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THALIDOMIDE PROTECTS AGAINST ISCHEMIC NEURONAL DAMAGE INDUCED BY FOCAL CEREBRAL ISCHEMIA IN MICE

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Abstract—We aimed to examine whether thalidomide might inhibit the neuronal damage resulting from focal cerebral ischemia, and if so to explore the neuroprotective mechanism. Focal cerebral ischemia was induced by permanent middle cerebral artery occlusion (MCAO) in mice, and thalidomide was intraperitoneally administered a total of three times (at 10 min before, just before, and 1 h after MCAO). Thalidomide significantly reduced (a) the infarct area and volume at 24 and 72 h after MCAO and (b) the neurological score at 72 h after MCAO. Brains were also histochemically assessed for apoptosis and lipid peroxidation using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining and an antibody recognizing 8-hydroxy-2′-deoxyguanosine (8-OHdG), respectively. Thalidomide reduced both the number of TUNEL-positive cells and the oxidative damage. However, post-treatment of thalidomide [20 mg/kg, three times (at just after, 1 h after, 3 h after MCAO)] did not reduce the infarct volume. In an *in vitro* study, we examined the effects of thalidomide on lipid peroxidation in mouse brain homogenates and on the production of various radical species. Thalidomide inhibited both the lipid peroxidation and the production of H_2O_2 and $O_2 \cdot^-$ (but not HO^-) radicals. We also measured the brain concentration of TNF-α by ELISA. The TNF-α level in the brain was significantly increased at 9–24 h after MCAO. However, thalidomide did not reduce the elevated TNF-α level at either 12 or 24 h after MCAO. These findings indicate that thalidomide has neuroprotective effects against ischemic neuronal damage in mice, and that an inhibitory action of thalidomide against oxidative stress may be partly responsible for these neuroprotective effects.

Key words: focal cerebral ischemia, middle cerebral artery occlusion, oxidative stress, thalidomide, TNF-α.

Stroke is the third most common cause of death after heart attack and cancer, and it has profound negative social and economic effects. The current treatment for complete stroke is only partially successful at reversing neurodegeneration and restoring premorbid function. Clinical and experimental data suggest that ischemic neuronal damage is at least partly caused by the free-radicals production and lipid peroxidation that occurs either during the ischemia itself or following reperfusion (Flamm et al., 1978; Hara et al., 1993; Toyoda et al., 2004). 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 et al., 2001). Hence, one of the prime goals of neuroprotective strategies is to reduce oxidative damage, and indeed edaravone, a radical scavenger, has been an approved neuroprotective agent for the treatment of acute cerebral infarction since 2001 in Japan.

Thalidomide [α-(N-phthalimido)-glutarimide] was first released in Europe and Canada as a rapidly acting and hangover-free sedative in 1956. Reports of phocomelia in the infants born to women who had taken thalidomide during pregnancy started to surface a few years later, leading to its withdrawal from the market. Despite its teratogenicity, thalidomide was approved by the United States Food and Drug Administration in 1998 as a treatment for erythema nodosum leprosum. The clinical efficacy of thalidomide against inflammatory and autoimmune diseases is attributed in part to its ability to inhibit TNF-α production (Sampaio et al., 1991; Moreira et al., 1993; Klausner et al., 1996). It has been reported that thalidomide reduces ischemic injury of the spinal cord in rabbits (Lee et al., 2007), and that it decreases polymorphonuclear leukocyte infiltration, retinal edema, and the synthesis of vascular endothelial growth factor (VEGF) and TNF-α following ischemia/reperfusion injury to the guinea-pig retina (Aydogan et al., 2007).

TNF-α, a cytokine involved in systemic inflammation, is a member of a group of cytokines that all stimulate the acute phase reaction. TNF causes apoptotic cell death, cellular proliferation, inflammation, and tumorigenesis. At 6 and 22 h after middle cerebral artery occlusion (MCAO), activated spleen cells are major secretors of TNF-α and some other cytokines (Offner et al., 2006). This response is associated with marked proinflammatory changes in the brain. Thus, the neuroimmune axis appears to have a feedback loop in which focal cerebral ischemia results in widespread activation of inflammatory cytokines in peripheral immune organs, and this in turn modulates CNS pathophysiology.

Given the background described above, we performed the present study to examine the neuroprotective effects of

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Abbreviations: DCFH, nonfluorescent di-chlorofluorescein; DMSO, dimethyl sulfoxide; DPPH, diphenyl-*p*-picrylhydrazyl; ESR, electron spin resonance; MABP, mean arterial blood pressure; MCAO, middle cerebral artery occlusion; PBS, phosphate-buffered saline; rCBF, regional cerebral blood flow; RGC-5, Retinal ganglion cells; ROS, reactive oxygen species; TBA, thiobarbituric acid; TTC, 2,3,5-triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; VEGF, vascular endothelial growth factor; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

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thalidomide against infarction, neurological deficits, and apoptosis in a murine focal cerebral ischemia model. In addition, we studied the mechanism of action of thalidomide, focusing on the extent to which ischemia induced TNF-α production in the ischemic brain (by ELISA) and oxidative stress [by assessing (i) radical-scavenging capacity in neuronal cells, (ii) immunohistochemical changes using 8-hydroxy-2′-deoxyguanosine (8-OHdG), (iii) lipid peroxidation in mouse brain homogenates using an assay for thiobarbituric acid (TBA) reactive substance, (iv) diphenyl-*p*-picrylhydrazyl (DPPH)-induced free radicals by electron spin resonance (ESR)-spin trapping and absorbance determinations].

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 efforts were made to minimize both suffering and the number of animals used. All *in vivo* experiments were performed using male ddY mice (4 weeks old; body weight 22–28 g; Japan SLC Ltd., Shizuoka, Japan). The animals were housed at 24±2 °C under a 12-h light/dark cycle (lights on from 07:00–19:00 h). Each animal was used for one experiment only.

Drugs

Thalidomide was synthesized at the Department of Materials Engineering (Prof. N. Shibata), Nagoya Institute of Technology (Nagoya, Japan). 2,3,5-Triphenyltetrazolium chloride (TTC), sodium pentobarbital, and isoflurane were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), Nissan Kagaku (Tokyo, Japan), and Merck Hoei Ltd. (Osaka, Japan), respectively. DPPH and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich and Koso Chemical (Tokyo, Japan), respectively.

Focal cerebral ischemia model in mice

Anesthesia was induced using 2.0–3.0% isoflurane and maintained using 1.0–1.5% isoflurane (both in 70% N_2O/30% O_2) by means of an animal general anesthesia machine (Soft Lander; Sin-ei Industry Co. Ltd., Saitama, Japan). Body temperature was maintained at 37.0–37.5 °C with the aid of a heating pad and heating lamp. After a midline skin incision, the left external carotid artery was exposed, and its branches were occluded (Hara et al., 1996, 1997). An 8–0 nylon monofilament (Ethicon, Somerville, NJ, USA) coated with a mixture of silicone resin (Xantopren; Bayer Dental, Osaka, Japan) was introduced into the left internal carotid artery through the common carotid artery so as to occlude the origin of the middle cerebral artery. Then, the left common carotid artery was occluded. After the surgery, the mice were kept in the preoperative condition (room temperature; 24±2 °C) until sampling.

Thalidomide treatment

Thalidomide or DMSO 0.8 ml/kg (vehicle) was administered intraperitoneally (10, 20 and 50 mg/kg in DMSO) a total of three times (at 10 min before, just after, and 1 h after MCAO, or at just after, 1 h after, and 3 h after MCAO).

Physiological monitoring

A polyethylene catheter inserted into the left femoral artery was used to measure arterial blood pressure (MABP) and heart rate (Power Laboratory/8SP; AD Instrument, Osaka, Japan) 20 min before and 30 min after MCAO. Blood samples (50 μl) were taken before and at 30 min after the onset of ischemia for pH, pCO_2, and pO_2 being measured (i-STAT 300F; Abbott Co., Abbott Park, IL, USA). Regional cerebral blood flow (rCBF) was monitored by Doppler flowmetry (Omegaflow flo-N1; Omegawave Inc., Tokyo, Japan). A flexible probe was fixed to the skull (2 mm posterior and 6 mm lateral to bregma).

Assessment of cerebral infarction

To analyze infarct volume, mice were euthanized using sodium pentobarbital at 24 h or 72 h after MCAO, and forebrains were sectioned coronally into five slices (2 mm thick). These were placed in 2% TTC at 37 °C for 30 min. The infarcted areas and volumes were recorded as images using a digital camera (Coolpix 4500; Nikon, Tokyo, Japan), then quantified using an Image J (http://rsb.info.nih.gov/ijdownload/) and calculated as in a previous report (Hara et al., 1997).

Neurological deficits

Mice were tested for neurological deficits at 72 h after MCAO. Scoring was done as described in our previous study (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 (K.H.) who rated the mice was masked as to the group to which each mouse belonged.

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

The 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). The brains were removed, fixed overnight in 4% paraformaldehyde, and immersed for 1 day in 25% sucrose with PBS. The brains were then embedded in a supporting medium for frozen-tissue specimens (OCT compound; Tissue-Tek). Cerebral sections 10 μm thick were cut on a cryostat at −25 °C, and stored at −80 °C until staining. After twice washing with phosphate-buffered saline (PBS), sections were incubated with terminal deoxyribonucleotidyl transferase (TdT) enzyme at 37 °C for 1 h. The sections were washed three times in PBS for 1 min at room temperature. Sections were subsequently incubated with an anti-fluorescein antibody–peroxidase conjugate at room temperature in a humidified chamber for 30 min, and then developed using DAB tetrahydrochloride peroxidase substrate.

Immunohistochemistry for 8-OHdG

Twenty-four hours after MCAO, mice were perfusion-fixed using heparinized saline followed by 4% paraformaldehyde in 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, then placed in PBS and blocked with 1% mouse serum for 30 min. A monoclonal antibody against 8-OHdG 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) for 2 min. Ischemic areas of cortical brain sections 0.4–1.0 mm anterior to