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PREVENTION OF *IN VITRO* AND *IN VIVO* ACUTE ISCHEMIC NEURONAL DAMAGE BY (2S)-1-(4-AMINO-2,3,5-TRIMETHYLPHENOXY)-3-{4-[4-(4-FLUOROBENZYL) PHENYL]-1-PIPERAZINYL}-2-PROPANOL DIMETHANESULFONATE (SUN N8075), A NOVEL NEUROPROTECTIVE AGENT WITH ANTIOXIDANT PROPERTIES

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Abstract—(2S)-1-(4-Amino-2,3,5-trimethylphenoxy)-3-{4-[4-(4-fluorobenzyl) phenyl]-1-piperazinyl}-2-propanol dimethanesulfonate (SUN N8075) is a novel antioxidant with neuroprotective properties. We examined whether SUN N8075 inhibited the neuronal damage resulting from permanent focal cerebral ischemia, and examined its neuroprotective properties *in vivo* and *in vitro* mechanism. Focal cerebral ischemia was induced by permanent middle cerebral artery occlusion in mice, and the resulting infarction, brain swelling, and neurological deficits were evaluated after 24 h or 72 h. Brain damage was assessed histochemically using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining and antibody recognizing 4-hydroxynonenal histidine adduct (4-HNE). In the *in vitro* study, we examined the effects of SUN N8075 on 1) lipid peroxidation in mouse brain homogenates and 2) cell viability and caspase-3 protease activity under a hypoxic insult or $FeSO_4$ in rat cultured cerebrocortical neurons. SUN N8075 administered either 10 min before or at 1 h after the occlusion reduced both infarction size and neurological deficits. SUN N8075 reduced brain swelling when administered 10 min before, 1 h, or 3 h after occlusion. Furthermore, only pretreatment (administered 10 min before) decreased infarct volume and brain swelling at 72 h after middle cerebral artery occlusion. SUN N8075 reduced the number of TUNEL-positive cells and decreased the level of oxidative damage, as assessed by immunopositive staining to 4-HNE. SUN N8075 inhibited lipid peroxidation, leakage of lactate dehydrogenase, caspase-3 activation induced by *in vitro* hypoxia, and the neuronal damage induced by *in vitro* $FeSO_4$ exposure. These findings indicate that SUN N8075 has neuroprotective effects against acute ischemic neuronal damage in mice and may prove promising as a therapeutic drug for stroke.

Key words: cerebral ischemia, lipid peroxidation, neuroprotection.

Stroke is the third commonest cause of death in industrialized countries. Among the many factors promoting brain damage after ischemia, oxidative stress plays a pivotal role through the formation of reactive oxygen species, leading to lipid peroxidation and DNA damage (Chan, 1996). Ischemia causes an imbalance between antioxidants and oxygen radicals, with an accumulations of toxic free radicals increasing the susceptibility of brain tissues to oxidative damage via inflammation, apoptosis, lipid peroxidation of membranes and DNA oxidation (Chan, 2001). Hence, a prime goal of neuroprotective strategies is to reduce oxidative damage. In fact, edaravone, a radical scavenger, has been approved as a neuroprotective agent for the treatment of acute cerebral infarction since 2001 in Japan.

SUN N8075, (2S)-1-(4-amino-2,3,5-trimethylphenoxy)-3-{4-[4-(4-fluorobenzyl) phenyl]-1-piperazinyl}-2-propanol dimethanesulfonate, is a novel antioxidant, and is currently in phase I clinical trials for stroke (Annoura et al., 2000). In the present study, we examined the neuroprotective effects of SUN N8075 on infarction, brain swelling, neurological deficits, and apoptosis in a murine permanent focal cerebral ischemia model. In addition, we studied the mechanism of action of SUN N8075, focusing on ischemia-induced oxidative stress, by assessing its effects on (i) lipid peroxidation in mouse brain homogenates, (ii) hypoxia or $FeSO_4$-induced cell damage in rat cerebrocortical cell culture, and (iii) the immunohistochemical evidence for lipid peroxidation.

EXPERIMENTAL PROCEDURES

Animals

The experimental designs and all procedures were in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Animal Care Guidelines issued by the Animal Experimental Committee of Gifu Pharmaceutical University. All the *in vivo* experiments were performed using male

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Abbreviations: Ac-DEVD-CHO, Ac-Asp-Glu-Val-Asp-H; AMC, 7-amino-4-methylcoumarin; HR, heart rate; LDH, lactate dehydrogenase; MABP, mean arterial blood pressure; MCA, middle cerebral artery; PBS, phosphate-buffered saline; rCBF, regional cerebral blood flow; SUN N8075, (2S)-1-(4-amino-2,3,5-trimethylphenoxy)-3-{4-[4-(4-fluorobenzyl) phenyl]-1-piperazinyl}-2-propanol dimethanesulfonate; TTC, 2,3,5-triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; 4-HNE, 4-hydroxynonenal histidine adduct.

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ddY mice (5-weeks old, Japan SLC, Ltd., Shizuoka, Japan). Attempts were made to limit the number, as well as pain and suffering, of animals used.

Drugs

SUN N8075 and lubeluzole were synthesized at Asubio Pharma Co. Ltd. (Osaka, Japan). Ebselen, riluzole, Trolox C, Ac-Asp-Glu-Val-Asp-H (Ac-DEVD-CHO), Hoechst 33342, 2,3,5-triphenyltetrazolium chloride (TTC), sodium pentobarbital, and isoflurane were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA), Tocris Cookson Ltd. (Bristol, UK), Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), Peptide Institute Inc. (Osaka, Japan), Invitrogen Molecular Probes (Eugene, OR, USA), Sigma-Aldrich Co. (St. Louis, MO, USA), Nissan Kagaku (Tokyo, Japan), and Merck Hoei Ltd. (Osaka, Japan), respectively.

Focal cerebral ischemia model in mice

Anesthesia was induced using 2.0–3.0% isoflurane, and maintained with 1.0–1.5% isoflurane in 70% N_2O and 30% O_2 via an animal general anesthesia machine (Soft Lander, Sin-ei Industry Co. Ltd., Saitama, Japan). The body temperature was maintained between 37.0 and 37.5 °C with the aid of a heating pad and heating lamp. In each mouse, regional cerebral blood flow (rCBF) was monitored by laser-Doppler flowmetry (Omegaflow flo-N1; Omegawave Inc., Tokyo, Japan) using a flexible probe fixed to the skull (2 mm posterior and 6 mm lateral to bregma). The left external carotid artery was exposed via a midline skin incision, and its branches were occluded in accordance with our previous reports (Hara et al., 1997).

SUN N8075 treatment

SUN N8075 or saline (vehicle) was administered i.v. (3 mg/kg, over 90 s, 0.1 ml/10 g) followed by a s.c. 10 mg/kg dose of SUN N8075 at 10 min before, 1 h after, or 3 h after middle cerebral artery (MCA) occlusion.

Physiological monitoring

A polyethylene catheter was inserted into the left femoral artery, and arterial blood pressure and heart rate were recorded (Power Laboratory/8SP; AD Instrument, Osaka, Japan) at 20 min before and 30 min after MCA occlusion. Blood samples (50 μl) were taken prior to and at 30 min after the onset of ischemia, and pO_2, pCO_2, and pH were measured using a blood gas analyzer (i-STAT 300 F; Abbot Co., Abbot Park, IL, USA).

Assessment of cerebral infarct and cerebral edema volumes

To analyze infarct volume, mice were killed using sodium pentobarbital at 24 h or 72 h after MCA occlusion, the brains sectioned coronally into five slices (2 mm thick), and the sections placed in 2% TTC at 37 °C for 30 min. The infarcted areas were recorded as images using a digital camera (Nikon COOLPIX 4500), and then quantitated using Image J and calculated as in a previous report (Hara et al., 1997). Brain swelling was calculated according to the following formula: (infarct volume+ipsilateral undamaged volume−contralateral volume)×100/contralateral volume (%) (Hara et al., 1997).

Neurological deficits

Mice were tested for neurological deficits at 24 h, 48 h, or 72 h after MCA occlusion and scored as described in our previous report (Hara et al., 1996) using the following scale: 0, no observable neurological deficits (normal); 1, failure to extend the right forepaw (mild); 2, circling to the contralateral side (moderate); 3, loss of walking or righting reflex (severe). The investigator who rated the mice was blind as to the group to which each mouse belonged.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining

TUNEL assay was performed according to the manufacturer's instructions (Roche Molecular Biochemicals Inc., Mannheim, Germany). Ischemic areas of cortical brain sections 0.4–1.0 mm anterior to bregma (through the anterior commissure) were excised and used. For the identification of brain structures, we referred to a mouse brain atlas (Paxinos and Franklin, 2001). Paraffin-embedded sections were dewaxed and rehydrated, then incubated in 20 mg/ml of proteinase K for 30 min. After immersion in 100 μml of 3% H_2O_2 for 30 min, sections were incubated in a terminal deoxynucleotidyl transferase (TdT) labeling reaction mixture (supplied with the kit) in a humidified chamber for 90 min at 37 °C, and then incubated in the stop buffer at 37 °C for 4 min.

Immunohistochemistry for 4-hydroxynonenal adducts (4-HNE)

Twenty-four hours after MCA occlusion, mice were perfusion-fixed using heparinized saline followed by 4% paraformaldehyde in phosphate-buffered saline (PBS), and the forebrain was processed and paraffin-embedded. After deparaffinization, sections were microwaved for 10 min at 121 °C in 10 μmol/l citric acid (pH 6.0), then allowed to cool to room temperature for 60 min. Sections were rinsed three times in PBS, incubated in 3% H_2O_2 in methanol for 30 min, and then placed in PBS and blocked with 1% mouse serum for 30 min. A monoclonal antibody against 4-HNE (25 mg/ml; JaICA, Shizuoka, Japan) was applied to sections overnight at 4 °C. Secondary antibody (M.O.M. biotinylated anti-mouse) was applied for 10 min. The avidin/biotinylated horseradish peroxidase complex (ABC Elite kit; Vector Laboratories, UK) was applied for 30 min, and the sections were allowed to develop chromogen in 3,3-diaminobenzidine+nickel solution (Sigma-Aldrich Co.) for 4 min.

Cell counting

To quantify DNA-fragmented cells after ischemia, the number of TUNEL-positive cells in the caudate-putamen [as the ischemic core (striatum) and in the cortex as the ischemic core (cortex) and as the ischemic penumbra; two areas, the superior and inferior cortical areas] were counted in a high-power field (×200) randomly chosen in a section through the anterior commissure. In addition, to assess oxidative damage, the distribution of immunopositive reactions for 4-HNE was delineated, the number of positive cells being counted in the same way as for TUNEL-positive cells. The histologist was blind as to the group to which each mouse belonged.

Lipid peroxidation in mouse forebrain homogenates

The supernatant fraction of forebrain homogenates from male adult ddY mice was prepared as described elsewhere (Hara and Kogure, 1990). Briefly, brain tissues were homogenized in a glass–Teflon homogenizer in four volumes of ice-cold phosphate-saline buffer (50 μM, pH 7.4), and the homogenate was stored at −80 °C. This stock brain homogenate was diluted 10-fold with the same buffer, and then 2 ml portions of the diluted homogenate were added to 10 ml of the test compound, and incubated at 37 °C for 30 min. The reaction was stopped by adding 400 ml of 35% $HClO_4$, followed by centrifugation at 2800 r.p.m. for 10 min. The supernatant (1 ml) was heated with 0.5 ml of thiobarbituric acid (TBA) solution (5 g/l in 50% acetic acid) for 15 min at 100 °C. The absorbance was then measured at 532 nm.