rats, or 3 times (30 min before occlusion, immediately and 2 h after reperfusion) in gerbils.

Seven days after the operation, the gerbils were anesthetized with chloral hydrate and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4). The brains were removed and placed in 30% sucrose at 4°C for 2 days. Transverse sections 30 μm thick were cut through the hippocampus with a cryostat and stained with cresyl violet. The hippocampal CA1 region was analyzed for quantification of ischemic neuronal injury. For quantitative analysis, the number of surviving pyramidal cells that had well preserved perikarya and nuclei was counted using an image analyzer (OPTIMAS 5.1, BioScan Inc., Edmonds, WA, USA). The density of surviving neurons was expressed as number of neuronal cells per 0.5 mm length in the middle of the CA1 region. Cell density in each animal was calculated as mean of the scores obtained from the right and left hemispheres.

Focal cerebral ischemia model

Rats weighing 270 ~ 290 g were initially anesthetized with 3.0% isoflurane in a 70% N_2O and 30% O_2 (v/v) mixture via face mask. Anesthesia was maintained with 2.0% isoflurane. A rectal temperature probe was introduced, and a heating pad maintained the body temperature at 37°C during the entire surgical period. Focal cerebral ischemia was achieved by right-sided endovascular middle cerebral artery occlusion (MCAO) [20]. Briefly, the right carotid arteries were exposed through a midline cervical incision. The right external carotid artery (ECA) was dissected free and isolated distally by coagulating its branches and placing a distal ligation prior to transection. A piece of 3-0-monofilament nylon suture (Ethicon, Johnson-Johnson, Brussels, Belgium), with its tip rounded by gentle heating and coated by 0.1% (w/v) poly-D-lysine, was inserted into the lumen of the right ECA stump and gently advanced 17.5 mm into the internal carotid artery (ICA) from the bifurcation to occlude the ostium

of MCAO. After 2 h of ischemia, the suture was pulled back and the animal was allowed to recover.

Measurement of infarct volume

Rats were anesthetized with chloral hydrate and decapitated 1 day after MCAO. Rat brains were cut into coronal slices of 2 mm in thickness using a rat brain matrix (Ted Pella, Redding, CA, USA). The brain slices were then incubated in 2% TTC at 37°C for 30 min to reveal the ischemic infarction. After TTC reaction, the brain slices were fixed with 4% paraformaldehyde (pH 7.4) in 0.1 M PB for 1 day and subsequently cryoprotected in PB containing 30% sucrose at 4°C for 2 days. The cross-sectional area of infarction between the bregma levels of +4 mm (anterior) and −6 mm (posterior) were determined with a computer-assisted image analysis (OPTIMAS 5.1, BioScan Ins, USA). On each slice, brain infarct size was measured manually by outlining the margins of infarct areas, and the infarct volume was calculated according to the slice thickness of 2 mm per section. Each side of the brain slices was measured separately, and mean values were calculated. The total volume of infarction was determined by integrating six chosen sections and expressed as percentage of the total brain volume. Because post ischemic brain edema will increase brain volume in the infarcted area, the corrected infarct volumes were calculated to compensate for brain edema. Thereafter the tissues were frozen and cut into 10 or 30 μm coronal sections on a cryostat (Leica 3050, Leica, Germany) and stored at 20°C.

Mixed glial cultures

Glial cells were cultured from the prefrontal cortices of 2–5 days old Sprague-Dawley rat pups as previously described [21]. For immunostimulation, astrocytes were treated for 24–48 h with IFN-γ (100 U/ml) and LPS (1 μg/ml). After immunostimulation, glucose deprivation was achieved by repeatedly rinsing and incubating in glucose-free DMEM.

Measurement of cell death and NO production

Cell death was quantified by measuring the amount of LDH released into the bathing medium. LDH activity was measured using a diagnostic kit (Sigma Chemical Co.). Cell viability was expressed as LDH activity. NO production from the immunostimulated cells was determined by measuring nitrite, a stable oxidation product of NO, as described previously [22].

Measurement of superoxide anion and peroxynitrite generation in vitro

As we described before [23], O_2^- generated from xanthine and xanthine oxidase was monitored with a Berthold LB9505 luminometer (Bad Wildbad, Germany). Extracellular $ONOO^-$ formation from the decomposition of SIN-1 was determined by $ONOO^-$-induced oxidation of dihydrorhodamine-123 according to our previous report [23].

Measurement of NO

Release of NO was measured with a commercially available Clark-type electrode (ISO-NO, Mark II, World Precision Instruments, Sarasota, FL, USA). NO was released from 200 μM *S*-nitroso-*N*-acetyl-penicillamine (SNAP) by incubation in phosphate-buffered saline (PBS) containing 4 mM cysteine as a reducing agent and various concentrations of SAC. All reagents were freshly prepared before use. The formation of NO was quantified from the initial rate of release using the data acquisition system Duo18 (World Precision Instruments).

Immunocytochemical staining

Astrocytes were fixed for 10 min at room temperature in PBS containing 4% paraformaldehyde and were then stored in cold 70% ethanol until use. To inactivate endogenous peroxidase, cells were incubated in PBS containing 3% H_2O_2 for 5 min at room temperature. Nonspecific staining was blocked by incubating cells in PBS containing 8% bovine serum albumin (BSA) for 30 min at room temperature. For detection of nitrotyrosine immunoreactivity, cells were incubated for 2 h at room temperature with PBS containing 1% BSA, 10 μg/ml of a rabbit anti-nitrotyrosine antibody. After extensive washing with PBS, cells were probed for 1 h at room temperature with a 1:200 dilution of an anti-rabbit horseradish peroxidase-conjugated secondary antibody. Cells were then exposed to a chromogenic mixture of diaminobenzidine.

Measurements of mitochondrial transmembrane potential

The mitochondrial transmembrane potential (MTP) was measured according to our previous report [24]. In brief, astrocytes cultured on 24-well culture plates were loaded for 20 min at 37°C with JC-1 (1.0 μg/ml) in culture medium. Depolarization of MTP was measured 3 h after JC-1 load by using a fluorescence microscope. Before the measurement, cells were protected from light and remained in a 5% CO_2/95% air chamber at 37°C.

Cerebrocortical cultures and oxygen-glucose deprivation

Cortical cultures of mixed neurons glia were prepared from embryonic (fetal day 15–17) Sprague-Dawley rats as described previously [25]. Briefly, following dissociation in 0.027% trypsin, cerebral cortical cells

were plated at a density of 4.5×10^5 per 35 mm dish containing poly-D-lysine-coated glass cover slips in Dulbecco's modified Eagle's medium with Ham's F12, and heat-inactivated iron-supplemented calf serum (HyClone) at a ratio of 8:1:1. After 15 days in culture (when the astrocyte layer had become confluent), the cultures were treated with cytosine arabinoside for 72 h. The culture medium was replenished three times weekly. Cultures were incubated at 36°C in a 5% CO_2/95% air humidified atmosphere. The cultures were used for experiments approximately 3 weeks after plating. Neurons could be reliably identified by morphological criteria under phasecontrast optics. Oxygen-glucose deprivation (OGD) was performed by placing the glucose-deprived cells inside an anaerobic chamber (Thermo-Forma, Marietta, OH, USA) maintaining an 85% N_2/5% CO_2/10% H_2 condition. One hour after starting the OGD, cells were returned to the normoglycemic (5.5 mM glucose) and normoxic condition for 2 h before determining the activity of LDH released into the medium.

Western blot analysis

Cells were lysed in lysis buffer and centrifuged at 14,000*g* for 10 min at 4°C. Protein concentration from the supernatant was determined and 10 μg of proteins were loaded for 10% SDS-polyacrylamide gel electrophoresis (PAGE). Electrophoresis was performed and proteins were transferred from the gel to the nitrocellulose membrane (Amersham Phamarcia Biotech Inc., Piscataway, NJ, USA) using an electroblotting apparatus. Membranes were blocked for 1 h in Tris-buffered saline (TBS) containing 0.05% Tween-20 and 5% skim milk, incubated with primary antibodies that recognize p-ERK (1:2000), ERK (1:1000), and β-actin (1:10,000) overnight, followed by horseradish peroxidase-conjugated secondary antibodies for 1 h. Protein bands were visualized by an ECL Plus Western blotting detection system (Amersham Pharmacia Biotech Inc.). For quantification, the density of phosphorylated ERK was normalized by corresponding non-phosphorylated forms of ERK. For western blot using rat brain tissue, rats were sacrificed with an overdose of chloral hydrate, and their brains were removed and sliced into six (2 mm thick) coronal sections using a brain matrix and then similarly treated as described before.

Statistical analysis

Data are expressed as the means ± SEM and analysed for statistical significance using repeated measures of ANOVA or two-way ANOVA by running the SAS Window v.9.1. program (SAS Institute, Cary, NC, USA). If needed, *post-hoc* Scheffe's test was performed for multiple comparisons. A p value <0.05 was considered significant.

Results

Alleviated ischemic injury by SAC

To test whether it reduced the ischemic injury, SAC (300 mg/kg) was intraperitoneally administrated. When it was administrated once right before MCAO or twice right before and after MCAO, SAC also reduced the size of infarct area after 2 h MCAO/22 h reperfusion (Figure 1A,B). When it was administrated twice 30 min before and 2 h after ischemia, SAC significantly blocked the death of neuronal cells in hippocampal CA1 region in gerbils (Figure 2A,B). NMDA excitotoxicity has been strongly associated with ischemia-evoked cerebral injury. SAC, however, did not alter the NMDA excitotoxicity (Figure 3).

Effect of SAC on scavenging ROS

In cerebral ischemic insults, a large amount of reactive molecules such as $ONOO^-$, H_2O_2, NO and O_2^- are generated. Thus, we characterized features of SAC as a free radical scavenger. SAC efficaciously scavenged the $ONOO^-$ generated from SIN-1, an $ONOO^-$ generator (Figure 4A): thus, the oxidation by SIN-1 of dihydrorhodamine-123 to rhodamine-123 was significantly inhibited by a low micromolar (10 μM) concentration of SAC. Previously, we have reported that the level of peroxynitrite markedly increased in immunostimulated or SIN-1-treated glial cells under glucose-deprived conditions [24,26,27]. SAC (10 μM) significantly reduced the nitrotyrosine immunoreactivity, a marker of $ONOO^-$, elevated in immunostimulated/glucose-deprived astrocytese (Figure 4B). Consistently, SAC (10 μM) decreased the oxidation of DCF-H (a marker of peroxynitrite level) markedly increased in immunostimulated or

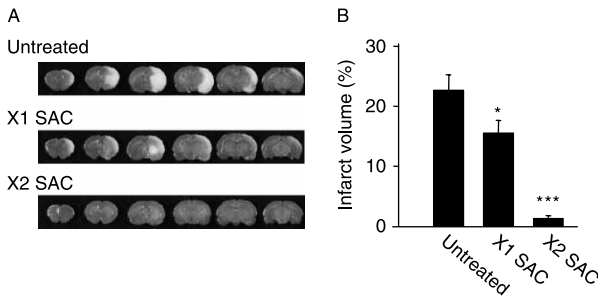

Figure 1. Neuroprotection by SAC in a focal ischemia rat model. (A) Representative photographs of the TTC-stained brain slices. SAC (300 mg/kg) was intraperitoneally administrated once right before occlusion (X1 SAC) or twice right before occlusion and after reperfusion (X2 SAC). (B) Quantitative analysis of infarct volume in untreated or SAC-treated groups. Data are expressed as means ± SEM of 19 ~ 20 independent experiments. $*p < 0.05$, $***p < 0.001$; significantly different from untreated group.

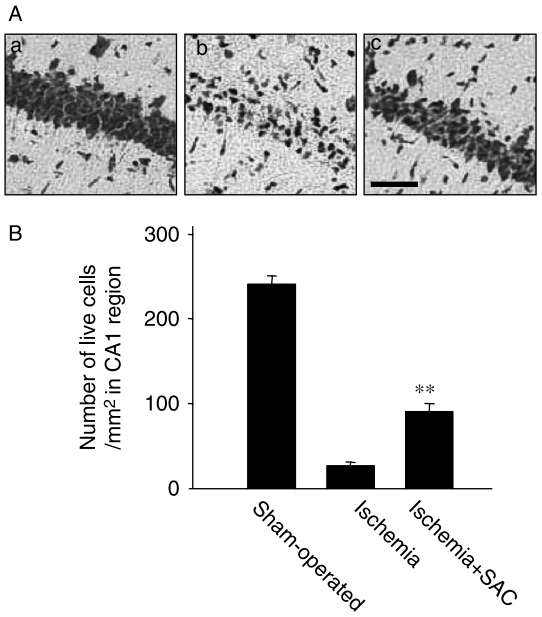

Figure 2. Neuroprotection by SAC in a global ischemia gerbil model. (A) Representative photomicrographs of the hippocampal CA1 region: (a) sham control, (b) ischemic control, (c) SAC-treated group. SAC (300 mg/kg) was intraperitoneally administrated twice 30 min before and 2 h after ischemia, respectively. (B) The number of surviving cells was determined in a square millimetre in the hippocampal CA1 region. Data are expressed as the means ± SEM of 17 ~ 20 independent experiments. $^{**}p < 0.01$; significantly different from ischemic control.

SIN-1-treated glial cells under glucose-deprived conditions (Figure 5A,B). Consequently, SAC (10 μM) prevented the depolarization of MTP (Figure 5C) and the augmented death (data not shown) in immunostimulated/glucose-deprived or SIN-1 treated/glucose-deprived astrocytes (data not shown). Previously, SAC was reported to scavenge hydrogen peroxide (H_2O_2) [6]. However, the efficacy of SAC for scavenging H_2O_2 appeared to be much less

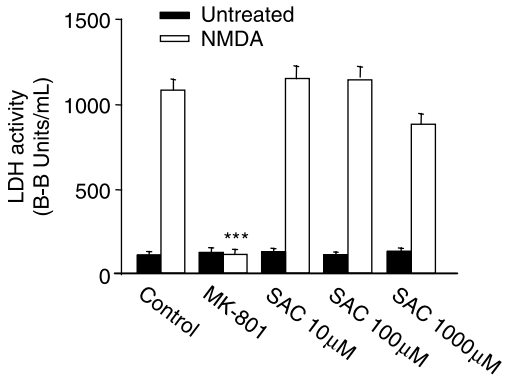

Figure 3. SAC does not inhibit the toxicity of NMDA. Cerebrocortical neurons were treated with 100 μM NMDA for 10 min and the release of LDH was determined 5 h later. Cells were treated with MK-801 (10 μM) or SAC (10, 100 or 1000 μM) 30 min before and during the NMDA treatment. Data are expressed as the means ± SEM. $n = 4$. $^{***}p < 0.001$; significantly different from the LDH release in the control group treated with NMDA.

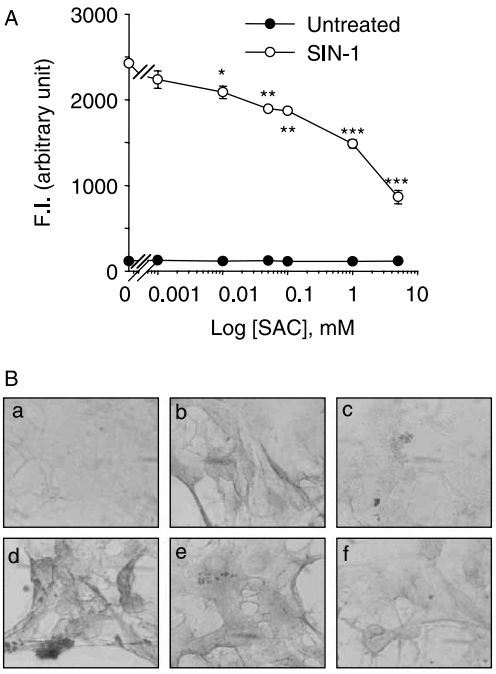

Figure 4. SAC effectively scavenges $ONOO^-$. (A) A concentration-response relationship of $ONOO^-$ scavenging by SAC. DHR-123 was incubated for 10 min with SIN-1 (200 μM) in the absence or presence of various concentrations of SAC. Data are expressed as mean of fluorescence intensities (FI) of 3 ~ 6 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; (repeated measures of ANOVA) compared with the value obtained in the absence of SAC. (B) SAC reduced the level of nitrotyrosine immunoreactivity, a marker of $ONOO^-$, in glucose-deprived immunostimulated glial cells. Cells were treated with IFN-γ (100 U/ml) and LPS (1 μg/ml) for 2 days and then deprived of glucose for 8 h in the absence and presence of SAC. (a) control, (b) glucose deprivation (GD), (c) immunostimulation, (d) immunostimulation + GD, (e) immunostiumulation + GD + SAC (10 μM), (f) immunostiumulation + GD + SAC (100 μM). Photomicrographs were taken 8 h after starting glucose deprivation and are representative of 4–5 separate experiments.

than that for $ONOO^-$. Thus, SAC (10 μM) did not significantly reduce the cytotoxicity of H_2O_2 (100 μM) (data not shown). The cytoprotective effect of SAC in H_2O_2 (100 μM)-treated astrocytes was observed only at 100 μM or higher concentrations. At even higher concentrations (>1 mM), furthermore, SAC did not significantly scavenge the NO produced from SNAP and the O_2^- generated from xanthine and xanthine oxidase (data not shown). The results indicate that SAC most effectively scavenged $ONOO^-$ among reactive molecules.

Inhibition of ERK activity by SAC

We further investigated the effect of SAC on the activity of MAPKs at 1, 2, or 3 h after OGD/re-oxygenation. The activity of ERK, one family of MAPKs, was progressively increased, peaking in intensity at 2 h re-oxygenation after OGD (data not shown). SAC significantly inhibited the activity of

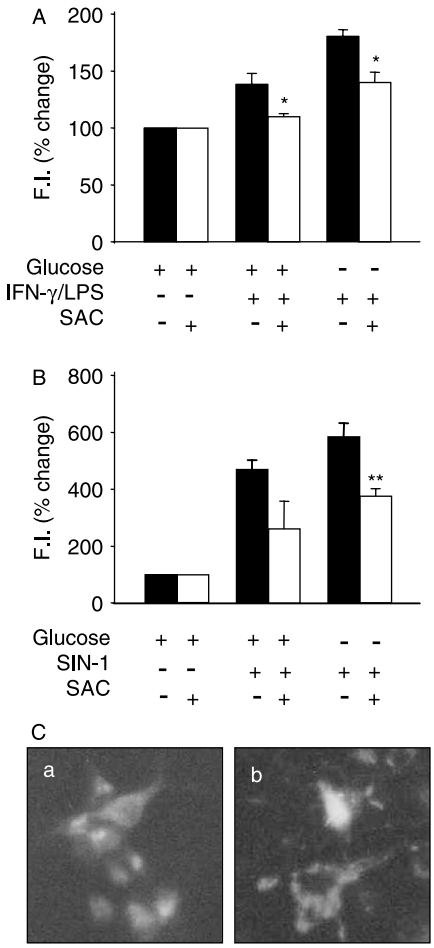

Figure 5. Effects of SAC on oxidation of DCF-H in immunostimulated (A) or SIN-1 (200 μM, (B)-treated/glucose-deprived glial cells. Cells were loaded with DCF-DA (30 μM) and the fluorescence of DCF was measured in the absence or presence of SAC (10 μM) at an excitation wavelength of 485 nm and emission wavelength of 530 nm using a fluorescence microplate reader. FI was corrected for autofluorescence (i.e. fluorescence of cells not loaded with DCF-DA). The fluorescence intensity in control cells that were not treated with SAC was assigned to 100%. Data were means ± SEM, and expressed as a percentage change from five separate experiments. $\star p < 0.05$, $\star\star p < 0.01$; significantly different from the relevant control. (C) Fluorescence microphotographs showing the changes of MTP using the fluorescence probe JC-1. Glial cells were deprived of glucose in the absence or presence of SIN-1 (200 μM) and SAC (10 μM): (a) SIN-1/GD, (b) SIN-1/GD + SAC. Microphotographs were taken 3 h after starting glucose deprivation and representative of four separate experiments.

ERK enhanced after 2 h of re-oxygenation (Figure 6A). However, the activities of other members of MAPKs, c-Jun N-terminal kinase (JNK) and p38 MAPK (p38), were not altered by OGD/re-oxygenation or SAC treatment (data not shown). Next, we investigated the changes of ERK activity in rat brain tissue after an exposure to transient MCAO and reperfusion. For this study, rat brains were taken out at 0.5, 1, 3, 6, 12 or 24 h after reperfusion. The most significant increase of ERK activity was obtained at 3 h after reperfusion (Figure 6B). Intraperitoneal adminis tration of SAC completely suppressed the increase of ERK activity induced after transient MCAO/reperfusion (Figure 6B). In contrast, the activities of JNK and p38 were not changed in rat brain tissue after exposure to a transient MCAO (data not shown).

In the present study, we further found that the ERK inhibitor U0126 partially decreased the LDH release caused by OGD/re-oxygenation (Figure 7A,B). Although SAC also significantly reduced the LDH release caused by OGD/re-oxygenation, there was no synergistic effect between SAC and U0126 (Figure 7B).

Discussion

In the present study, SAC significantly decreased the infarct volume in rat brain exposed to transient MCAO-reperfusion and the neuronal damage in the hippocampal CA1 region after global ischemia in gerbils. These results are in good agreement with previous reports [9,10]. One of the major causes of neuronal death during or after OGD/re-oxygenation includes excitotoxicity through over-stimulation of glutamate receptor subtypes including *N*-methyl-D-aspartate (NMDA, an analogue of excitatory amino acid neurotransmitters) [28,29]. Our present results, however, demonstrated that SAC did not repress the excitotoxicity caused by NMDA.

In cerebral hypoxic/ischemic insults, ROS are produced in a large amount, leading to massive injury of brain tissue [30,31]. In agreement, various kinds of antioxidants have been found to prevent brain ischemic damage [1–4]. In *in vitro* and *in vivo* experimental models, SAC ameliorated ischemia-induced neuronal cell death possibly due to its antioxidant activity [5,9,32]. Many previous studies, however, indicated that SAC scavenged O_2^- and H_2O_2 only at high micromolar to millimolar concentrations [5,6]. In the preset study, we also obtained weak scavenging effect of SAC on O_2^- and H_2O_2. Furthermore, SAC neither directly scavenged the NO produced from SNAP nor suppressed the production of NO in immunostimulated mixed glial cells. Therefore, these findings indicate that the antioxidant properties of SAC against NO, O_2^- and H_2O_2 may not play crucial role in its neuroprotective action in neuronal cells exposed to ischemia/reperfusion and OGD/re-oxygenation.

In general, the cytotoxicity of NO is increased by reaction with O_2^- in a 1:1 cytochiometry to form the strong oxidant $ONOO^-$ [33]. The level of $ONOO^-$ has been shown to markedly increase during ischemic insult [34,35]. Many organosulfur compounds including SAC were shown to scavenge radicals such as $ONOO^-$ [9]. In the present study, at a concentration as low as 10 μM SAC significantly reduced the level of $ONOO^-$ generated exogenously (from SIN-1) or endogenously (by LPS/IFN-γ treatment). Our finding may be supported by the previous report showing that SAC inhibited lipid peroxidation caused by

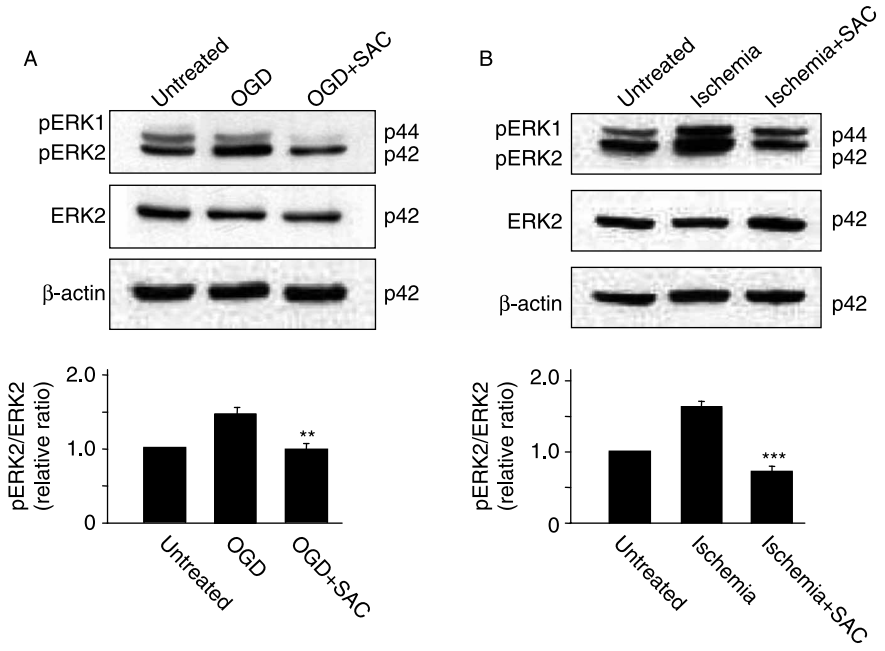

Figure 6. SAC inhibits the activation of ERK. (A) OGD-induced ERK activation. Cortical neurons were treated with OGD for 1 h and further incubated in a normoxic condition for 2 h. Cells were then harvested and lysed. The levels of unphosphorylated and phosphorylated p38 MAPK in total cell lysates were measured using western blotting with a specific antibody. The fold difference of relative phosphorylation ratio is shown in a bar graph. Results are expressed as the means ± SEM from five separate experiments. $^{**}p < 0.01$, significantly different from the group exposed to OGD without SAC (10 μM). (B) Focal ischemia-induced ERK activation. SAC (300 mg/kg) was intraperitoneally administrated twice right before and after MCAO. The rat brain was taken out 3 h after reperfusion and brain total extracts were analyzed by western blotting with a specific antibody. The fold difference of relative phosphorylation ratio is shown in a bar graph. Results are expressed as the means ± SEM from four separate experiments. $^{***}p < 0.001$, significantly different from the ischemia group that was not treated with SAC.

$ONOO^-$ [9]. Thus, the scavenging effect of SAC against $ONOO^-$ may be at least in part associated with the reduction of ischemic injury.

Few studies have been done of the interaction of SAC with intracellular signalling molecules. Although the precise role of MAPKs has not been well established, much evidence has been accumulated that MAPKs become activated in focal cerebral ischemia. Of MAPKs, ERK has been shown to be activated in neuronal cultures after oxidative stress induced by glutamate or hypoxia [36] and in hippocampal organotypic cultures treated with OGD [19,37]. The activation of ERK has also been reported after focal [18,19,37–39] or global cerebral ischemia

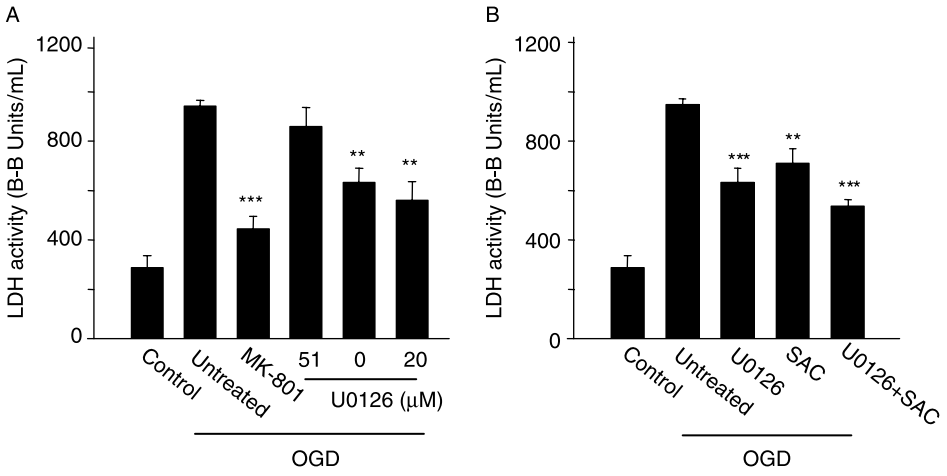

Figure 7. The ERK inhibitor U0126 reduced the neuronal cell death caused by OGD/re-oxygenation. (A) and (B) Neuronal cells in culture were exposed to OGD/re-oxygenation in the absence or presence of U0126 (10 μM) and/or SAC (10 μM). Results were expressed as means ± SEM of four separate experiments. $^{**}p < 0.01$, $^{***}p < 0.001$; significantly different from OGD-treated group in the absence of both U0126 and SAC.

[19,40]. Furthermore, inhibition of ERK activation by different MEK specific inhibitors U0126 or PD98059 reduced neuronal cell death induced by oxidative glutamate toxicity or hypoxia/ischemia-reoxygenation in cultured cells [36] and animal models [18,19]. In the present study, the activity of ERK was similarly increased in cerebral cortical neurons after OGD and in rat brain following transient focal ischemia. SAC significantly inhibited the activation of ERK in both *in vitro* OGD/re-oxygenation and *in vivo* ischemia/reperfusion models. Therefore, it is likely that SAC ameliorates the ischemia-induced injury in part by inhibiting the activity of ERK.

$ONOO^-$ was previously reported to activate MAPK pathways in neuronal cells [41,42]. Therefore, the inhibition of ERK activity by SAC could be simply due to its antioxidant activity. However, our preliminary study shows that U0126 does not have antioxidant activity, as tested in models for endogenously (i.e. immunostimulation under glucose-deprived condition) or exogenously (i.e. SIN-1 treatment under glucose-deprived condition) generated oxidative stress (data not shown). Therefore, although antioxidant effect of SAC may be associated with inhibition of ERK activity, direct inhibition of ERK activity may also at least in part contribute for the neuroprotective effect of SAC. At present, however, further studies are needed to elucidate the exact mechanism(s) underlying the inhibition of oxidative stress-induced ERK activation by SAC.

Previously, SAC showed high bioavailability, reabsorption, and stability in blood in human and rat, and its acute/subacute toxicity was very minor in mice and rat (LD_{50} value >54.7 mM/kg po; >20 mM/kg ip) [43,44]. Therefore, understanding the mechanism of SAC for its neuroprotective effect would provide a therapeutic strategy for the treatment of cerebral ischemic injury.

Acknowledgements

This study was supported by a research project on Functional Food Development from the Office for Government Policy Coordination, a grant from the Agriculture R&D Promotion Center, under the Ministry of Agriculture and Forestry, a grant (M103KV010005 03K2201 00510) from Brain Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, and in part a grant from the Korea Health 21 R&D Project (02-PJ1-PG6-AG01-0003) of the Ministry of Health & Welfare, the Republic of Korea.

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