

**Edaravone protects against hypoxia/ischemia-induced
endoplasmic reticulum dysfunction**

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Abbreviation: edaravone, 3-methyl-1-phenyl-pyrazolin-5-one; NAC, N-acetyl-L-cysteine; ebselen, 2-phenyl-1,2-benzisoselenazol-3(2H)-one; ER, endoplasmic reticulum; UPR, unfolded protein response; TTC, 2, 3, 5-triphenyltetrazolium chloride; RT-PCR, reverse transcription-polymerase chain reaction; GRP78, glucose regulated protein 78; eIF2 α , eukaryotic initiation factor α ; CHOP, C/EBP homologous protein; ROS, reactive oxygen species; CM-H₂DCFDA, 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate

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Abstract

Endoplasmic reticulum (ER) stress-induced cell death plays an important role in cerebral ischemia. In the present study, we investigated whether edaravone (3-methyl-1-phenyl-pyrazolin-5-one), a free radical scavenger, can protect against ER damage induced by cerebral ischemia. In a mouse model of hypoxia/ischemia, treatment with edaravone reduced edema-corrected infarction volume, attenuated hemispheric swelling and improved neurological status. Moreover, edaravone suppressed ER stress-mediated apoptosis by inhibiting eIF-2 α phosphorylation, CHOP induction and caspase-12 activation. In mouse primary cultured glial cells, edaravone attenuated ER stress as evidenced by inhibition of the induction of GRP78 and CHOP and XBP-1 splicing under treatment with tunicamycin (Tm) which induces ER stress. Tm did not induce the production of reactive oxygen species (ROS) in primary cultured glial cells. In addition, the free radical scavengers NAC (N-acetyl-L-cysteine) and ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one) did not affect ER stress response caused by Tm. These results demonstrated a novel action of edaravone which can protect against ER dysfunction in cerebral ischemia.

Introduction

The endoplasmic reticulum (ER) regulates protein synthesis, protein folding and trafficking, cellular responses to stress and intracellular calcium (Ca^{2+}) levels (Rao et al., 2004). The conditions which impair the function of the ER, designated 'ER stress', can lead to an accumulation of unfolded or malformed proteins in the ER lumen (Kaufman, 1999). In response to ER stress, cells have developed a self-protective signal transduction pathway termed the unfolded protein response (UPR), which includes the induction of molecular chaperones in the ER, translational attenuation and ER-associated degradation (ERAD) (Cudna and Dickson, 2002). However, if the damage is too severe to repair, the UPR ultimately initiates an apoptotic pathway (Cudna and Dickson, 2002; Oyadomari et al., 2002; Breckenridge et al., 2003). ER stress-induced cell death has been shown to involve the activation of caspase-12 (Nakagawa et al., 2000; Nakagawa and Yuan, 2000). Another component of the ER stress-mediated apoptotic pathway is C/EBP homologous protein (CHOP), also known as growth arrest- and DNA damage-inducible gene 153 (GADD153) (Wang et al., 1996).

Cerebral ischemia is a pathophysiological ER stressor (Paschen and Doutheil,

1999; Kumar et al., 2003). Several studies have shown that ischemic injury causes a severe impairment of ER function, which in turn triggers shutdown of protein translation and apoptosis (Tajiri et al., 2004; Kumar et al., 2003), suggesting that the ER plays an important role in cerebral ischemia. Thus, reducing ER stress may provide a therapeutic way to block the pathological process induced by cerebral ischemia.

Edaravone (3-Methyl-1-phenyl-2-pyrazolin-5-one) is a potent and novel scavenger of free radicals inhibiting not only hydroxyl radicals but also free radical-mediated lipid peroxidative damage (Watanabe et al., 1997). It has been reported that edaravone changes into 2-oxo-3-(phenylbudrazono)-butanoic acid after reacting with peroxy radicals in vitro (Yamamoto et al., 1996). Edaravone has protective effects on cerebral edema and tissue injury after ischemia-reperfusion in rats (Abe et al., 1998) and patients with acute cerebral ischemia (Houkin et al., 1998; Mitsumori et al., 1998). Additionally, edaravone protects against mitochondrial injury in hepatic and myocardial ischemia/reperfusion (Okatani et al., 2003; Rajesh et al., 2003), and inhibits inflammatory reaction (Kono et al., 2003). These effects were predominantly thought to be a result of the protective action of edaravone against lipid

peroxidation.

In the present study, we focused on the possible protective effect of edaravone against ER dysfunction. We obtained evidence that edaravone significantly inhibited ER stress-mediated apoptotic signals induced by hypoxia/ischemia and attenuated ER stress *in vivo* and *in vitro*.

Materials and Methods

Edaravone was a gift from Mitsubishi Chemical Industries, Japan. The drug was dissolved in NaOH (1 M), and adjusted to pH 7.4 according to the manufacturer's instructions. Tunicamycin was purchased from Wako Chemical Industries, Japan. NAC (N-acetyl-L-cysteine) and ebselen were purchased from Sigma.

Induction of hypoxia/ischemia

Male C57BL mice (6 weeks old) 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. The water bath temperature was maintained at 35.5°C, which in previous experiments was shown to maintain the animals' core body temperature at 37.5-37.7°C throughout the hypoxic interval (Vannucci et al., 2001). Animals were allowed to recover in room air for 30 min and then returned to their cages with free access to food and water. The rectal temperature was measured at several points during the initial hours after hypoxia/ischemia, and the longer intervals until they are killed. 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 NIH Guide for Care and Use of Laboratory animals and were approved by the animal care and use committee of Hokkaido University.

Determination of infarct volume and hemispheric swelling

Animals were sacrificed 3 days after hypoxia/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, Japan) in normal saline at room temperature in a dark place for 30 min and then washed in 5% PBS (phosphate buffer saline) twice. The slices were fixed in 10% formalin for photography and the infarction area was measured. An image analysis system (NIH 1.61 software) was used to determine the infarcted 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, the infarct volume in each mouse was corrected as described (Aspey et al., 1998; Swanson et al., 1990) 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 hypoxia/ischemia were assessed and scored as described (Huang et al., 1994) as follows: 0, no observable neurological deficit (normal); 1, failure to extend left forepaw on the lifting of the body by the tail (mild); 2, circling to the contralateral side (moderate); 3, leaning to the contralateral side at rest or no spontaneous motor

activity (severe). Animals not showing neurological deficits were excluded from the study.

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 confluency in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal calf serum, 100 units/ml of penicillin G, and 100 µg/ml of streptomycin (Invitrogen). All cultured cells were maintained at 37°C in 5% CO₂-95% air.

RT-PCR

Total RNA was isolated from mouse brain or cultured cells using TRI REAGENT (Sigma, St. Louis, MO). RT-PCR was performed as described previously (Hosoi et al., 2002a). The following primers were used: CHOP forward, 5'-ccc tgc ctt tca cct tgg-3'; CHOP reverse, 3'-ccg ctc gtt ctc ctg ctc-5'; XBP-1 forward, 5'-cct tgt ggt tga gaa cca gg-3'; XBP-1 reverse, 3'-cta gag gct tgg tgt ata c-5'; GAPDH forward, 5'-gat gga gca tca tac tga tcc-3'; GAPDH

reverse, 3'-aaa ccc atc acc atc ttc cag-5'.

Western blotting

Primary antibodies used for Western blotting analysis were as follows: anti-phospho-eIF2 α (Ser51) polyclonal antibody (Cell Signaling Technology, Tokyo, Japan), 1:1000; anti-GADD153-polyclonal antibody (Santa Cruz Biotechnology, California, U.S.A), 1:1000; anti-Caspase-12-polyclonal antibody (ProSci incorporated, Poway, CA), 1:1000; anti-KDEL monoclonal antibody (StressGen Biotechnologies Corp), 1:1000. Western blotting analysis was performed as described previously (Hosoi et al., 2002b).

Measurement of intracellular reactive oxygen species (ROS)

A cell membrane-permeable and oxidant-sensitive fluorescent dye 5-(6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) was used to measure ROS. Tunicamycin (10 μ M)-treated or untreated glial cells were washed with pre-warmed (37 °C) phosphate-buffered saline once and then incubated in culture medium, together with CM-H₂DCFDA (10 μ M). After 30 min of incubation, the cells were washed with pre-warmed medium

twice before being distributed into a 96-well plate in 200 μ l. Fluorescence intensity in each well was measured on a fluorescent microplate reader (Tecan, Durham, NC) at excitation and emission wavelengths of 485 nm and 538 nm, respectively. Finally, the number of cells in each well was counted with Erma Cell Counting Chamber (Tokyo, Japan). The data were expressed as fluorescent intensity per 2×10^4 cells.

Results

Edaravone protects against cerebral injury caused by hypoxia/ischemia

Treatment with edaravone (1~10 mg/kg, i.p., once a day for three days) immediately after hypoxia/ischemia dose-dependently decreased infarct volume on the third day following hypoxia/ischemia. Remarkable protection was achieved with 3 and 10 mg/kg of edaravone by 30% and 60% reduction in edema-corrected infarct volume, respectively (Fig. 1b). However, no significant reduction in infarct volume was observed in mice dosed with 1 mg/kg (Fig. 1b). Hemispheric swelling was also significantly decreased with a dose of 3 (40% reduction) and 10 mg/kg (60% reduction) under the same treatment conditions (Fig. 1c).

In addition, we examined the effect of edaravone on the rectal temperature of mice in the model of hypoxia/ischemia. No significant change in body temperature was observed in saline- and edaravone (10 mg/kg)-treated groups during the experiment (Fig. 2). These results indicate that the protective effect of edaravone on hypoxia/ischemia is not caused by hypothermia.

Edaravone improves neurological function

Treatment with edaravone (10 mg/kg) significantly reduced neurological deficits and provided functional recovery from hypoxia/ischemia when examined at 24, 48 and 72 h, compared with the saline group (Fig. 3).

Edaravone represses ER stress-mediated apoptotic signals

A key feature of ER stress induced by cerebral ischemia is the blocking of translation at the initiation step, as indicated by increased phosphorylation of eIF2 α (Mengesdorf et al., 2002; DeGracia et al., 2002). Therefore, we first examined whether edaravone affects the phosphorylation of eIF2 α which has also been demonstrated to be an inducer of the transcription factor CHOP (Oyadomari and Mori, 2003). The level of phospho-eIF2 α in injured cortex

was markedly increased following hypoxia/ischemia and detectable until 12 h, whereas the administration of edaravone at 10 mg/kg noticeably reduced the levels from 6 to 12 h (approx 35% to 50% reduction, compared with the saline-treated group) (Fig. 4a). No change in the level of phosphor-eIF2 α was observed in the brains of animals only injected with edaravone (Fig. 4d). In the previous study, we have observed that CHOP was significantly up-regulated in the hypoxia/ischemic cortex, hippocampus and striatum, and inductions of CHOP in the three regions show similar extent (unpublished observation). Quantification revealed a remarkable increase in the protein level of CHOP in the injured cortex 12 h after hypoxia/ischemia, compared with that in sham-operated animals. However, injection of edaravone remarkably down-regulated CHOP protein level (approx 90% reduction), compared with that in the saline-treated group (Fig. 4b). The protein level of CHOP was not affected by treatment with edaravone in normal brains (Fig. 4d).

Furthermore, Western blotting showed that hypoxia/ischemia led to the activation of caspase-12 as evidenced by a decrease in the level of the pro-caspase-12. The activation of caspase-12 was largely restored by edaravone (almost 30% restoration, compared with the saline-treated group) (Fig.

4c). No difference in the activation of caspase-12 was observed between the saline-treated and edaravone-treated groups in normal brains (Fig. 4d).

Edaravone attenuates ER stress in primary cultured glial cells

To elucidate the mechanism underlying the protection against ER dysfunction, first we evaluated the effect of edaravone on ER stress in primary cultured glial cells through treatment with ER stressor, tunicamycin (Tm, an inhibitor of N-glycosylation). Western blotting analysis showed that treatment with Tm remarkably up-regulated the expression of GRP78 and CHOP, markers of ER stress response, whereas edaravone (1~10 μ M) dose-dependently inhibited GRP78 and CHOP induction (Fig. 5a and b).

XBP-1 mRNA has been shown to be spliced upon ER stress (Yoshida et al., 2001; Calton et al., 2002). We observed the splicing of XBP-1 mRNA 6 h after treatment with Tm (1 μ g/ml). Edaravone inhibited or even abrogated XBP-1 mRNA splicing (Fig. 5c), as reflected by a decrease in the ratio of spliced to unspliced forms of XBP-1.

In contrast to edaravone, NAC and ebselen, both of which have been demonstrated to be free radical scavengers, did not affect the induction of

GRP78 and CHOP under treatment with Tm in primary cultured glial cells (Fig. 6a and b). Thus, the effect of inhibiting ER stress may be specific to edaravone.

Tm did not induce the increase in ROS levels in primary cultured glial cells

To determine whether the protective effect of edaravone on the ER results from resistance to ROS, which has been suggested to damage the ER and lead to cell death (Hayashi et al., 2003), we assessed the levels of ROS under treatment with Tm in primary cultured glial cells. Tm did not generate ROS in glial cells (Fig. 6c), suggesting that the inhibition of ER dysfunction by edaravone under conditions of Tm treatment may not be based on the radical scavenging action.

Discussion

In the present study, we used a murine model of hypoxia/ischemia. Thirty minutes of hypoxia/ischemia produced markedly damage to the ipsilateral cortex, hippocampus and striatum, as determined by TTC-staining. On the other hand, we showed that peripheral administration of edaravone protected brain from hypoxia/ischemic injury as evidenced by reduction in infarct volume and

hemispheric swelling. Improvement of neurological score was also observed in the same animals that showed reduction in infarct volume and hemispheric swelling. Simultaneously, we found that edaravone can effectively suppress ER dysfunction *in vivo* and *in vitro*. Moreover, the present findings indicate that the protective effect of edaravone on ischemic injury may be mediated in part by restoration of ER dysfunction.

In cerebral ischemia, excessive generation of oxygen free radicals followed by lipid peroxidation is thought to play an important role in the secondary injury (Seki et al., 1998). Exogenous radical scavengers have been demonstrated to suppress oxidative stress and thus prevent ischemic injury (Crompton, 1999; Zoratti and Szabo, 1995). Edaravone has been shown to exert a protective effect against cerebral ischemic injury by inhibiting OH⁻ and iron-dependent lipid peroxidations, both of which are essential in mediating ischemic cell damage (Watanabe et al., 1994). On the other hand, ample evidence suggested that ER damage is involved in neuronal cell death induced by cerebral ischemia (Tajiri et al., 2004; Kumar et al., 2003). Thus, in the present study, we investigated the effect of edaravone on ER dysfunction under pathological conditions.

In mice subjected to 30 min hypoxia/ischemia followed by a given period of recovery, we observed an increase in the level of the transcription factor CHOP and activation of caspase-12 in the ischemic cortex. The results indicate that hypoxia/ischemia caused severe ER damage and triggered ER stress-associated apoptosis. On the other hand, treatment with edaravone significantly inhibited caspase-12 activation and CHOP induction. In addition, we observed suppression of phospho-eIF2 α , upstream of CHOP. Therefore, the protective effects of edaravone in hypoxia/ischemic injury may be partly due to inhibition of ER stress and subsequent apoptotic signaling pathway.

We found that edaravone also inhibited ER stress even if the condition was non-ischemic. Treatment of cells with Tm, an inducer of ER stress, caused ER dysfunction as indicated by the induction of representative genes related to ER stress. However, Tm did not cause the production of ROS in primary cultured glial cells. Thus it was suggested that ER stress does not induce ROS production in glial cells. In addition, other free radical scavengers, such as NAC and ebselen, provided no protection against ER dysfunction. The results indicated that the property of inhibiting ER stress is specific to edaravone. Thus, the protective effect of edaravone against hypoxia/ischemia may involve a

novel pathway which directly attenuates ER stress in addition to suppressing ROS production. Considering the findings of the present study, we speculate that edaravone has multiple effects, although the mechanism of its actions needs to be elucidated further.

In summary, the present results suggest that edaravone provided protection against ER dysfunction induced by hypoxia/ischemia and the protective effect may be mediated by an inhibition of ER stress as well as the radical scavenging action. Therefore, the restoration of ER dysfunction by edaravone may be a novel neuroprotective mechanism. Further, on the basis of its protection against ER damage, edaravone may also be useful for treating other neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease.

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Figure legends

Fig. 1. Treatment with edaravone attenuated hypoxia/ischemia-induced brain injury. Mice were injected intraperitoneally with saline or edaravone immediately after hypoxia/ischemia and sacrificed 3 days later. Brain coronal sections (2 mm) were stained with 2% TTC. (a) Representative data from ischemic brain. Treatment with edaravone dose-dependently reduced edema-corrected infarction volume (b) and hemispheric swelling (c). Data are means \pm S.E. from 10 mice in each group. *, $p < 0.05$; **, $p < 0.01$ compared with the saline-injected group, one-way ANOVA followed by Dunnett's test.

Fig. 2. Edaravone did not affect the body temperature of animals in hypoxia/ischemia. Saline or edaravone (10 mg/kg) was administered (i.p.) immediately after hypoxia/ischemia. The rectal temperature was measured at the indicated time points. Data are means \pm S.E. from 5 mice in each group.

Fig. 3. Treatment with edaravone improved neurological function. Saline or edaravone was administered (i.p.) immediately after hypoxia/ischemia. The

neurological deficits were assessed at 1, 2 and 3 days of recovery. Data are means \pm S.E. from 10 mice in each group, respectively. *, $p < 0.05$ compared with the neurological score of the saline-treated group on the corresponding day, one-way ANOVA followed by Dunnett's test.

Fig. 4. Treatment with edaravone suppressed the ER stress-mediated apoptosis. Mice were subjected to 30 min hypoxia/ischemia followed by recovery until sacrifice. Saline or edaravone (10 mg/kg) was injected immediately after hypoxia/ischemia. The tissue samples were from the cerebral cortex. (a) Administration of edaravone decreased the phosphorylation of eIF2 α . Phospho-eIF2 α was examined at the indicated time points by Western blotting. (b) The change in the CHOP protein level determined at the indicated time points after hypoxia/ischemia by Western blotting. (c) Treatment with edaravone reduced the activation of caspase-12. The upper panel is a representative Western blotting of procaspase-12. The lower panel shows the quantification of procaspase-12 levels. The tissue samples were from 24 h after ischemia. (d) Western blotting analysis of phospho-eIF2 α , CHOP and caspase-12 in extracts from the right hemisphere in

normal animals treated with saline or edaravone (10 mg/kg). The data are expressed as the mean \pm S.E. from 6 mice in each group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the saline-treated group, Fisher test.

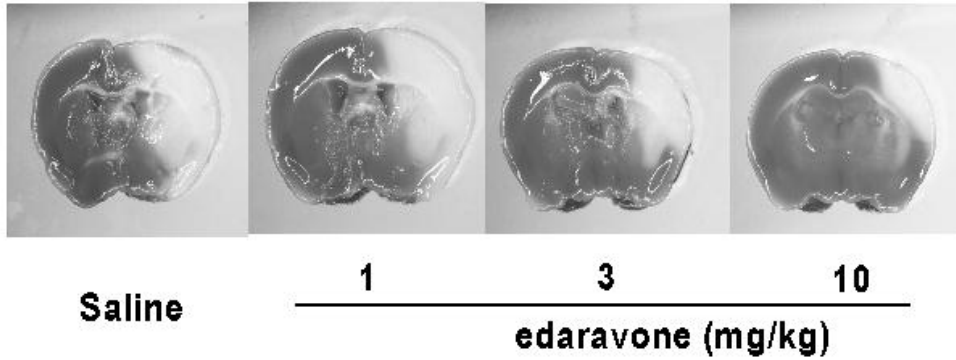
Fig. 5. Edaravone inhibited ER stress response in primary cultured glial cells. Primary cultured glial cells were preincubated with edaravone for 1 h and then treated with Tm for 24 h, and protein levels were assessed by Western blotting. Edaravone inhibited the induction of GRP78 (a) and CHOP (b). The quantification is expressed as the mean \pm S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ compared with Tm-treated group, Fisher test. The spliced form of XBP-1 was analyzed by RT-PCR (c). The data are representative of three independent experiments.

Fig. 6. NAC and ebselen provided no protection against ER stress. Primary cultured glial cells were preincubated with NAC or ebselen at the indicated doses for 1 h and then treated with Tm for 24 h. The protein levels were assessed by Western blotting. NAC (a) and ebselen (b) did not affect the protein levels of GRP78 and CHOP under treatment with Tm. The data are

representative of two independent experiments. (c) Tm did not induce the production of ROS in primary cultured glial cells. Primary cultured glial cells were treated with Tm for the indicated period or H₂O₂ (1 mM) for 48 h, and then incubated with CM-H₂DCA (10 μM) for 30 min. The cells were distributed to a 96-well plate in 200 μl. The fluorescent intensity per 2 × 10⁴ cells was measured. The data represent the mean ± S.E. of three independent experiments.

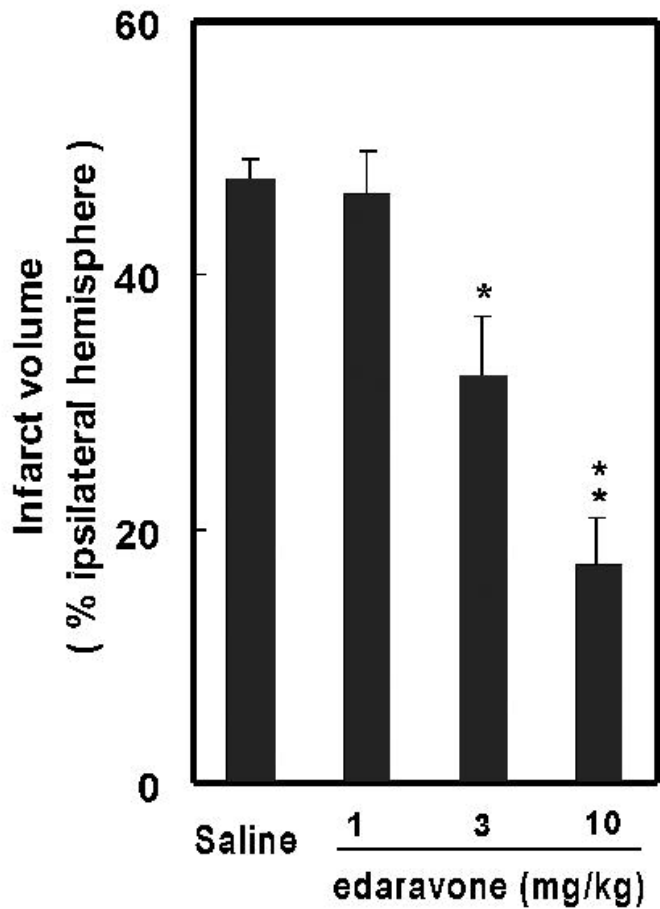
Fig. 1

a



b

Edema-corrected infarction volume



c

Hemispheric swelling

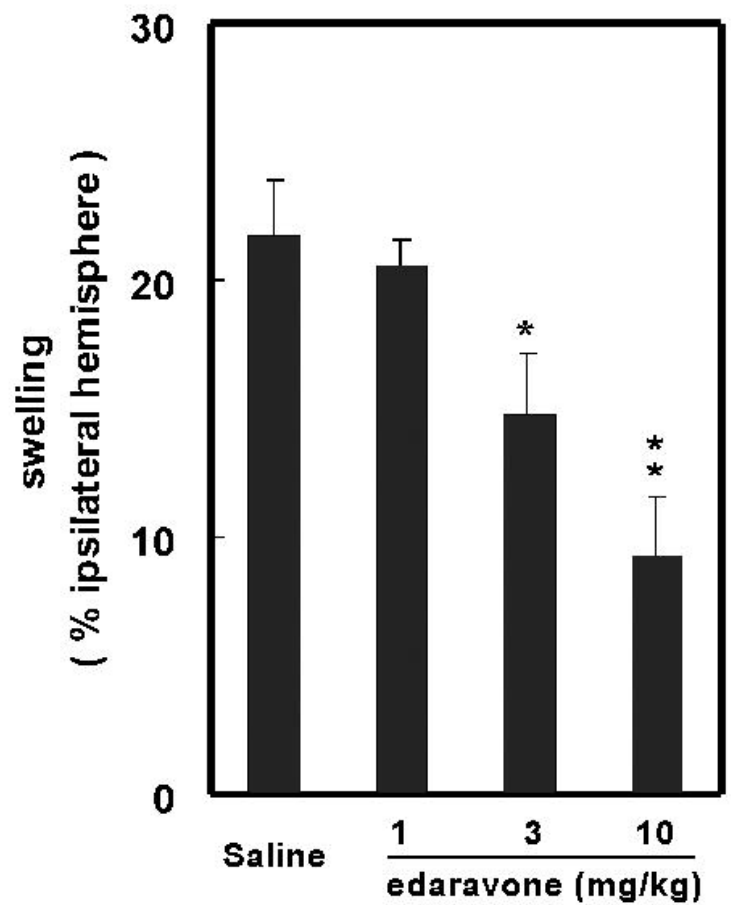


Fig. 2

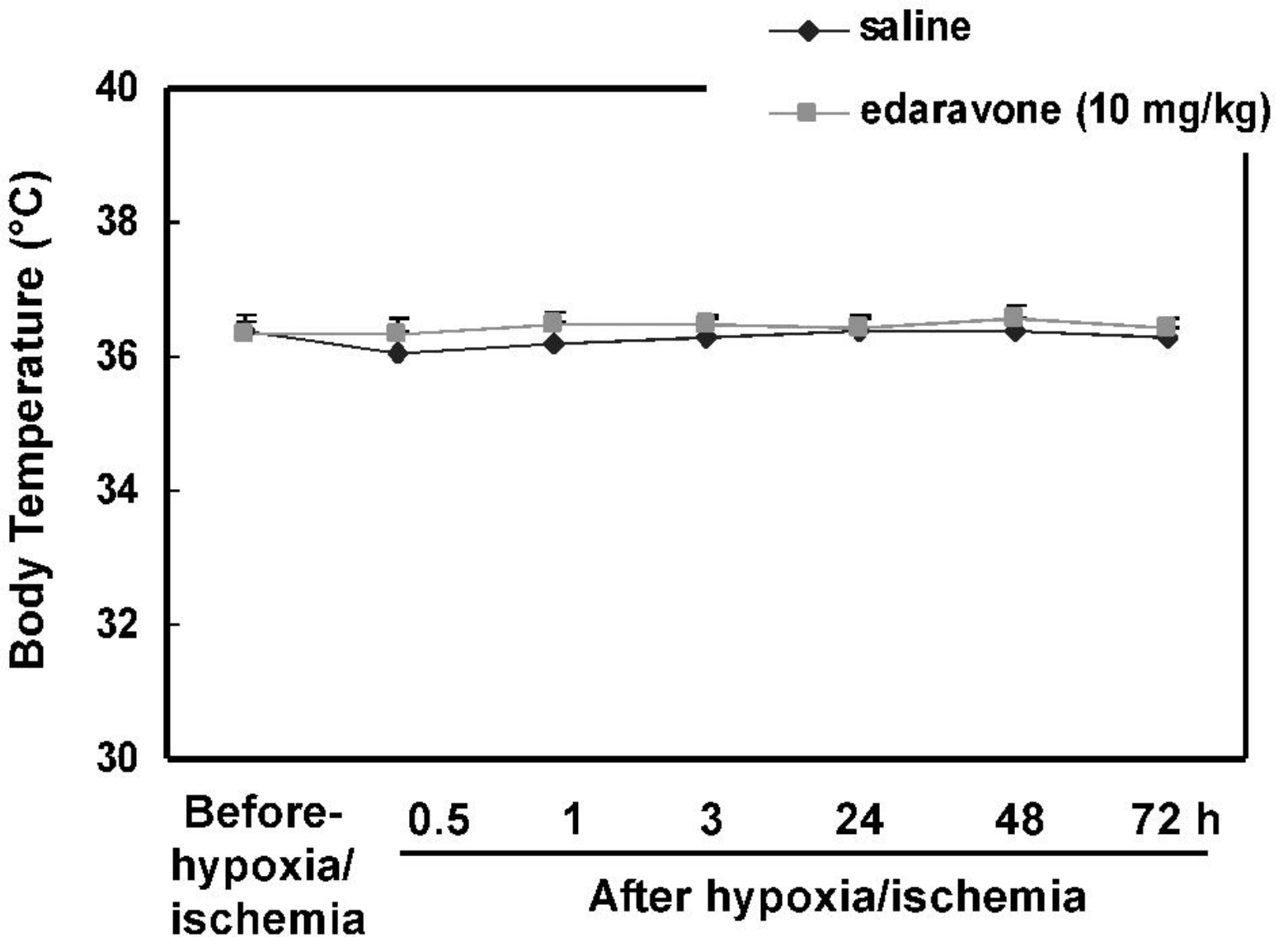


Fig. 3

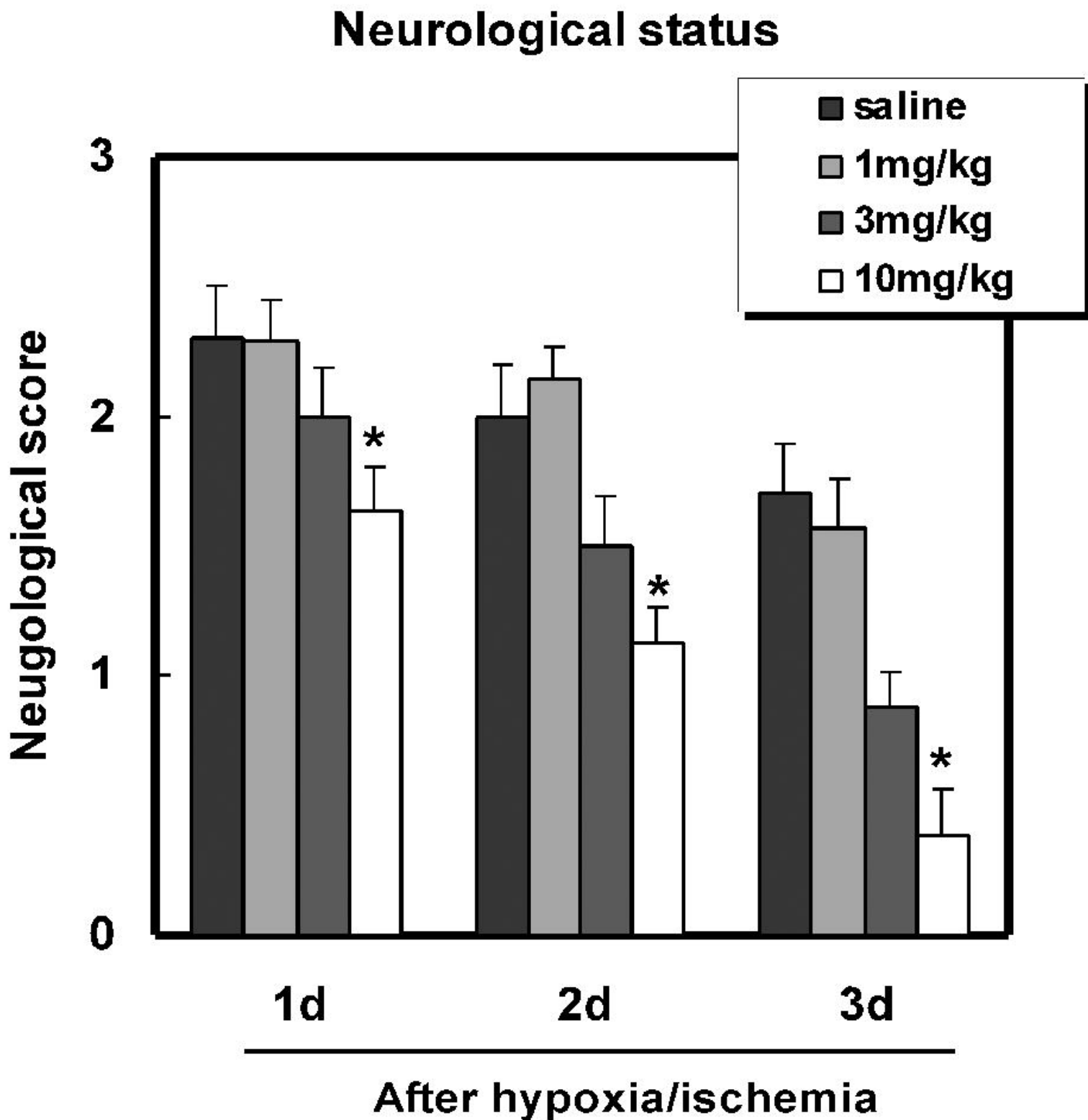


Fig. 4

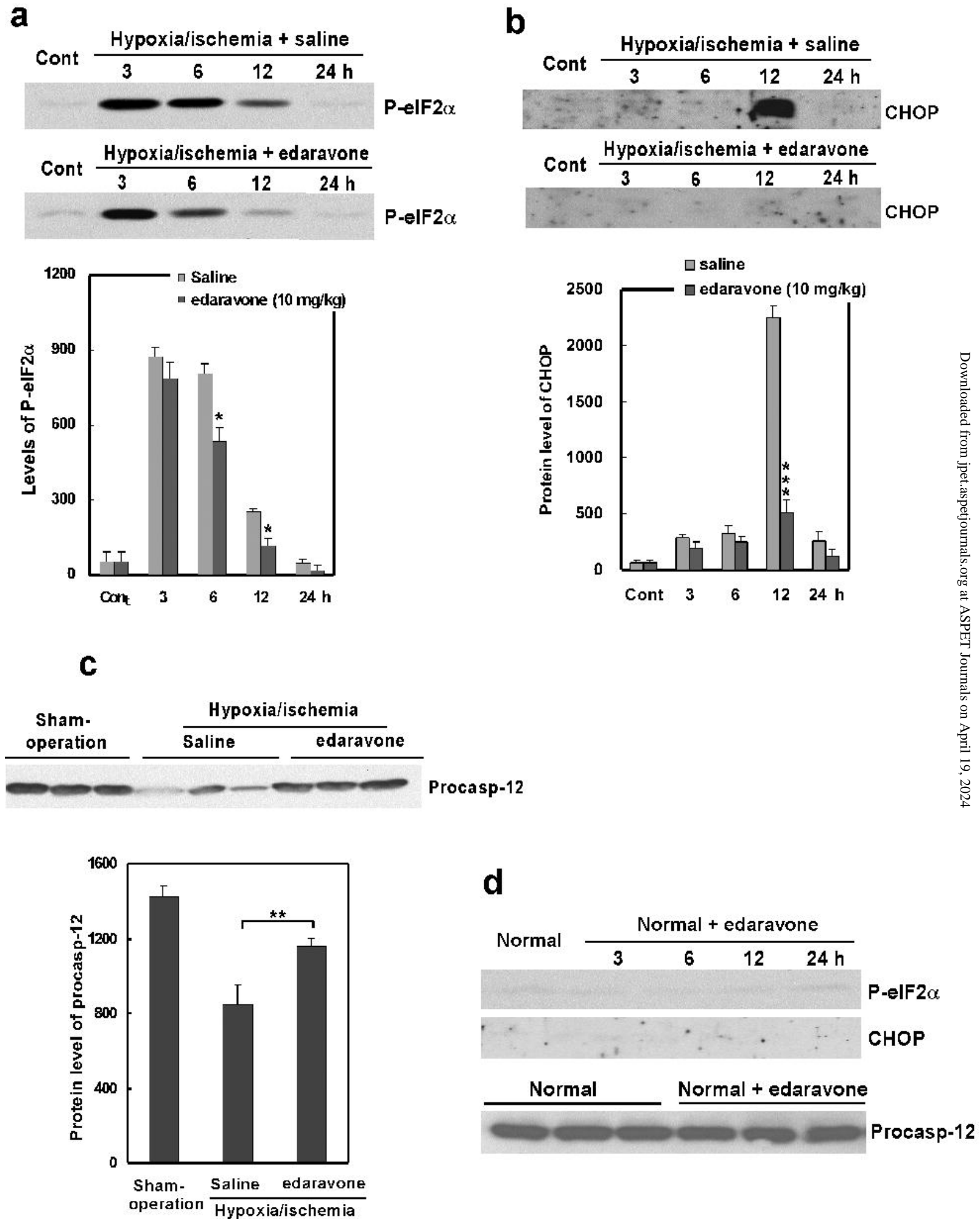


Fig. 5

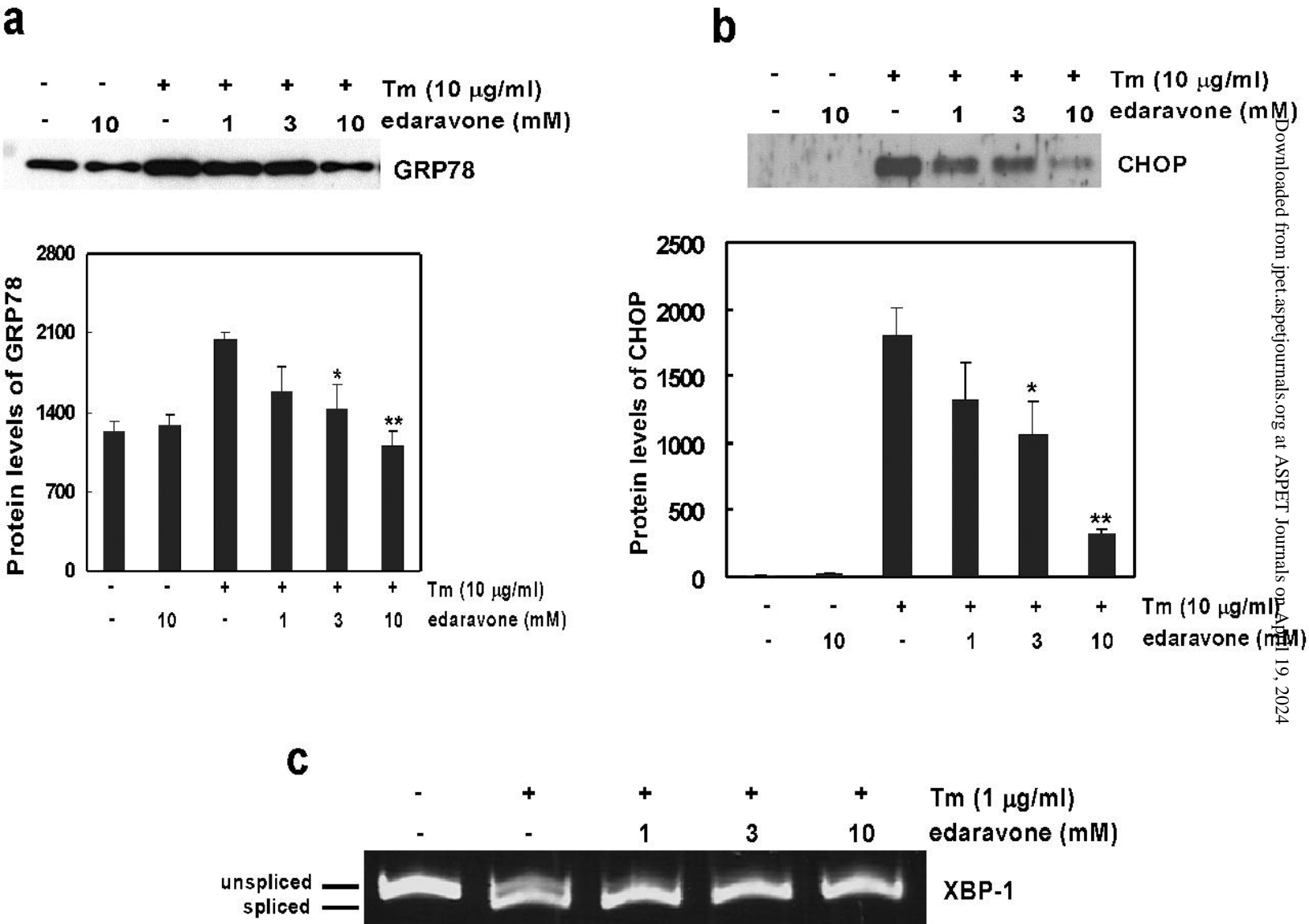
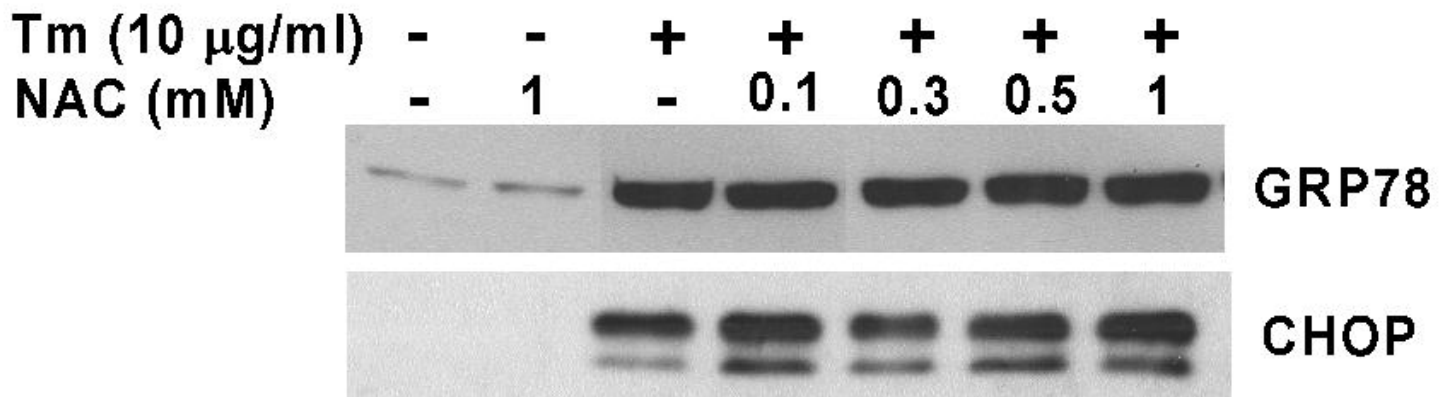
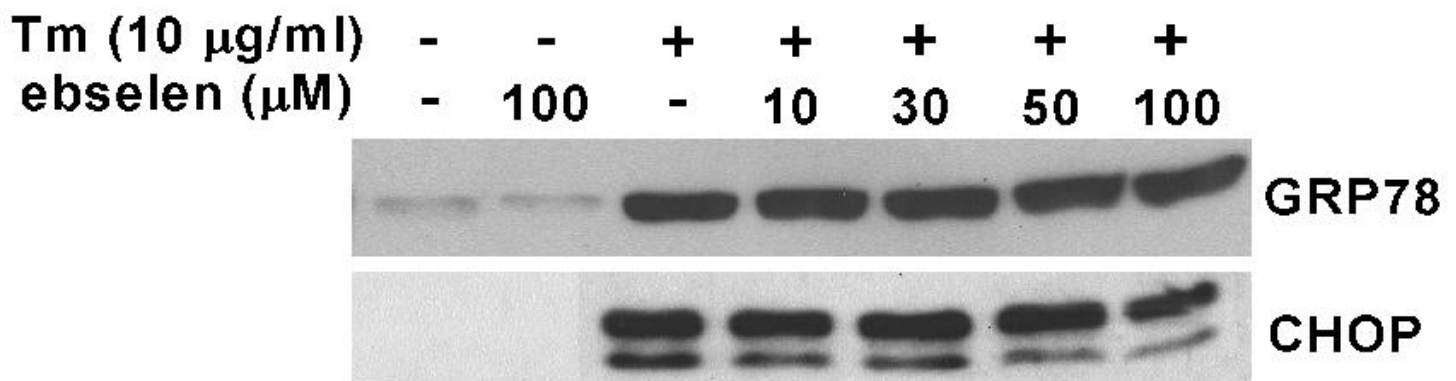


Fig. 6

a



b



c

