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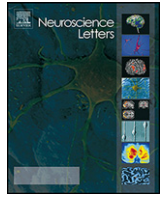
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Post-treatment of a BiP inducer prevents cell death after middle cerebral artery occlusion in mice

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ABSTRACT

We previously reported the effect of a selective inducer of BiP (a BiP inducer X; BIX) after permanent middle cerebral artery occlusion (MCAO) in mice. However, in acute stroke, almost all drugs have been used clinically after the onset of events. We evaluated the effect of post-treatment of BIX after permanent MCAO in mice, and examined its neuroprotective properties in *in vivo* mechanism. BIX (intracerebroventricular injection at 20 μ g) administered either at 5 min or 3 h after occlusion reduced both infarct volume and brain swelling, but at 6 h after occlusion there was no reduction. BIX protected against the decrease in a dose-dependent manner. Furthermore, BIX reduced the number of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)-positive cells induced by the ischemia in ischemic penumbra. These findings indicate that post-treatment with BIX after ischemia has neuroprotective effects against acute ischemic neuronal damage in mice even when given up to 3 h after MCAO. BIX may therefore be a potential drug for stroke.

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Stroke is the third most common cause of death after heart attack and cancer, and it has profound negative social and economic effects. The only preventive treatment for stroke is anti-platelet therapy for patients with transient ischemic attack or stroke, which produces a modest but clinically worthwhile benefit [3]. In acute stroke, only a small fraction of patients benefit from intravenous administration of recombinant tissue plasminogen activator (t-PA), which is the only drug with proven effectiveness in reducing the size of infarct in humans [1,17].

BiP is one of the molecular chaperones localized to the ER membrane, and is a highly conserved member of the 70-kDa heat shock protein family [10,11]. It has been reported that the expression of BiP, an endoplasmic reticulum (ER) molecular chaperone, was upregulated by ischemia in focal and global transient ischemia models [7,16,19]. Furthermore, previous reports showed that the induction of BiP prevents neuronal death induced by ER stress [8,14,15,21]. By contrast, inhibition of GRP78 (78 kDa glucose-regulated protein) mRNA induction increases cell death in response to calcium release from the ER, oxidative stress, hypoxia, and T-

cell-mediated cytotoxicity [4,13,18]. Therefore, BiP activators will be effective agents against cerebral ischemia.

ER stress, which is caused by an accumulation of unfolded proteins in the ER lumen, is associated with stroke and with neurodegenerative diseases such as Parkinson's and Alzheimer's. An earlier study showed that pretreatment with BIX (intracerebroventricular injection at 5 or 20 μ g) protects cells from ER stress [9]. BIX is an inducer of BiP mRNA found by using a BiP reporter assay system [9]. Our previous report showed that BIX selectively induces BiP in SK-N-SH cells and pretreatment with BIX (intracerebroventricular injection at 5 or 20 μ g) reduces the area of infarction and the neuronal cell death due to focal cerebral ischemia in mice [9].

In the present study, we examined the neuroprotective effects of post-treatment with BIX on infarction, brain swelling, neurological deficits, and apoptosis in a murine permanent focal cerebral ischemia model.

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, and approved by the Animal Experimental Committee of Gifu Pharmaceutical University. All *in vivo* experiments were per-

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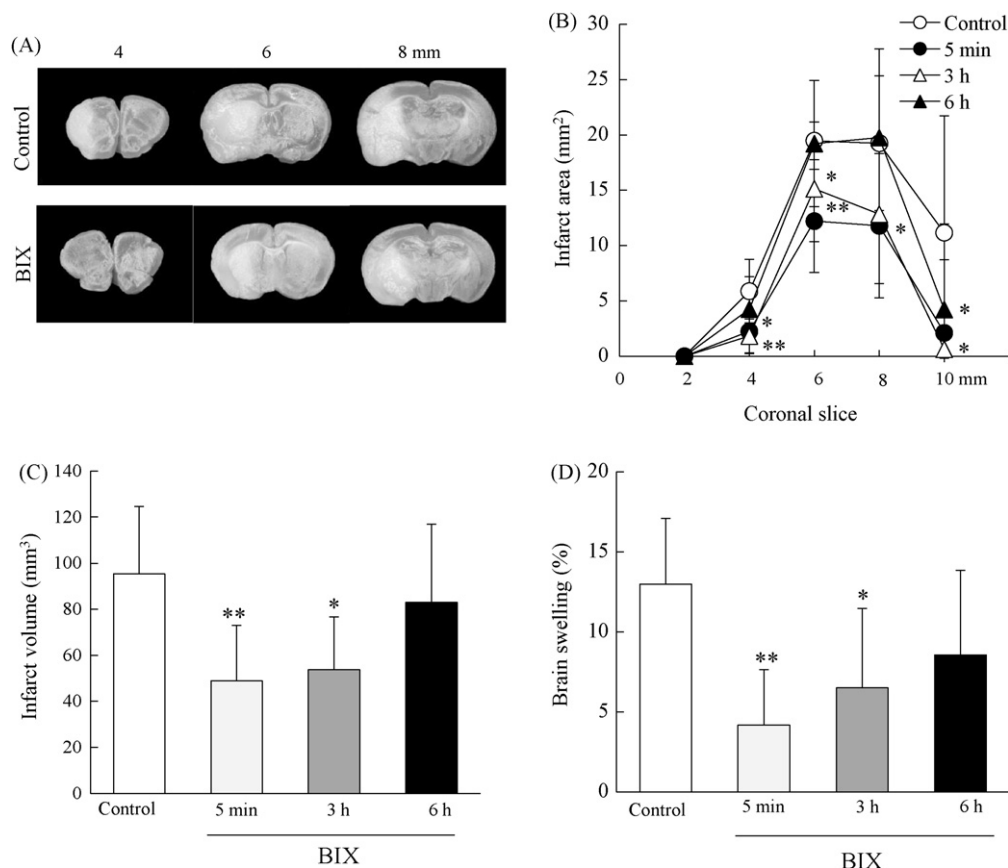


Fig. 1. Effects of BIX administered after ischemia on a therapeutic window after MCAO in mice. (A) TTC staining of coronal brain sections (2 mm thick) at 24 h after permanent MCAO in representative mice. Upper panels, vehicle-injected (control) mice. Lower panels, BIX (intracerebroventricular injection at 20 µg)-treated mice. (B) Brain infarct area measured at 24 h after MCA occlusion. Brains were removed and the forebrains sliced into five coronal 2 mm sections. (C and D) Effects of BIX on infarct volume and brain swelling (measured at 24 h after MCAO). Values are expressed as the mean ± S.D. * $P < 0.05$, ** $P < 0.01$ vs. control ($n = 7-11$).

formed using male adult ddY mice (body weight 26–32 g; Japan SLC Ltd., Shizuoka, Japan). The animals were housed at $24 \pm 2^\circ\text{C}$ under a 12 h light/dark cycle (lights on from 07:00 to 19:00). Each animal was used for one experiment only.

Anesthesia was induced using 2.0–3.0% isoflurane, and maintained using 1.0–1.5% isoflurane (both in 70% $\text{N}_2\text{O}/30\% \text{O}_2$) by means of an animal general anesthesia machine (Soft Lander; Sin-ei Industry Co. Ltd., Saitama, Japan). Body temperature was maintained at $37.0-37.5^\circ\text{C}$ 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 [5,6]. 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 occluded 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; $24 \pm 2^\circ\text{C}$) until sampling.

BIX was dissolved in 10% DMSO, and fresh solution was made daily. Two microliters of vehicle (10% DMSO in saline) or BIX 1, 5 or 20 µg was administered intracerebroventricularly at 5 min, 3 h, and 6 h after ischemia. Used animals were divided into each group so as not to make significant differences in average body weight.

To analyze infarct volume, mice were euthanized using sodium pentobarbital at 24 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 Image J, and calculated as in our previous report [5]. Brain swelling was calculated according

to the following formula: $(\text{infarct volume} + \text{ipsilateral undamaged volume} - \text{contralateral volume}) \times 100 / \text{contralateral volume} (\%)$ [6]. To minimize potential bias in infarct volume assessment, the investigator who analyzed the cerebral infarction was blinded.

Mice were tested for neurological deficits at 24 h after MCAO. Scoring was done as described in our previous study [6], 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); and (3) loss of walking or righting reflex (severe). The investigator who rated the mice was blinded as to the group to which each mouse belonged.

The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (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. The brains were removed, fixed overnight in 4% paraformaldehyde, and immersed for 1 day in 25% sucrose with phosphate-buffered saline (PBS). The brains were then embedded in a supporting medium for frozen-tissue specimens (OCT compound; Tissue-Tek). Cerebral sections 20 µm thick were cut on a cryostat at -25°C , and stored at -80°C until staining. After washing twice with 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. To quantify the number of DNA-fragmented cells present after MCAO, the numbers of

TUNEL-positive cells such as necrotic and apoptotic cells) in the caudate-putamen (as the ischemic core) and cortex (as the ischemic penumbra, two areas) were counted in a high-power field ($\times 200$) on a section through the anterior commissure by a blinded investigator. Each count was expressed as number/mm² ($n=7$).

Data are presented as the mean $\pm$ S.D. Statistical comparisons were made using a one-way ANOVA followed by Dunnett's test and Mann–Whitney *U*-test (using STAT VIEW version 5.0: SAS Institute, Cary, NC). $P<0.05$ was considered to indicate statistical significance.

Using TTC staining, we examined whether BIX would reduce infarct volume. Twenty-four hours after MCAO, the mice had developed infarcts affecting the cortex and striatum (Fig. 1A). When administered at 5 min or 3 h after MCAO, BIX significantly reduced the infarct area, infarct volume, and brain swelling, but had no such effect when administered at 6 h (Fig. 1B and D). Our previous results indicate that the induction of BiP by BIX was transient, peaking at 4 h after treatment, but the levels of BiP protein continued to increase until 12 h [9]. Although some drugs for cerebral infarction are permitted for clinical use, most drugs aim to improve the CBF. These drugs must be administered in the early phase of infarction; otherwise they cause adverse effects. For example, t-PA, a most remarkable therapeutic agent for cerebral infarction, must be administered within 3 h after the onset of the infarction, but sometimes causes serious complications such as cerebral hemorrhage [20]. In our previous report, we checked the physiological parameters between control and BIX-treated in permanent MCAO model, and there were no significant differences [9]. It would appear that BIX could save neurons from cell death even if it was given as late as 3 h after the onset of ischemia.

At 24 h after MCAO, an ischemic zone was consistently identified in the cortex (penumbra area) and subcortex (core area) of the left cerebral hemisphere. By measuring infarction, we noted that BIX significantly reduced both the infarct area and the volume in a dose-dependent manner (Fig. 2A and B). Moreover, BIX (intracerebroventricular injection at 20 μ g) improved neurological deficits (Fig. 2C). When BIX was administered at 5 min after MCAO, it exhibited dose-dependent neuroprotective effects and, with a dose of 20 μ g, reduced both the infarct volume and the neurological deficits significantly. BIX induced BiP mRNA in a concentration-dependent manner; its effects were significant at 1–50 μ M [9]. Furthermore, BIX does not induce other ER stress-associated signals (such as XBP-1 splicing or CHOP), and does not evoke ER stress. In cultured human neuroblastoma SK-N-SH cells, BIX at 5 μ M inhibited tunicamycin (Tm)-induced cell death [9]. Even a high dosage of BIX at 50 μ M did not induce BiP mRNA mediated by non-activating transcription factor 6 pathways [9]. These results imply that the mechanism of BiP induction utilized by BIX may be different from those used by ER stressors, such as thapsigargin and Tm. It has been reported that the activation of transducers of ER stress is caused by dissociation of BiP from luminal domains of PKR (protein kinase regulated by RNA)-like ER-associated kinase and inositol-requiring kinase 1 [2]. It may be assumed that the artificial induction of BiP induced by BIX disturbs the activation of transducers of ER stress, because abundant BiP remains bound to these transducers preventing their activation.

The morphological features of TUNEL-stained cells (indicative of the ischemic damage and apoptotic cell death induced by 24 h MCAO) are shown in Fig. 3B. Cells exhibiting shrunken cell bodies and condensed nuclei were distributed in both the ischemic core and penumbra of the territory affected by MCAO, with the TUNEL-positive cells being among the population displaying such features. Lei et al. [12] reported that numerous cells in the penumbra were TUNEL-positive at 24 h after MCA occlusion, while only a small number of cells in the core were TUNEL-positive at that time. In this study, TUNEL-positive cells (necrotic and apoptotic cells) were

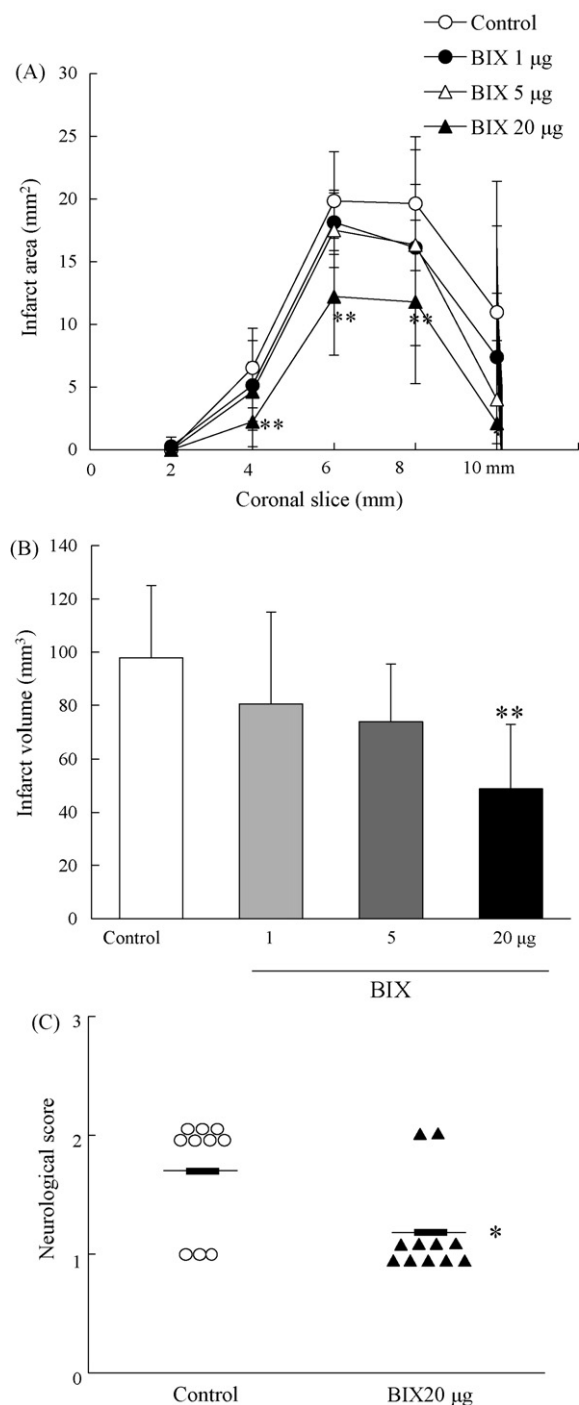


Fig. 2. Effects of BIX administered at 5 min after ischemia on infarction and neuronal damage at 24 h after MCAO in mice. (A) Brain infarct area measured at 24 h after MCA occlusion. Brains were removed and the forebrains sliced into five coronal 2 mm sections. $*P<0.05$, vs. control ($n=7-11$). (B) Effects of BIX on infarct volume (measured at 24 h after MCAO). BIX protected against the decrease in a dose-dependent manner. Values are expressed as the mean $\pm$ S.E. $**P<0.01$ vs. control ($n=7-11$). (C) Effects of BIX on neurological score (assessed at 24 h after MCAO). Values are expressed as the mean $\pm$ S.D. $*P<0.05$ vs. control ($n=10-11$).

predominantly located in the ischemic core region rather than in the ischemic penumbra, and BIX significantly reduced the number of TUNEL-positive cells in the ischemic penumbra (Fig. 3C). We next distinguished apoptotic cells from necrotic cells, and each type was counted. Only densely labeled cells showing cell shrinkage, chromatin condensation, and fragmented nuclei indicating apoptosis were considered to be apoptotic cells, whereas cells with light dif-

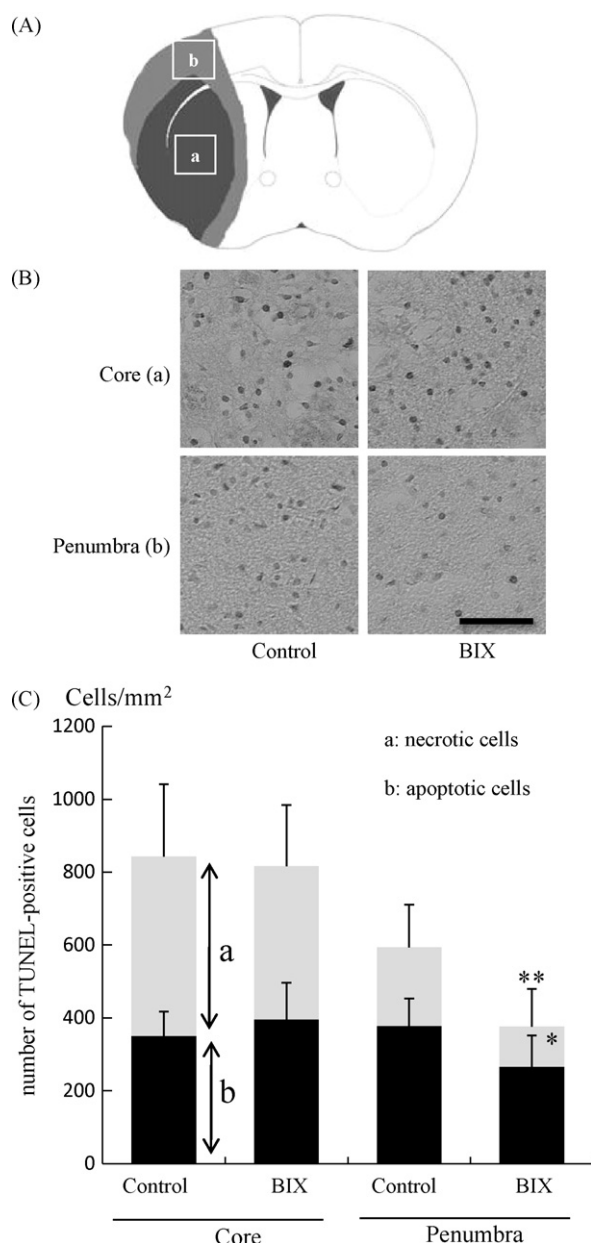


Fig. 3. Effects of BIX on TUNEL staining at 24 h after MCAO in mice. (A) Schematic drawing showing brain regions at 0.4–1.0 mm anterior to bregma (through the anterior commissure); measurement areas (a and b) in Fig. 1A. (B) Representative photomicrographs of TUNEL-positive cells in ischemic core and penumbra. Scale bar = 50 μ m. (C) Quantitative analysis of TUNEL-positive cells (necrotic and apoptotic cells). TUNEL-positive cells were significantly reduced in ischemic penumbra at 24 h after MCAO. (a and b): the number of necrotic cells and apoptotic cells, respectively, among all positive cells. Values are expressed as the mean $\pm$ S.D. * P < 0.05, ** P < 0.01 vs. control (n = 7).

fuse labeling were taken as necrotic. BIX reduced the number of necrotic and apoptotic cells significantly in the ischemic penumbra area at 24 h after MCAO (Fig. 3C).

In conclusion, we suggest that post-treatment of BIX, even if it was administered at 3 h after ischemia, provides significant protection against focal ischemia and has a wide therapeutic window. Hence, drugs which selectively induce BiP may exert a

neuroprotective effect and may be the seeds of new treatment of stroke.

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