

Sodium 4-Phenylbutyrate Protects against Cerebral Ischemic Injury

Xin Qi, Toru Hosoi, Yasunobu Okuma, Masayuki Kaneko, and Yasuyuki Nomura

Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan

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ABSTRACT

Sodium 4-phenylbutyrate (4-PBA) is a low molecular weight fatty acid that has been used for treatment of urea cycle disorders in children, sickle cell disease, and thalassemia. It has been demonstrated recently that 4-PBA can act as a chemical chaperone by reducing the load of mutant or mislocated proteins retained in the endoplasmic reticulum (ER) under conditions associated with cystic fibrosis and liver injury. In the present study, we evaluated the neuroprotective effect of 4-PBA on cerebral ischemic injury. Pre- or post-treatment with 4-PBA at therapeutic doses attenuated infarction volume, hemispheric swelling, and apoptosis and improved neurological status in a mouse model of hypoxia-ischemia. Moreover, 4-PBA suppressed ER-mediated apoptosis by inhibiting eukaryotic initiation factor 2 α phosphorylation, CCAAT/enhancer-

binding protein homologous protein induction, and caspase-12 activation. In neuroblastoma neuro2a cells, 4-PBA reduced caspase-12 activation, DNA fragmentation, and cell death induced by hypoxia/reoxygenation. It protected against ER stress-induced but not mitochondria-mediated cell death. Additionally, 4-PBA inhibited the expression of inducible nitric-oxide synthase and tumor necrosis factor- α in primary cultured glial cells under hypoxia/reoxygenation. These results indicate that 4-PBA could protect against cerebral ischemia through inhibition of ER stress-mediated apoptosis and inflammation. Therefore, the multiple actions of 4-PBA may provide a strong effect in treatment of cerebral ischemia, and its use as a chemical chaperone would provide a novel approach for the treatment of stroke.

Ischemic stroke is the third leading cause of death in industrially advanced countries and a major cause of long-lasting disability. Even though a large number of compounds have been proven to reduce ischemic injury, clinical trials have been unsuccessful because of toxic side effects. Thus, the development of new drugs and discovery of novel mechanisms for treating cerebral ischemia are needed. Evidence obtained in recent years has demonstrated that endoplasmic reticulum (ER)-mediated cell death plays an important role in cerebral ischemia. Therefore, targeting the ER may provide a therapeutic approach for blocking the pathological process induced by cerebral ischemia. However, no pharmacological approach for treating ischemia-induced ER dysfunction has been reported so far.

Sodium 4-phenylbutyrate (4-PBA) is a low molecular weight fatty acid that has been approved for clinical use as an ammonia scavenger in children with urea cycle disorders

(Maestri et al., 1996) and for treatment of sickle cell disease and thalassemia on the basis of its capacity to activate transcription of β - and γ -globin (Dover et al., 1994; Collins et al., 1995). 4-PBA has been found to prolong life (Kang et al., 2002) and contribute to therapy for spinal muscular atrophy (Andreassi et al., 2004) by altering the pattern of gene expression. Moreover, 4-PBA can reduce the neuroinflammation and disease process in multiple sclerosis (Dasgupta et al., 2003). Furthermore, a number of studies published in the last several years have described the use of 4-PBA as a chemical chaperone to reverse the mislocalization and/or aggregation of proteins associated with human disease (Burrows et al., 2000; Rubenstein and Zeitlin, 2000; Perlmutter, 2002). Investigations revealed that 4-PBA can reverse the misfolded proteins in the ER, including the Δ F508 cystic fibrosis transmembrane conductance regulator (Rubenstein and Zeitlin, 2000) mutant α 1-antitrypsin (Burrows et al., 2000).

Apoptosis plays a pivotal role in neuronal cell death from ischemic stroke. There is evidence to suggest that the acti-

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ABBREVIATIONS: ER, endoplasmic reticulum; 4-PBA, sodium 4-phenylbutyrate; TTC, 2,3,5-triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; RT-PCR, reverse transcription-polymerase chain reaction; eIF2 α , eukaryotic initiation factor 2 α ; CHOP, C/EBP homologous protein; iNOS, inducible nitric-oxide synthase; TNF- α , tumor necrosis factor- α ; CCCP, carbonyl cyanide-*m*-chlorophenyl hydrazone; NaN₃, sodium azide; Tm, tunicamycin; LDH, lactate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

variation of apoptosis is initiated by cell-surface receptors or by mitochondrial stress (Budihardjo et al., 1999). Recently, another apoptotic-regulatory pathway involved in ER stress has been receiving attention. The conditions that impair the function of the ER, designated "ER stress", can lead to an accumulation of unfolded or misfolded proteins in the ER lumen (Kaufman, 1999). In the case of mild ER stress, cells have developed a self-protective signal transduction pathway termed the unfolded protein response, which includes the induction of molecular chaperones in the ER, translational attenuation, and ER-associated degradation (Cudna and Dickson, 2003). However, if the damage is too severe to repair, the unfolded protein response ultimately initiates an apoptotic pathway (Oyadomari et al., 2002; Cudna and Dickson, 2003). ER stress-induced cell death has been shown to involve the activation of caspase-12 (Nakagawa et al., 2000). Another component of the ER stress-mediated apoptotic pathway is the C/EBP homologous protein (CHOP), also known as growth arrest- and DNA damage-inducible gene 153 (Wang et al., 1996). 4-PBA can reduce the load of mutant or mislocated proteins retained in the ER under conditions associated with cystic fibrosis and liver injury. Despite the fact that cerebral ischemia has been shown to cause ER dysfunction, it is not clear whether 4-PBA can protect against ischemia-induced neurodegeneration. Therefore, in the present study, we investigated the protective effect of 4-PBA on cerebral ischemia and the mechanism underlying the neuroprotection.

Cerebral ischemia is also associated with a marked inflammatory reaction that contributes to the evolution of tissue injury (del Zoppo et al., 2000). Evidence suggests that the induction of inducible nitric-oxide synthase (iNOS) is one of the mechanisms by which postischemic inflammation exerts its deleterious effects (Iadecola et al., 1997). The expression of proinflammatory cytokines such as TNF- α and interleukin-1 β in ischemic injury results in an up-regulation of iNOS that produces large amounts of NO and thereby causes neurotoxicity in the central nervous system (del Zoppo et al., 2000). Mice with a null mutation of the iNOS gene showed remarkable resistance to ischemic brain injury (Iadecola et al., 1997). Moreover, iNOS expression is also involved in the mechanisms of cerebral ischemia in humans (Forster et al., 1999). These findings indicate that iNOS expression is deleterious to the postischemic brain and raise the possibility that inhibition of iNOS is of therapeutic value to stroke victims. Because 4-PBA functions as a chemical chaperone in the ER and also has an anti-inflammatory effect, we hypothesized that 4-PBA has clinical efficacy for the treatment of cerebral ischemia.

Materials and Methods

Induction of Hypoxia-Ischemia and Drug Treatment. Male C57BL mice (6 weeks old, 16–20 g) were anesthetized with halothane (2% in 70% N₂O:30% O₂), and the right carotid artery was isolated and double-ligated with 4-0 surgical thread. The incision was sutured, and the animals were allowed to recover with access to food and water for 3 h. To induce hypoxia, each animal was placed in a 500-ml glass jar partially submerged in a temperature-controlled water bath and exposed to a humidified gas mixture of 6% O₂/balance N₂ for specific intervals of 30 min. Animals were allowed to recover in room air for 30 min and then were returned to their cages with free access to food and water. Animals in which the right

common carotid artery was separated but no ligation and hypoxia were performed were used as sham-operated controls. All animal experiments were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the animal care and use committee of Hokkaido University.

4-PBA was prepared by titrating equimolar amounts of 4-phenylbutyric acid (Wako Pure Chemicals, Tokyo, Japan) and sodium hydroxide to pH 7.4. In *in vivo* experiments, 4-PBA was administered intraperitoneally at a volume of 5 ml/kg.

Determination of Infarct Volume and Hemispheric Swelling. Animals were killed 3 days after ischemia, and the brain was removed and cut into four 2-mm sections. The brain slices were immersed in a 2% solution of TTC (Wako Pure Chemicals) in normal saline at room temperature in a dark place for 30 min and then washed in phosphate-buffered saline twice. The slices were fixed in 10% formalin for photography, and the infarction area was measured. An image-analysis system (NIH Image Software version 1.61) was used to determine the infarct volume (MV), right hemisphere volume (RV), and left hemisphere volume (LV). To compensate for the effect of brain edema on the measured infarct volume in the ischemic hemisphere, infarcted volumes in each mice were corrected with the following formula: corrected infarct volume (%) = [LV – (RV – MV)]/LV \times 100. Hemispheric swelling in the ischemic hemisphere was also calculated: swelling (%) = RV – LV/LV \times 100.

Evaluation of Neurological Status. Neurological deficits of mice at 1, 2, and 3 days after ischemia were assessed and scored (Huang et al., 1994) as follows: 0, no observable neurological deficit (normal); 1, failure to extend the left forepaw on the lifting of the whole body by the tail (mild); 2, circling to the contralateral side (moderate); and 3, leaning to the contralateral side at rest or no spontaneous motor activity (severe). Animals not showing neurological deficits at the above time points were excluded from the study.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Staining. The mice were treated with either saline or 120 mg/kg *i.p.* 4-PBA immediately after hypoxia-ischemia. After 3 days of recovery, the brain was removed and cut into frozen slices (40 μ m). Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining was performed using a kit for programmed cell death (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan) according to the manufacturer's directions. The two fixed areas of each section were examined by microscopy in the ischemic cortex or the contralateral hemisphere. The TUNEL-positive cells were counted with NIH Image Software version 1.61.

Cell Culture. Primary glial cells were prepared from the whole brain of neonatal (<24 h) C57BL/6 mice as described previously (Hosoi et al., 2000). The cells were allowed to grow to confluence in Dulbecco's modified Eagle's medium with 10% (v/v) fetal calf serum, 100 U/ml penicillin G, and 100 μ g/ml streptomycin (Invitrogen, Carlsbad, CA). Mouse neuroblastoma Neuro2a cells were maintained in modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum. All cultured cells were maintained at 37°C in 5% CO₂/95% air.

Exposure to Hypoxic Environment. When the cells became subconfluent, they were cultured for a given period in 5% CO₂/95% nitrogen. The balance was maintained in a humidified incubator (ANX-1; HIRASAWA, Tokyo, Japan) at 37°C within a sealed, anaerobic, gloved cabinet containing a catalyst to scavenge free oxygen.

Lactate Dehydrogenase Leakage Assays. The viability of neuro2a cells after hypoxia or treatment with tunicamycin (Tm), carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) and sodium azide (NaN₃; all from Sigma-Aldrich Japan K.K., Tokyo, Japan) was estimated by the lactate dehydrogenase (LDH) leakage method using a cytotoxicity detection kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol. LDH activity was measured as the optimal density at 492 nm, and the percentage of LDH leakage was defined as the ratio of LDH activity in the

culture medium to the total activity: % = (extracellular activity)/(extracellular activity + remaining cellular activity).

DNA Fragmentation. The Neuro2a cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, and 0.5% Triton X-100) and incubated for 20 min at 4°C. The samples were centrifuged at 27,000g for 15 min at 4°C. The supernatants were incubated with 40 µg/ml of proteinase K for 30 min at 37°C and extracted with equal volumes of phenol and phenol/chloroform (1:1 v/v). The DNA was precipitated from the supernatants with a 1/10 volume of 3 M sodium acetate, pH 5.2, and 2 volumes of ethanol and treated with 40 µg/ml RNase A for 1 h at 37°C. The recovered DNA was then analyzed by electrophoresis on a 1.5% agarose gel and visualized with 0.5 µg/ml ethidium bromide.

RT-PCR. Total RNA was isolated from mouse brain or cultured cells using TRI Reagent (Sigma-Aldrich, St. Louis, MO). RT-PCR was performed as described previously (Hosoi et al., 2002). The following primers were used: CHOP, 5'-ccc tgc ctt tca cct tgg-3' and 3'-ccg ctc gtt ctc ctg ctc-5'; TNF-α, 5'-cac gtc gta gca aac cac caa-3' and 3'-ccc att ccc ttc aca gag caa-5'; and GAPDH, 5'-gat gga gca tca tac tga tcc-3' and 3'-aaa ccc atc acc atc ttc cag-5'.

Western Blotting. Primary antibodies used for Western blotting were as follows: anti-phospho-eIF2α (Ser51) polyclonal antibody (Cell Signaling Technology, Tokyo, Japan), 1:1000; anti-growth arrest- and DNA damage-inducible gene 153 (CHOP) polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 1:1000; anti-caspase-12 polyclonal antibody (ProSci Incorporated, Poway, CA), 1:1000; anti-iNOS-monoclonal antibody (BD Biosciences Transduction Laboratories, Lexington, KY), 1:1000; and anti-KDEL monoclonal antibody (StressGen Biotechnologies, San Diego, CA), 1:1000. Western blotting analysis was performed as described previously (Hosoi et al., 2002).

Results

4-PBA Protects against Ischemic Injury. Hypoxia-ischemia was induced by unilateral common carotid artery occlusion combined with systemic hypoxia. In a preliminary experiment, to evaluate the degree of brain damage, we measured the area of infarction after 3 days of recovery followed by hypoxia for 20, 30, and 40 min. In TTC staining, no infarction appeared in the brains subjected to only hypoxia or only right carotid artery ligation. Large individual variation in infarction area in the mice that had been subjected to 20 min of hypoxia-ischemia and severe damage and high mortality in the mice that had been subjected to 40 min of hypoxia-ischemia were observed. Thus, we chose 30 min of hypoxia-ischemia, which produced consistent brain damage, as an elementarily experimental condition in this model.

To investigate whether 4-PBA can protect the brain from damage caused by hypoxia-ischemia, we administered 4-PBA at various doses. First, we tested the treatment with 4-PBA (15, 40, and 120 mg/kg i.p. once a day for 3 days) 30 min before hypoxia-ischemia. Pretreatment with 4-PBA dose-dependently decreased infarct volume on the third day after ischemia. A remarkable reduction in edema-corrected infarct volume was achieved with 40 and 120 mg/kg 4-PBA (40% and 70%, respectively) (Fig. 1a). However, no significant reduction in infarct volume was observed in mice dosed with 15 mg/kg (Fig. 1a). Hemispheric swelling was also significantly decreased with the doses of 40 mg/kg (21% reduction) and 120 mg/kg (52% reduction) under the same treatment conditions (Fig. 1a).

Next, we examined whether post-treatment with 4-PBA (40 and 120 mg/kg i.p. once a day for 3 days) can protect against ischemic injury. We observed a potent protective

effect when the treatment was started 1 h after the ischemia. The infarct volume was markedly diminished by both 40 mg/kg (48% reduction) and 120 mg/kg (70% reduction) (Fig. 1b). Hemispheric swelling was also significantly decreased (45% reduction for 40 mg/kg, and 56% reduction for 120 mg/kg). Treatment with 4-PBA 3 h after ischemia had a tendency to protect the injured brain (Fig. 1b).

To determine whether the neuroprotection from 4-PBA is associated with antiapoptotic activity, we evaluated apoptosis using the TUNEL assay. We observed an increase in TUNEL-positive cells 3 days after hypoxia-ischemia (Fig. 1c). Administration of 4-PBA (120 mg/kg) significantly suppressed the number of TUNEL-positive cells in the ischemic cortex by almost 50% compared with the vehicle-treated group in the corresponding brain sections (Fig. 1d). No TUNEL-positive cells were observed in the contralateral hemisphere (Fig. 1c).

4-PBA Improves Neurological Function. Pre- or post-treatment with 4-PBA (40 and 120 mg/kg) significantly reduced neurological deficits and provided a functional recovery from ischemia when examined at 24, 48, and 72 h, compared with the saline group. Maximal improvement was achieved with 120 mg/kg 4-PBA (Fig. 2).

4-PBA Represses ER Stress-Mediated Apoptotic Signals. A key feature of cerebral ischemia is the blocking of translation at the initiation step, as indicated by increased phosphorylation of eIF2α (DeGracia et al., 2002). Therefore, we first assessed whether 4-PBA could affect the phosphorylation of eIF2α, which has also been demonstrated to be an inducer of the transcription of CHOP (DeGracia et al., 2002). The levels of phospho-eIF2α in ischemic cortex were markedly increased after hypoxia-ischemia and were detectable until 12 h, whereas the administration of 4-PBA at 120 mg/kg noticeably down-regulated the level of phospho-eIF2α from 6 to 12 h (approximately 35–80% reduction compared with the saline group) (Fig. 3a). No change in the level of phospho-eIF2α was observed in the brains of animals only injected with 4-PBA (Fig. 3e). Furthermore, quantification revealed a remarkable increase in the mRNA and protein levels of CHOP in the injured cortex 12 h after ischemia. However, injection of 4-PBA reduced the CHOP mRNA (approximately 40% reduction) and protein (approximately 90% reduction) levels (Fig. 3, b and c). The protein expression of CHOP was not affected by treatment with 4-PBA in normal brain (Fig. 3e).

Furthermore, Western blotting analysis showed that caspase-12 was activated 24 h after hypoxia-ischemia, as evidenced by a decrease in the level of the procaspase-12, which was largely restored by 4-PBA (almost 30% restoration compared with the saline group) (Fig. 3d). No difference in the activation of caspase-12 was observed between the saline and 4-PBA-treated groups of normal mice (Fig. 3e).

4-PBA Suppresses ER Stress-Induced but Not Mitochondria-Derived Cell Death. First, we evaluated the effect of 4-PBA on cell death in the Neuro2a cells line under conditions of hypoxia/reoxygenation. The cells were pretreated by 4-PBA (3 mM) for 1 h and then exposed to 24 h of hypoxia followed by 12 h of reoxygenation. The cell viability was quantified by assaying the release of LDH. There was a significant increase in the release of LDH into the culture medium after hypoxia/reoxygenation compared with the normoxic group, whereas the treatment with 4-PBA signifi-

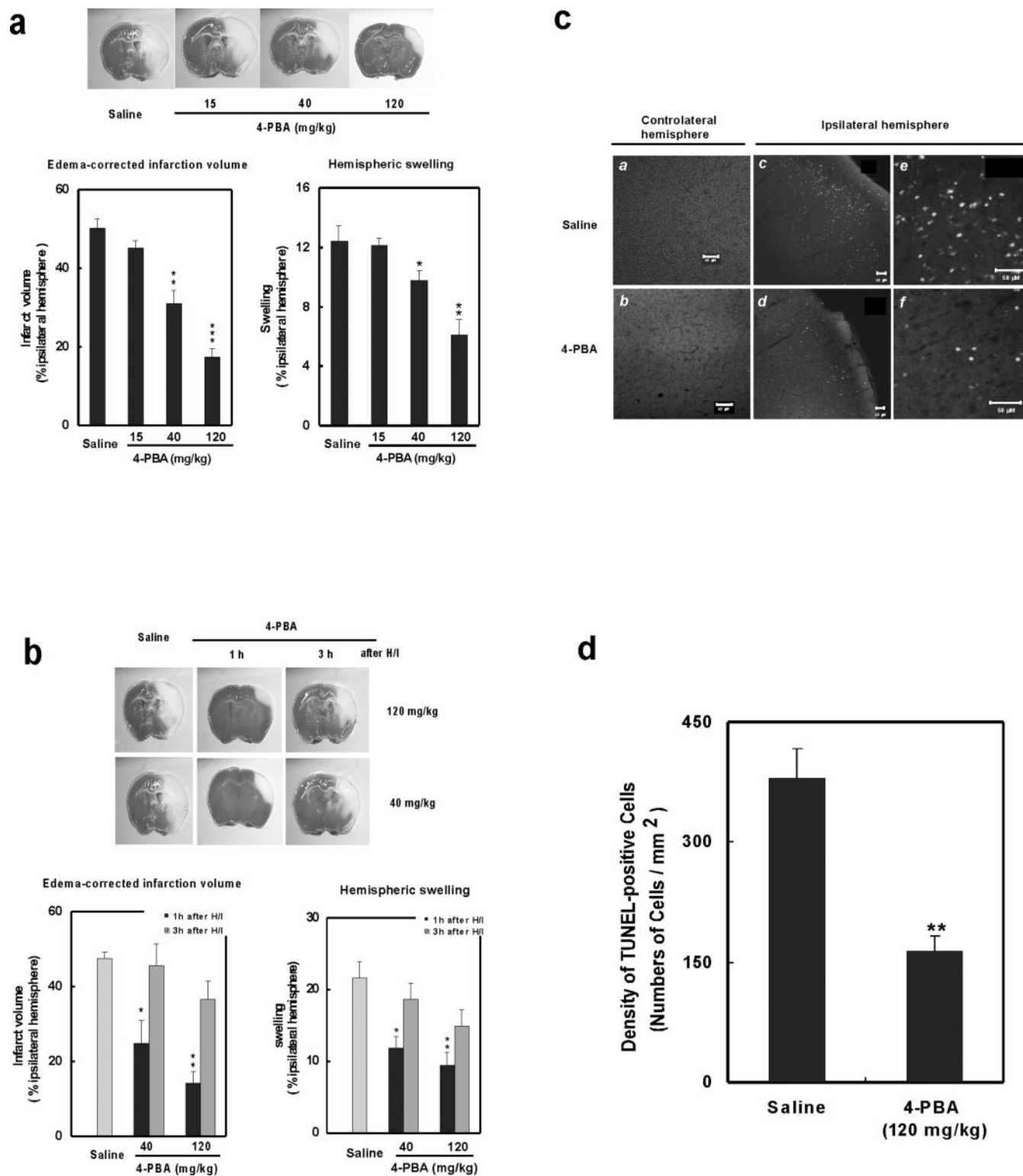


Fig. 1. Treatment with 4-PBA attenuated brain injury induced by hypoxia-ischemia. Mice were injected intraperitoneally with saline or 4-PBA and killed 3 days later. **a**, treatment with 4-PBA 30 min before hypoxia-ischemia dose-dependently reduced edema-corrected infarction volume and hemispheric swelling. Brain coronal sections (2 mm) were stained with 2% TTC. Data are means \pm S.E. from 10 mice in each group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with the saline-injected group, Dunnett's test. **b**, administration of the indicated dose of 4-PBA 1 h after ischemia diminished the infarction and hemispheric swelling. Data are means \pm S.E. from eight mice in each group. *, $p < 0.05$; **, $p < 0.01$, compared with the saline-injected group, Dunnett's test. **c**, administration of 4-PBA (120 mg/kg) suppressed apoptosis induced by hypoxia-ischemia. Saline or 4-PBA (120 mg/kg) was injected immediately after ischemia. Cerebral slices (40 μ m) were stained with TUNEL. No TUNEL-positive cells were seen in the contralateral hemisphere with saline (**a**) and 4-PBA (**b**). A remarkable increase was observed in the ischemic cortex of saline-injected mice (**c**) and at higher magnification ($\times 40$) (**e**). 4-PBA significantly reduced TUNEL-positive cells (**d**) and at higher magnification ($\times 40$) (**f**). **d**, quantification of the density of TUNEL-positive cells. Data are means \pm S.E. of TUNEL-positive cells from three mice in each group. **, $p < 0.01$, compared with the corresponding cortex in the saline-injected group, Student's *t* test.

cantly repressed the release (Fig. 4a). The antiapoptotic effect of 4-PBA was confirmed by a reduction of DNA fragmentation under 24 h of hypoxia (Fig. 4b).

To further investigate the direct effect of 4-PBA on the ER, the cells were treated with Tm (10 μ g/ml, an inhibitor of protein *N*-glycosylation and an ER stress inducer). Cells were pretreated with 4-PBA (3 mM) for 1 h before adding Tm. The release of LDH induced by Tm was significantly decreased by 4-PBA (Fig. 4c). Moreover, 4-PBA remarkably suppressed the activation of caspase-12 caused by hypoxia or Tm (Fig. 4, d and e).

In contrast, 4-PBA did not provide protection against the cell death induced by CCCP (a mitochondrial uncoupler) and NaN_3 (an inhibitor of mitochondrial complex IV), which can result in mitochondrial dysfunction (Fig. 5, a and b). We observed no changes of glucose-regulated protein 78, CHOP, and caspase-12 activation, the representative genes in ER stress response, after CCCP and NaN_3 treatment (Fig. 5c), indicating that CCCP and NaN_3 do not cause the ER stress.

4-PBA Inhibits iNOS and TNF- α Induction in Primary Cultured Glial Cells. Hypoxia for 24 h followed by 3 h of reoxygenation induced a remarkable expression of iNOS in primary cultured glial cells compared with the control group. Treatment with 4-PBA in doses of 3 and 10 mM significantly inhibited the induction of iNOS protein. 4-PBA also significantly down-regulated the mRNA level of TNF- α under hypoxia for 6 h (Fig. 6).

Discussion

In the present study, we show for the first time that the peripheral administration of 4-PBA at therapeutic doses protected the brain from ischemic injury, as evidenced by reductions in infarct volume and hemispheric swelling. The pro-

TECTIVE effect of 4-PBA was further demonstrated by a decrease in the number of apoptotic cells. Most importantly, administration of 4-PBA provided a wide therapeutic window. That is, the treatment was effective not only before but also after ischemia. In clinical trials, functional outcome rather than histological evaluation is a relevant parameter in patients (Barber et al., 1998). In the hypoxia-ischemia, neurological deficits declined progressively during a period of 1 to 3 days after ischemia. The spontaneous neurological recovery is likely to have been caused by oxygen recovery and blood reperfusion after hypoxia-ischemia and is similar to observations in a clinical study (Barber et al., 1998). It should be noted that treatment with 4-PBA 1 or 3 h after ischemia notably improved the neurological outcome in comparison with infarction volume. Such facilitation of functional recovery suggests that 4-PBA may protect against not only infarcted neuronal cell death but also axonal injury, which contributes to the resulting neurological deficits (Imai et al., 2002). 4-PBA has low toxicity and can penetrate well into cerebrospinal fluid (Berg et al., 2001). It has been used for clinical treatment of urea cycle disorders in children, sickle cell disease, thalassemia, and cystic fibrosis (Perlmutter, 2002). Results of clinical trials have shown that 4-PBA has few side effects and is safe for patients (Carducci et al., 2001). Therefore, the findings of the present study suggest that 4-PBA as a therapeutic agent may have great potential for patients with acute stroke.

Cerebral ischemia is a pathophysiological ER stressor (Paschen and Doutheil, 1999; Kumar et al., 2003). ER dysfunction induces apoptosis in neuronal cells (Oyadomari et al., 2002; Cudna and Dickson, 2003). Therefore, targeting the ER-associated apoptotic pathway might be an effective way to treat cellular injury. It has been indicated that ER dysfunction is responsible for the shutdown of translation initi-

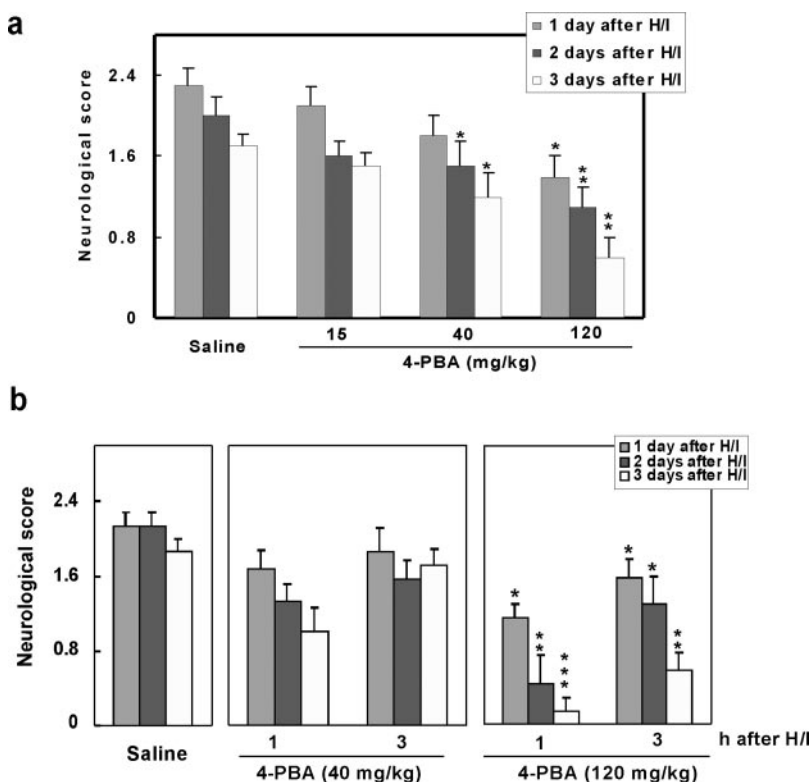


Fig. 2. Treatment with 4-PBA improved neurological function. Saline or 4-PBA was administered (intraperitoneally) 30 min before or 1 or 3 h after hypoxia-ischemia (H/I). The neurological deficits were assessed on days 1, 2, and 3 of recovery. **a**, pretreatment with 4-PBA reduced the neurological deficits at the doses of 40 and 120 mg/kg from 1 to 3 days of recovery. **b**, delayed treatment with 4-PBA improved the neurological status at 120 mg/kg from 1 to 3 days of recovery. Data are means \pm S.E. from 8 to 10 mice in each group, respectively. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the neurological score for the saline-treated group on the corresponding day, Dunnett's test.

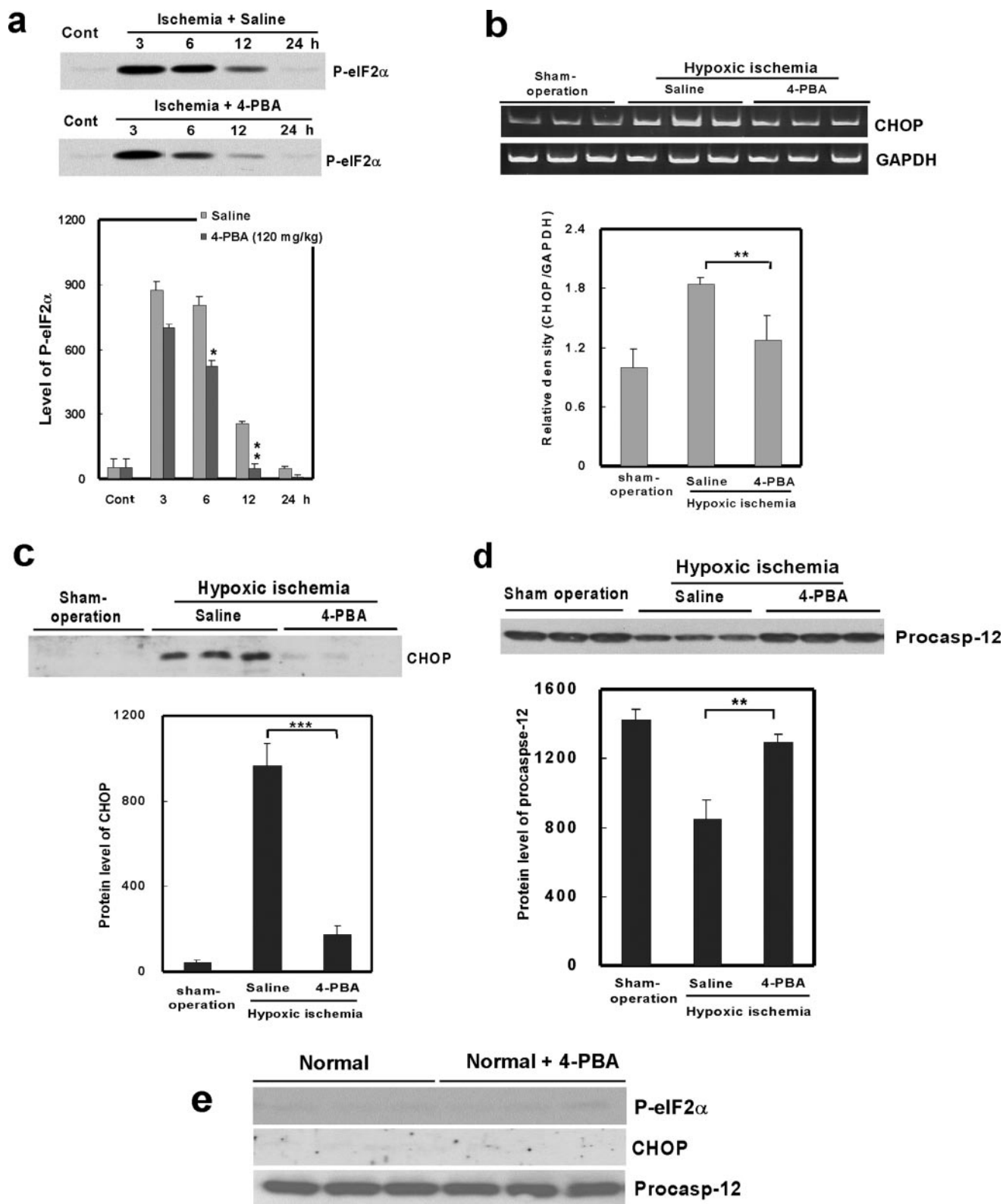


Fig. 3. Treatment with 4-PBA suppressed the ER stress-mediated apoptosis. Mice were subjected to 30 min of hypoxia-ischemia followed by recovery until death. Saline or 4-PBA (120 mg/kg) was injected immediately after ischemia. The tissue samples were from the cerebral cortex. **a**, administration of 4-PBA decreased the phosphorylation of eIF2 α . Phospho-eIF2 α was examined at the indicated time points by Western blotting. The lower panel is quantification of phospho-eIF2 α . **b**, expression and quantification of CHOP mRNA in the indicated group by RT-PCR. Tissue samples were from 12 h after ischemia. **c**, the change in protein level of CHOP determined by Western blotting. Tissue samples were from 12 h after ischemia. **d**, treatment with 4-PBA reduced the activation of caspase-12. The upper panel is a representative Western blotting analysis of procaspase-12. The lower panel is the quantification of procaspase-12 levels. The tissue samples were from 24 h after ischemia. **e**, Western blotting analysis of phospho-eIF2 α , CHOP, and caspase-12 in extracts from the right cortex of normal animals treated with saline or 4-PBA (120 mg/kg). The data are expressed as the mean \pm S.E. of six mice in each group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared with the saline-treated group, Fisher's test.

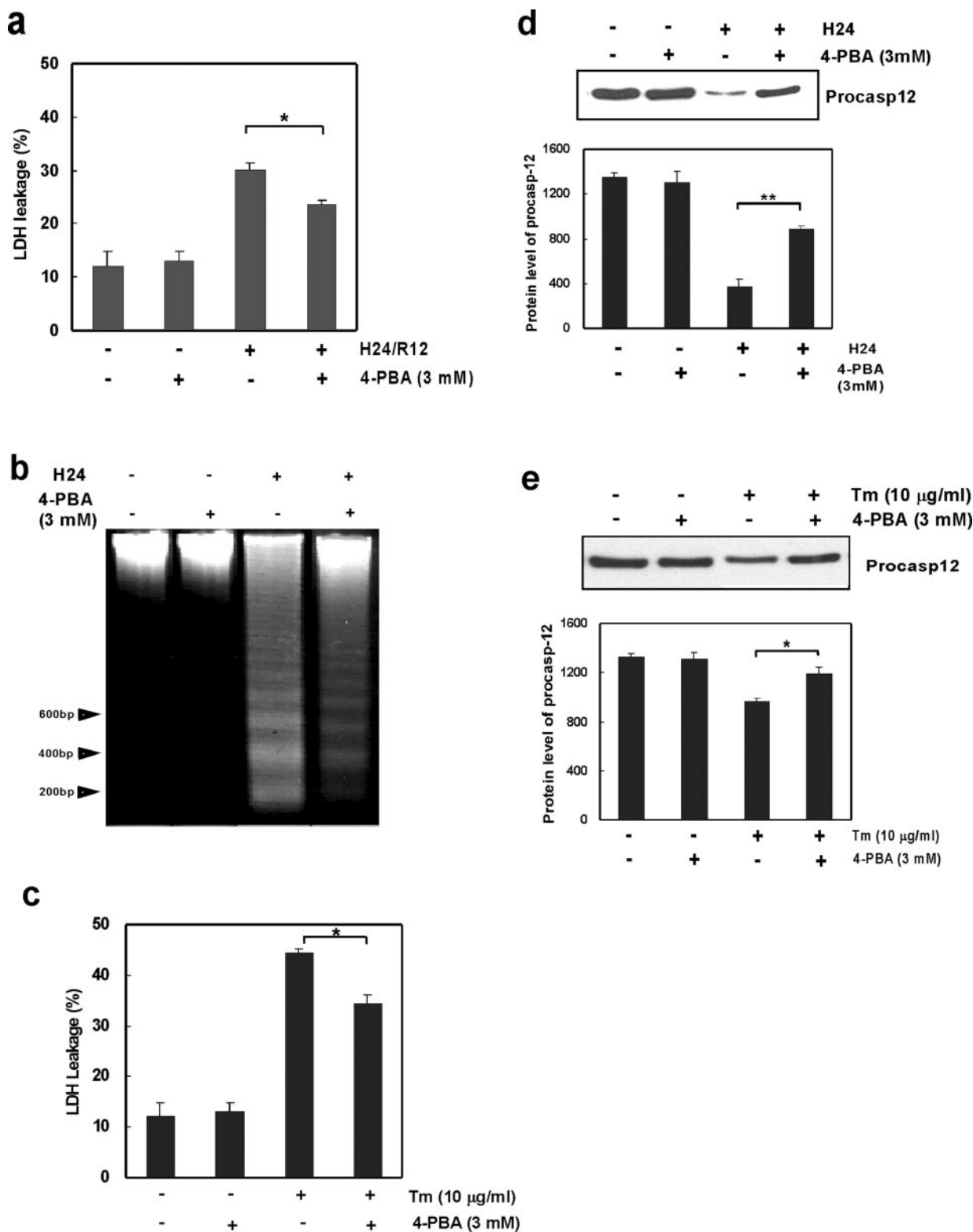


Fig. 4. 4-PBA inhibited cell death in Neuro2a cells. The cells were pretreated with 4-PBA (3 mM) for 1 h and then further treated with the indicated conditions. **a**, 4-PBA reduced the release of LDH under condition of 24 h of hypoxia followed by 12 h of reoxygenation (H24/R12). Data represent the average of four independent experiments for each sample. *, $p < 0.05$, compared with the H/R group, Fisher's test. **b**, 4-PBA attenuated apoptosis caused by 24 h of hypoxia (H24). The DNA fragmentation was measured to evaluate apoptosis. The data are representative of three independent experiments. **c**, 4-PBA inhibited the cell death caused by treatment with Tm for 48 h. Data represent the average of three independent experiments for each sample. *, $p < 0.05$, compared with the Tm-treated group, Fisher's test. **d**, 4-PBA inhibited the activation of caspase-12 under the condition of 24 h of hypoxia (H24). The protein level of caspase-12 was analyzed by Western blotting. The quantification is expressed as the mean \pm S.E. of three independent experiments. **, $p < 0.01$, compared with the hypoxia-treated group, Fisher's test. **e**, 4-PBA suppressed the activation of caspase-12 caused by Tm treatment for 48 h. Western blotting analysis was performed to detect the protein levels of caspase-12. Data represent the mean \pm S.E. of three independent experiments. *, $p < 0.05$, compared with the Tm-treated group, Fisher's test.

ated by phosphorylation of eIF2 α under conditions of cerebral ischemia (Kumar et al., 2001). Consistent with the results reported in recent years (Althausen et al., 2001; Kumar et al., 2003), we observed a rapid increase in our ischemia model in eIF2 α phosphorylation in the early postischemic brain, demonstrating that suppression of protein synthesis is a common response of cells to severe forms of stress. Elevated phospho-eIF2 α level results in an increase in the bypass scanning translation product CHOP (DeGracia et al., 2002). Transcriptional induction of the CHOP gene is one of main pathways leading to apoptosis (Mori, 2000). Induction of CHOP was observed in rat hippocampus that had been subjected to global cerebral ischemia (Paschen et al., 1998). A recent study has shown that CHOP $-/-$ mice have smaller infarcts than wild-type animals subjected to bilateral carotid artery occlusion (Tajiri et al., 2004). These findings suggest that CHOP might be an important target for therapeutic intervention to prevent the progression of ischemic brain injury. In the present study, we found that CHOP was remarkably induced 12 h after hypoxia-ischemia, which is similar to findings in other ischemia models. Treatment with 4-PBA markedly inhibited the induction of CHOP in the ischemic

region. Thus, suppression of CHOP induction by 4-PBA treatment could result in increased cell survival in the ischemic brain. Moreover, phospho-eIF2 α , an inducer of CHOP, was suppressed at a later point (6 to 12 h after hypoxia-ischemia). It seems reasonable to assume that 4-PBA inhibits the induction of CHOP partly because of suppression of eIF2 α phosphorylation.

ER stress-induced cell death has been shown to involve the activation of caspase-12, which subsequently activates executor caspases such as caspase-9 and caspase-3 (Morishima et al., 2002). Caspase-12 is localized on the cytosolic side of the ER membrane; however, unlike other caspases, caspase-12 is specific to insults that elicit ER stress and is not proteolytically activated by other death stimuli (Nakagawa et al., 2000). Mice that are deficient in caspase-12 are more resistant to ER stress-induced apoptosis (Nakagawa et al., 2000). Previous studies have shown that caspase-12 is activated after permanent and transient middle cerebral artery occlusion, and many caspase-12-positive cells exhibited DNA fragmentation (Mouw et al., 2003; Shibata et al., 2003), suggesting that the processing and activation of caspase-12 is involved in ischemia-induced apoptosis. We found that

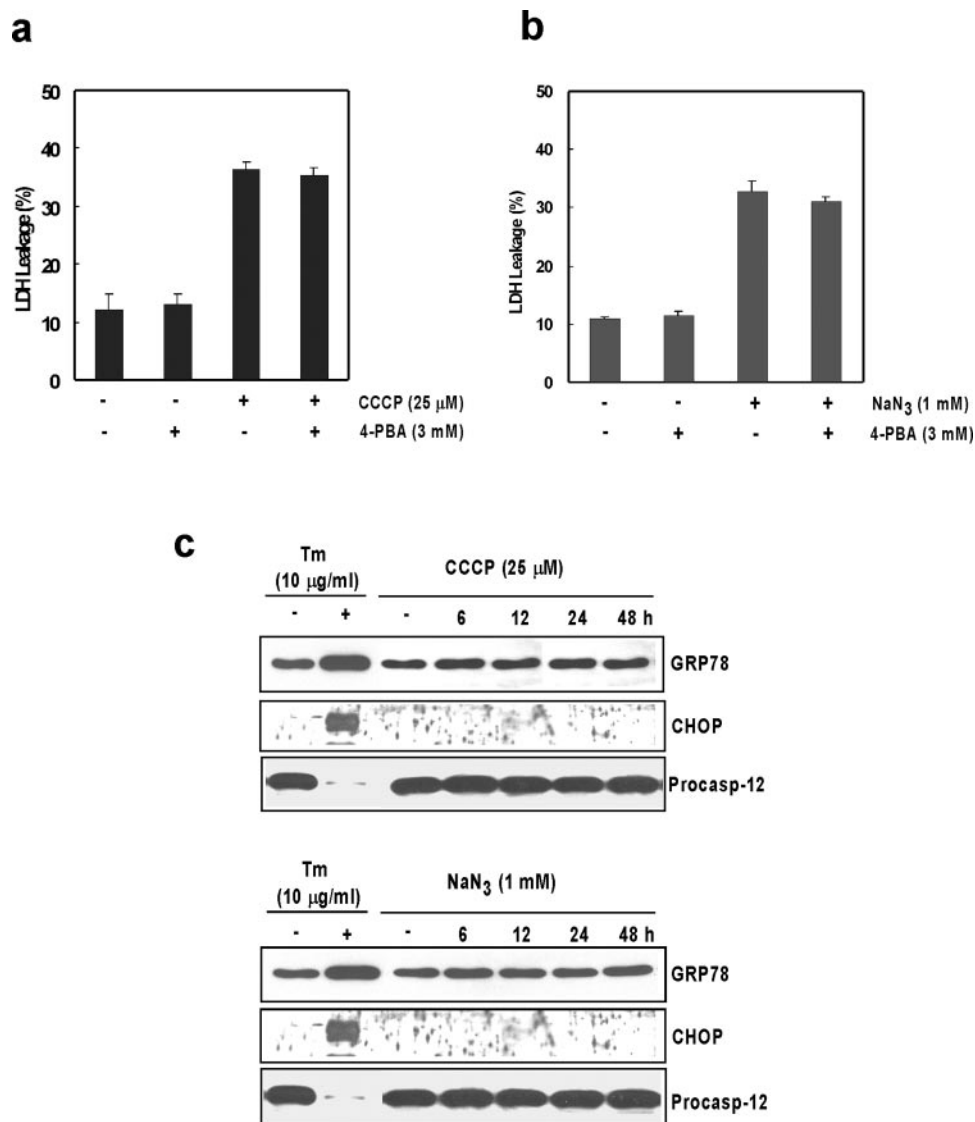


Fig. 5. 4-PBA afforded no protection against mitochondrial damage. **a**, 4-PBA did not protect against the cell death caused by CCCP or NaN₃ treatment. Neuro2a cells were pretreated with 4-PBA (3 mM) for 1 h and then further treated with CCCP (25 μ M) and NaN₃ (1 mM) for 48 h. The cell death was evaluated with use of the LDH assay. Data represent the average of three independent experiments with each sample. **b**, CCCP and NaN₃ could not induce the ER stress response. Neuro2a cells were treated with CCCP or NaN₃ at the indicated time points and the changes of glucose-regulated protein 78 (GRP78), CHOP, and caspase-12 were determined by Western blotting. Data are representative of two independent experiments.

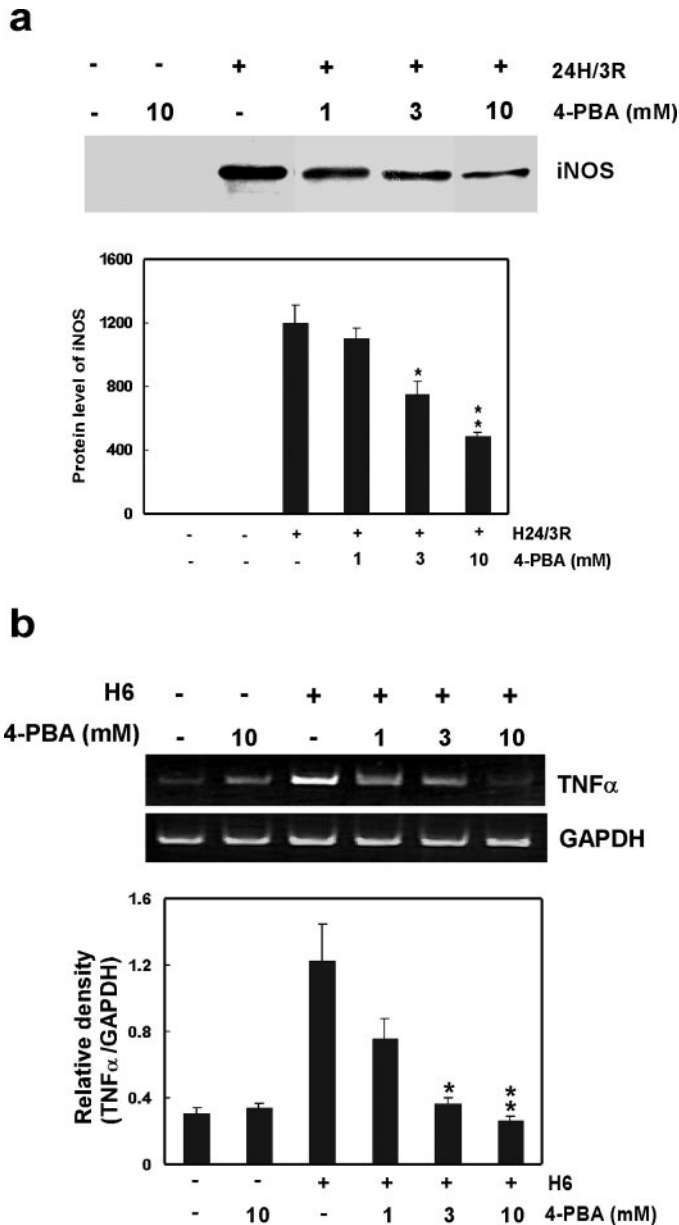


Fig. 6. 4-PBA inhibited inflammatory reaction in primary cultured glial cells. The cells were pretreated with 4-PBA at the indicated doses for 1 h and then further treated with hypoxia/reoxygenation. **a**, 4-PBA diminished the induction of iNOS under conditions of 24 h of hypoxia followed by 3 h of reoxygenation (H24/3R). The protein level of iNOS was detected by Western blotting. **b**, 4-PBA decreased TNF- α mRNA expression under hypoxia for 6 h (H6) as determined by RT-PCR. Quantification presents the mean \pm S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$, compared with the hypoxia-treated group, Dunnett's test.

caspase-12 was activated 24 h after hypoxia-ischemia and that 4-PBA remarkably suppressed the activation. The finding indicates that 4-PBA inhibits caspase-12-dependent apoptotic pathway as well as the apoptotic pathway involved in CHOP. Recent studies have shown that caspase-3 activation is involved in caspase-12-mediated apoptotic cascade in a cytochrome-*c*-independent manner (Morishima et al., 2002; Hitomi et al., 2004). Thus, it is possible that caspase-3 activation is inhibited by suppression of caspase-12. Activated caspase-3 is directly responsible for DNA fragmentation (Liu et al., 1997). 4-PBA treatment significantly inhibited apoptosis under in vivo and in vitro ischemic conditions, as indi-

cated by the results of TUNEL assays and by DNA fragmentation. Thus, 4-PBA might inhibit caspase-3 activation by suppressing ER stress-mediated apoptotic signaling and therefore reduce the extent of apoptosis.

According to these findings in our study, we speculate that the protective effects of 4-PBA on cerebral ischemic injury may be to inhibit ER stress and the subsequent apoptotic signaling pathway. Furthermore, 4-PBA inhibited neuronal cell death caused by hypoxia, an in vitro model of cerebral ischemia, or the ER stress-inducer Tm. The protective effect was associated with suppressing the activation of caspase-12. Moreover, 4-PBA inhibited ER damage but was ineffective against mitochondria-derived cell death in Neuro2a cells. These findings collectively indicate that the ER may be a primary target of 4-PBA.

We found that 4-PBA seems to widely suppress ER stress-mediated apoptotic signaling under conditions of ischemia. 4-PBA has been demonstrated to act as a chemical chaperone to reduce overload of ER (Burrows et al., 2000; Rubenstein and Zeitlin, 2000). Therefore, the chaperone-like activity of 4-PBA may function in the pathogenesis of cerebral ischemia, which has been suggested to cause severe ER dysfunction. Considering the findings of the present study, it is reasonable to conclude that the protective effect of 4-PBA on ER stress-mediated injury may involve the activity of chemical chaperone. Furthermore, the use of a chemical chaperone as a strategy for the treatment of neurodegenerative disease may be attractive because of its applicability to a wide range of pathological conditions, such as Alzheimer's disease, Parkinson's disease, and Poly Q disease, which are believed to be caused by an accumulation of unfolded or misfolded proteins in the ER.

Focal cerebral ischemia elicits a strong inflammatory response. Elevation of TNF- α and induction of iNOS have been demonstrated in various experimental models of brain injury. Proinflammatory cytokines are mainly produced by astrocytes and microglia in inflammatory conditions of the central nervous system (Merrill and Jonakait, 1995). Moreover, reactive astrocytes induce iNOS and produce NO after transient global ischemia (Endoh et al., 1994). iNOS is not normally present in cells, but its expression is induced in a pathological state, typically in association with inflammation. NO, which is produced by iNOS, is believed to be cytotoxic to neighboring neurons and oligodendrocytes (del Zoppo et al., 2000). To maintain cellular function and protect neuronal cells against neurotoxicity, it is thus important to inhibit the induction of TNF- α and iNOS in glial cells under pathological conditions. In the present study, 4-PBA inhibited the induction of iNOS and expression of TNF- α in primary cultured glial cells under conditions of hypoxia/reoxygenation, suggesting that 4-PBA suppresses ischemia-related inflammatory reaction. Therefore, the reduction of ischemic injury by 4-PBA may be partly caused by inhibition of TNF- α and iNOS induction. Furthermore, as a result of inhibition of iNOS induction, 4-PBA may contribute to the decrease in NO production and subsequent neurotoxicity to neighboring neurons.

Taken together, we found that 4-PBA protects against ischemic brain injury, the mechanism of which involves inhibition of ER stress-mediated apoptosis and inflammation. We presented evidence that targeting the ER may provide a therapeutic approach for blocking apoptotic process induced

by cerebral ischemia. Finally, we proposed that the therapeutic potential of 4-PBA as a chemical chaperone may extend to other ER stress-related neurodegenerative disease.

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Address correspondence to: Dr. Yasuyuki Nomura, Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan. E-mail: nomura@pharm.hokudai.ac.jp