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Isoflurane Preconditioning Induces Neuroprotection against Ischemia via Activation of P38 Mitogen-Activated Protein Kinases

Shuqiu Zheng and Zhiyi Zuo

Department of Anesthesiology, University of Virginia Health System, Charlottesville, Virginia

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ABSTRACT

A brief exposure to the volatile anesthetic isoflurane (preconditioning) induces ischemic tolerance in rat brain. However, whether isoflurane preconditioning improves long-term neurological outcome after brain ischemia and the mechanisms for this neuroprotection are not known. Here, we report that isoflurane preconditioning (2% isoflurane for 30 min at 24 h before brain ischemia) reduced brain infarct sizes and improved neurological deficit scores assessed 6, 24, and 72 h after permanent right middle cerebral arterial occlusion (MCAO) in adult male rats. More morphologically intact neurons and fewer dying cells existed in the ipsilateral frontal cortex area 1 and rostral subventricular zone of caudate putamen of isoflurane-preconditioned rats than rats undergoing MCAO alone at 14 days after the MCAO. This neuroprotection was abolished by an inhibitor of p38 mitogen-activated protein kinases (MAPK), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580) (the percentages of infarct volumes in the ipsilateral hemisphere volumes were 34 ± 7% for MCAO, 24 ± 6% for isoflurane preconditioning plus MCAO, and 30 ± 6% for SB203580 plus isoflurane preconditioning plus MCAO, $n = 8$, $P < 0.05$ for isoflurane preconditioning plus MCAO to compare with MCAO alone or with SB203580 plus isoflurane preconditioning plus MCAO) and mimicked by an activator of these kinases, anisomycin. Isoflurane induced a rapid and prolonged increase of the phosphorylated p38 MAPK in cerebral neocortex. These active kinases distributed mainly in perikaryal regions of neurons. These results suggest that isoflurane preconditioning may improve long-term neurological outcome after focal brain ischemia and that the effects may be mediated by activating p38 MAPK.

Ischemic brain injury is involved in the pathophysiology of many common human diseases such as stroke and head trauma. Although multiple strategies or interventions have been proposed or used to reduce ischemia-induced cell death, clinically practical methods to reduce ischemic brain injury and to improve long-term outcome are not yet well established. Isoflurane, a commonly used volatile anesthetic, has been demonstrated to be neuroprotective when applied during ischemia (Miura et al., 1998), and this neuroprotection has been a research focus for the past 2 decades (Warner, 2000). However, a recent study has questioned the usefulness of this method to improve long-term neurological outcome after brain ischemia, because the isoflurane-induced reduction of brain infarct volume was observed at 2 days but not at 14 days after transient middle cerebral arterial occlusion (MCAO) in rats (Kawaguchi et al., 2000). Recently, in a rat brain slice model, we have demonstrated that isoflurane exposure before oxygen-glucose deprivation (to simulate ischemia) saved more Purkinje neurons from death than the application of isoflurane during oxygen-glucose deprivation (Zheng and Zuo, 2003). This isoflurane pre-exposure–induced ischemic tolerance (isoflurane preconditioning) in neurons was also observed in rats when the brain infarct volume was evaluated 4 days after permanent MCAO (Kapinya et al., 2002).

In both in vivo and in vitro ischemia models, mitogen-activated protein kinases (MAPK), characterized as proline-directed serine-threonine protein kinases, have been demonstrated to play a crucial role in regulating brain cell death and survival after ischemia (Nozaki et al., 2001; Irving and Bamford, 2002). MAPK consists of three families: extracellu-

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ABBREVIATIONS: MCAO, middle cerebral arterial occlusion; MAPK, mitogen-activated protein kinase; ERK, extracellular signaling-regulated kinase; JNK, c-Jun N-terminal kinase; TUNEL, terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labeling; MKK, mitogen-activated protein kinase kinase; MAPKAP, mitogen-activated protein kinase-activated protein; CNS, central nervous system; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; Fr1, frontal cortex area 1; SVZ, subventricular zone; PD98059, 2′-amino-3′-methoxyflavone.

lar signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 MAPK. After activation by phosphorylation on both Thr and Tyr residues, MAPK phosphorylates important intracellular enzymes and transcription factors (Nozaki et al., 2001; Irving and Bamford, 2002).

In this study, we used permanent MCAO in adult male rats to test the hypotheses that isoflurane preconditioning improves long-term neurological outcome (14 days after the MCAO) and that the isoflurane preconditioning-induced neuroprotection is dependent on MAPK activation.

Materials and Methods

The institutional Animal Care and Use Committee at the University of Virginia approved the animal protocol. All reagents were obtained from Sigma Chemical (St. Louis, MO) unless specified in the text.

Permanent Middle Cerebral Arterial Occlusion. We permanently occluded the right middle cerebral artery with an intraluminal filament in adult rats and awakened the animals immediately thereafter to simulate the clinical situation. Briefly, male Sprague-Dawley rats weighing 250 to 280 g were anesthetized with isoflurane. They were then intubated and mechanically ventilated with air containing 2% isoflurane. The inhaled and exhaled gases were monitored with a Datex infrared analyzer (Capnomac, Helsinki, Finland) that monitored O_2, CO_2, and volatile anesthetic concentrations. The right femoral artery was cannulated for monitoring of arterial blood pressure, blood gases, and glucose. Rectal and temporalis muscle temperatures (to reflect brain temperature) were monitored and maintained with a heating blanket and a lamp at 37 to 38°C and 36 to 37°C, respectively, during anesthesia.

Permanent MCAO was accomplished as described previously (Rao et al., 2001). A 3-0 monofilament nylon suture with a rounded tip was gently advanced to the right internal carotid artery via the external carotid artery until slight resistance was felt and a reduction in regional cerebral blood flow (monitored as described below) was observed. Before the insertion, the suture was pretreated with poly-L-lysine [0.1% (w/v), in deionized water] and then dried in a 60°C oven for 1 h (Belayev et al., 1996). Isoflurane anesthesia was stopped once the suture was in place. After recovery from anesthesia, rats were placed back in their cages with ad libitum access to food and water.

Regional cerebral blood flow was monitored using a laser Doppler flowmeter (Rao et al., 2001). After a right craniectomy (4 mm in diameter, 2–4 mm lateral and 1–2 mm caudal to bregma), a laser Doppler flowmeter probe (model DP3) was placed on the surface of the right cortex. The probe was linked to a laser flowmeter device (Laser Doppler Perfusion and Temperature Monitor, Moor Instruments, Ltd., Devon, England). Once MCAO was achieved, the probe was removed and the cranial defect was covered with dental acrylic.

Isoflurane Preconditioning. Isoflurane preconditioning was performed 24 h before the permanent MCAO. Anesthesia was induced with isoflurane and then maintained with 2% isoflurane via an endotracheal tube for 30 min. Respiration was controlled by a ventilator to maintain normal end-tidal O_2 and CO_2 concentrations. Rectal and temporalis muscle temperatures were maintained at 37 to 38°C and 36 to 37°C, respectively, during anesthesia.

Application of p38 MAPK inhibitor or Activator. SB203580 (100 or 200 μg/kg) and anisomycin (10, 20, or 40 mg/kg) were i.p. injected alone (24 h before the permanent MCAO) or 30 min before the application of isoflurane preconditioning.

Evaluation Of Infarct Volume, Neuronal Loss, and Neurological Deficit Scores. The evaluation of infarct volume at 6, 24, and 72 h after the permanent MCAO was performed after 2,3,5-triphenyltetrazolium chloride staining as described previously (Alessandrini et al., 1999). The infarct areas were quantified using NIH Image 1.60. To account for the cerebral edema and differential shrinkage resulting from brain ischemia and tissue processing and to correct for the individual difference in brain volumes, the percentage of infarct volume in the ipsilateral hemisphere volume was calculated (Swanson et al., 1990).

Because 2,3,5-triphenyltetrazolium chloride staining will not adequately differentiate the infarcted area from the area that is not infarcted in brains at 14 days after ischemia, neuronal loss in these brains was evaluated as follows. After being perfused with saline, rats were perfused transcardially with 200 ml of phosphate-buffered paraformaldehyde. Brains were removed and stored in the fixative for 2 days at 4°C. Coronal paraffin sections, 5 μm thick, at about 1.5 mm rostral and 3 mm caudal to bregma were obtained and subjected to Nissl staining. These sections were examined by an observer blinded to the group assignment of the sections. A reticle ($\sim$720 μm^2) was used to count cells in the same size area. Morphologically intact and Nissl staining positive cells were counted in the frontal cortex area 1 (Fr1), rostral subventricular zone (SVZ) of caudate putamen (in the sections at bregma + 1.5 mm) and CA3c of hippocampus (in the sections at bregma −3 mm). Three determinations, each on different locations in these three brain regions, were performed and averaged to yield a single number (density of the cells) for each brain region of individual rat.

To determine the density of dying cells in the Fr1, SVZ, and CA3c at 14 days after the permanent MCAO, sections were stained by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labeling (TUNEL) using a kit from Serologicals Co. (Norcross, GA). Briefly, sections were incubated with 20 μg/ml proteinase K for 10 min at room temperature. After being washed in an equilibration buffer for 10 min at room temperature, sections were incubated with dUTP-digoxigenin and terminal deoxynucleotidyl transferase for 1 h at 37°C. The detection of the reaction was performed according to the protocol provided by the manufacturer. Negative controls included sections treated similarly but without incubation with terminal deoxynucleotidyl transferase, dUTP-digoxigenin, or anti-digoxigenin antibody, respectively. The density of TUNEL-positive cells was determined as described above for morphologically intact neurons after Nissl staining.

Neurological deficit scores after right MCAO were evaluated daily based on an eight-point scale by a person blinded to the group assignment. Rats were scored as follows: 0, no apparent deficits; 1, failure to extend left forepaw fully; 2, decreased grip of the left forelimb; 3, spontaneous movement in all directions, contralateral circling only if pulled by the tail; 4, circling or walking to the left; 5, walking only if stimulated; 6, unresponsiveness to stimulation and with depressed level of consciousness; and 7, dead (Rogers and Hunter, 1997).

Western Analysis. Parietal neocortex was homogenized in ice-cold buffer containing 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DL-dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, and 20 μg/ml aprotinin. The prepared samples then were subjected to Western blotting as described previously (Zuo and Johns, 1997). Anti-phosphorylated p38, JNK, ERK, mitogen-activated protein kinase kinase (MKK) 3/6, and mitogen-activated protein kinase-activated protein 2 (MAPKAP2) antibodies were rabbit polyclonal IgG raised against human phosphorylated p38, JNK, ERK, MKK3/6, and MAPKAP2 peptides, respectively, with cross-reactivity to the corresponding rat proteins. Anti-phosphorylated p38, JNK, and ERK antibodies were from Santa Cruz (Santa Cruz, CA). Anti-phosphorylated MAPKAP2 antibody was from Cell Signaling Technology, Inc. (Beverly, MA). Anti-β-actin was rabbit affinity-purified polyclonal antibody raised against the C-terminal actin fragment attached to multiple antigen peptide backbone. Immunoreactivity was visualized by enhanced chemiluminescence. The volume of the protein bands was quantitated by an ImageQuant 5.0 densitometer (Amersham Biosciences, Piscataway, NJ). The data of MAPK, MKK3/6, and MAPKAP2 were then normalized to those of β-actin to control for errors in protein sample loading and transferring during the Western blotting.

Immunohistochemistry. Parietal neocortex was fixed as described in the section *Evaluation of Infarct Volume, Neuronal Loss, and Neurological Deficit Scores* and was cut into 5 μm thick coronal paraffin sections. Immunohistochemistry was performed as described before (Schlag et al., 1998). The antibody for the phosphorylated p38 was the same antibody used for Western blotting. Immunoreactivity was visualized with an avidin-biotinylated horseradish peroxidase reaction with an avidin-biotinylated enzyme complex staining system from Vector Laboratories, Inc. (Burlingame, CA). Control incubations leaving out the primary or secondary antibodies were performed.

Statistical Analysis. Results are means ± S.D. ($n \geq 6$ animals for each group). Data of physiological parameters, neuronal and TUNEL-positive cell density, and Western blot signals were analyzed by Student's t test. Results of neurological deficit scores and infarct sizes were analyzed by Mann-Whitney rank sum test or by Kruskal-Wallis analysis of variance on ranks followed by Student-Newman-Keuls method as appropriate. A $P \leq 0.05$ was accepted as significant.

Results

Isoflurane Preconditioning Induces Ischemic Tolerance. Compared with rats subjected to the MCAO alone, rats exposed to isoflurane before the MCAO had significantly smaller brain infarct sizes and better neurological deficit scores at 6 h, 24 h, and 3 days after the permanent MCAO (Fig. 1). Consistent with our results, a recent study has shown that rats pretreated with 1.4% isoflurane for 3 h at 24 h before permanent MCAO (achieved by craniotomy and coagulation of middle cerebral artery) had smaller infarct sizes when evaluated 4 days after the MCAO (neurological deficit scores were not evaluated in the study) (Kapinya et al., 2002).

To determine whether isoflurane preconditioning could result in improved long-term neurological outcome, we evaluated rats 14 days after the permanent MCAO. Although the neurological deficit scores of rats with or without isoflurane preconditioning were not statistically different, rats with isoflurane preconditioning tended to have better neurological functions than rats with MCAO only (Fig. 1). In addition, rats with isoflurane preconditioning had more morphologically intact neurons in the ipsilateral Fr1 and SVZ compared with MCAO-alone rats (Fig. 2). Interestingly, there were more TUNEL-positive cells in the ipsilateral Fr1 and SVZ than in the corresponding regions of the side contralateral to ischemia in the same rats (Fig. 3), suggesting that ischemia-induced cell death continues to occur even at 14 days after the MCAO. Importantly, this delayed cell death was also reduced by isoflurane preconditioning (Fig. 3).

Because physiological parameters such as blood pressures, glucose level, and brain temperature can affect brain infarct sizes and functional outcome after ischemia, they were closely monitored and controlled during the period of surgery. There were no differences in these physiological parameters among rats in control and isoflurane preconditioning groups (Table 1).

Isoflurane Preconditioning Induces a Time-Dependent Change in the Phosphorylated P38 MAPK and ERK (Active Forms of the MAPK). Because MAPK is important in regulating cell death and survival after ischemia (Nozaki et al., 2001; Irving and Bamford, 2002), we tested whether isoflurane preconditioning altered the activity of MAPK in brain cells. We found that cerebral neocortex of Sprague-Dawley rats, after being exposed to 2% isoflurane for 30 min, had a higher level of the phosphorylated p38 MAPK than that of control rats. This increase in the active p38 MAPK was rapid (it was statistically significant at 60 min after the isoflurane exposure) and peaked at 6 h after the exposure (Fig. 4). These active p38 MAPKs were mainly localized in the perikaryal regions of neurons (Fig. 5). It is noteworthy that the isoflurane-induced increase in the phosphorylated p38 MAPK lasted for at least 24 h after the isoflurane exposure (Figs. 4 and 5). On the contrary, the level of phosphorylated ERK decreased gradually and was significantly lower than in control rats at 6 h after the isoflurane exposure. This decreased level of the active ERK was still apparent 14 days after the isoflurane exposure (Fig. 4). It was interesting that isoflurane exposure did not affect the level of the phosphorylated JNK (Fig. 4). Thus, the three known MAPK families responded differently to isoflurane exposure. In addition, our results reveal rarely described prolonged effects of volatile anesthetics on intracellular signaling molecules in the CNS.

SB203580, a p38 Inhibitor, Blocks Isoflurane Preconditioning-Induced Neuroprotection. Activation of p38 MAPK has been shown to precondition myocardium against

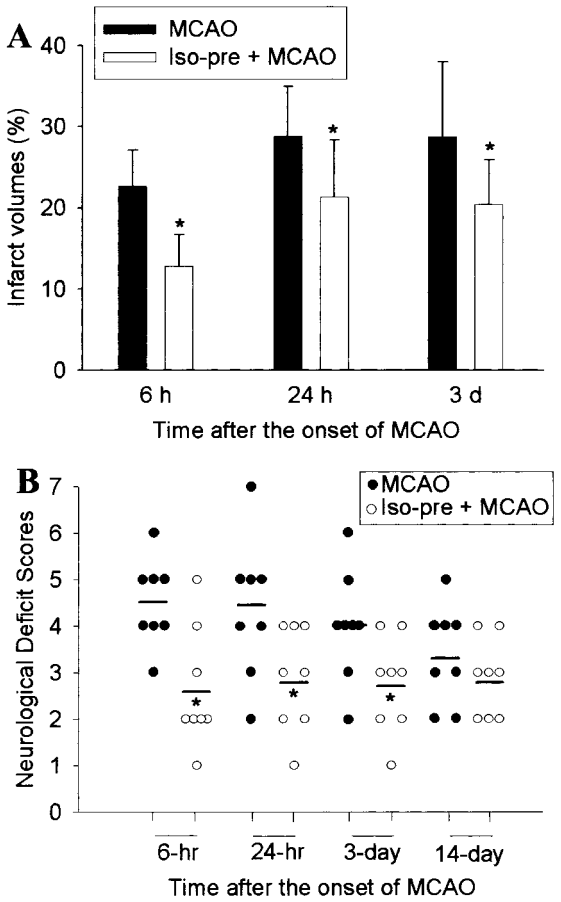

Fig. 1. Isoflurane preconditioning improves neurological outcome after permanent right middle cerebral arterial occlusion (MCAO). A, percentage of infarct volume in ipsilateral hemisphere volume. Results are the means ± S.D. ($n = 8$). B, neurological deficit scores that were evaluated immediately before the animals were sacrificed for the assessment of infarct sizes (data are presented in Fig. 1A) are shown. Each circle represents the score for a single rat. A horizontal bar indicates the mean value for each group. *, $P \leq 0.05$ compared with the corresponding MCAO group. Iso-pre, isoflurane preconditioning.

ischemia (Zhao et al., 2001). Does isoflurane-induced p38 MAPK activation mediate the neuroprotection by isoflurane preconditioning? We administrated the p38 MAPK inhibitor SB203580 (alone or 30 min before the isoflurane exposure) 24 h before the permanent MCAO. Although SB203580 alone did not affect the neurological deficit scores or brain infarct sizes compared with those of MCAO, it abolished the isoflurane preconditioning-induced neuroprotection (Fig. 6). These results suggest that the activation of p38 MAPK by isoflurane pretreatment is critical for the isoflurane preconditioning-induced neuroprotection.

The effects of SB203580 on the amount of the phosphorylated p38, JNK, and ERK under basal condition and after isoflurane exposure are shown in Fig. 7. SB203580 did not affect the amount of the phosphorylated ERK and JNK under basal condition and after isoflurane exposure. However, SB203580 decreased the phosphorylated p38 MAPK after isoflurane exposure.

Activation of P38 MAPK before Ischemia Induces Neuroprotection. To substantiate our argument that isoflurane preconditioning induced an increase in the activity of p38 MAPK before ischemia to provide neuroprotection, we tested whether the p38 MAPK activator anisomycin would also induce brain ischemic tolerance. The rats treated with anisomycin 24 h before ischemia had significantly smaller infarct sizes than rats without anisomycin pretreatment (Fig. 6). This anisomycin pretreatment-induced neuroprotection was inhibited by SB203580 (Fig. 6). These results suggest that activation of p38 MAPK before ischemia is neuroprotective. We then studied whether anisomycin indeed activated p38 MAPK in rat brain. Consistent with a previous report on effects in heart (Zhao et al., 2001), anisomycin also increased the phosphorylated p38 MAPK in brain (Fig. 8). These effects are specific because anisomycin at the doses used in our study did not affect the amount of the phosphorylated ERK and JNK (Fig. 8). Similar to the case of isoflurane preconditioning, the phosphorylated p38 reactivity induced by anisomycin was localized in the perikaryal regions of neurons (Fig. 8).

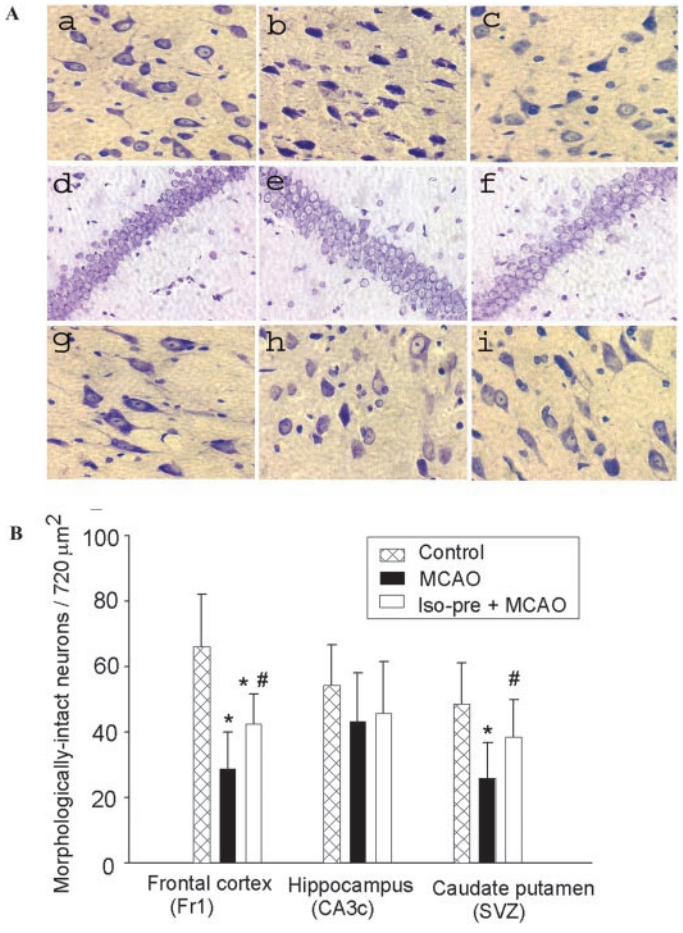

Fig. 2. Isoflurane preconditioning reduces ischemia-induced neuronal loss. Cells in the Fr1, SVZ of caudate putamen, and CA3c of hippocampus were examined after Nissl staining at 14 days after the permanent right middle cerebral arterial occlusion (MCAO). A, representative sections of Fr1 (a–c), CA3c (d–f), and SVZ (g–i) are from contralateral (a, d, and g) or ipsilateral MCAO brains of rats without (b, e, and h) or with (c, f, and i) isoflurane preconditioning (magnification, 400×). Quantitative data are presented in B. Results are the means ± S.D. (n = 16 for control, the contralateral brains, and n = 8 for MCAO and iso-pre + MCAO groups of the ipsilateral brains). *, $P < 0.05$ compared with control; #, $P < 0.05$ compared with MCAO alone. Iso-pre, isoflurane preconditioning.

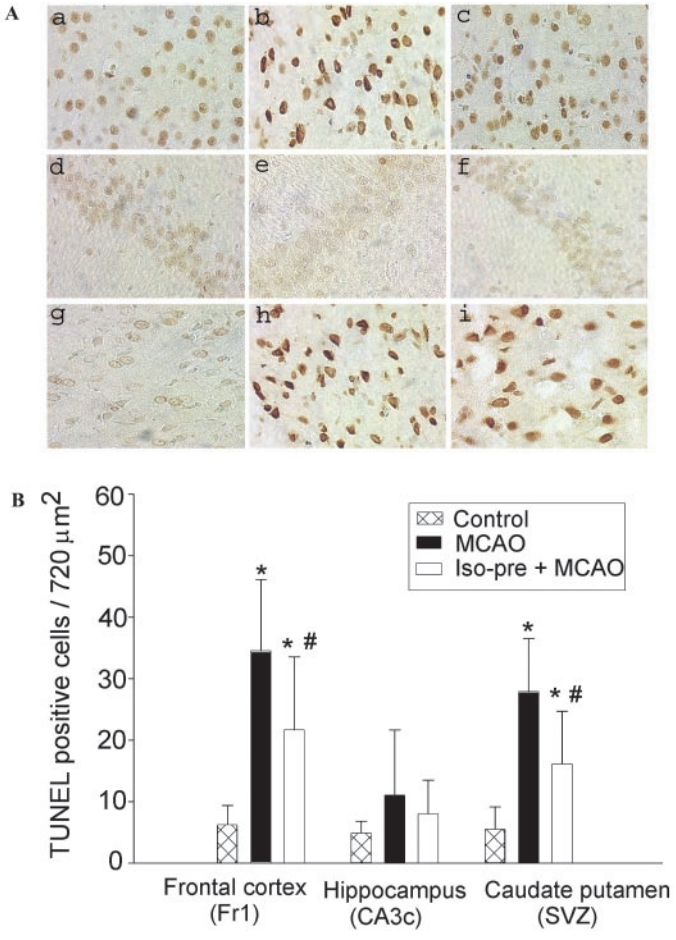

Fig. 3. Isoflurane preconditioning reduces dying cells evaluated at 14 days after the permanent right MCAO. Cells in the Fr1, SVZ of caudate putamen, and CA3c of hippocampus were examined after TUNEL staining. A, representative sections of Fr1 (a–c), CA3c (d–f), and SVZ (g–i) are from contralateral (a, d, and g) or ipsilateral MCAO brains of rats without (b, e and h) or with (c, f and i) isoflurane preconditioning (magnification, 400×). Quantitative data are presented in B. Results are the means ± S.D. (n = 16 for control, the contralateral brains, and n = 8 for MCAO and iso-pre + MCAO groups of the ipsilateral brains). *, $P < 0.05$ compared with control; #, $P < 0.05$ compared with MCAO alone. Iso-pre, isoflurane preconditioning.

Autophosphorylation of p38 MAPK Plays a Role in Isoflurane-Induced p38 MAPK Phosphorylation. Two pathways have been found to activate/phosphorylate p38 MAPK: 1) the classic pathway by p38 MAPK kinases (MKK3/6) (Nozaki et al., 2001; Irving and Bamford, 2002) and 2) autophosphorylation of p38 MAPK after it has been bound to transforming growth factor-β–activated protein kinase 1-binding protein 1 (Ge et al., 2002). SB203580, an inhibitor of p38 MAPK but not of MKK3/6 (Kumar et al., 1999; Ge et al., 2002), at least partly inhibited the isoflurane-induced p38 MAPK phosphorylation in the cerebral cortex of rats (Fig. 9). Associated with this inhibition, the phosphorylated MAPKAP2, a substrate of p38 MAPK, was also decreased. In contrast, the phosphorylated MKK3/6 were not changed. These results suggest that the autophosphorylation pathway of p38 MAPK may be activated by isoflurane to increase the phosphorylated p38 MAPK in the brain.

Discussion

In addition to confirming the short-term neuroprotective effects of isoflurane preconditioning in vivo (Kapinya et al., 2002; Xiong et al., 2003), our study has four significant findings: 1) isoflurane preconditioning may improve long-term neurological outcome because more morphologically intact neurons were found in the ischemic penumbral brain regions Fr1 and SVZ of rats preconditioned with isoflurane than control rats at 14 days after the MCAO; 2) isoflurane preconditioning reduced ischemia-induced delayed cell death; 3) isoflurane induced phosphorylation/activation of p38 MAPK, which was critical in isoflurane preconditioning-induced neuroprotection; and 4) autophosphorylation of p38 MAPK may be involved in the isoflurane-induced increase in the phosphorylated p38 MAPK in rat brain.

In our study, rats were preconditioned with 2% isoflurane for 30 min because 1) in our previous brain slice study, the EC_{50} for isoflurane preconditioning-induced neuroprotection was 1.17 ± 0.31% and the maximally protective effects were achieved at isoflurane concentrations of 3% or higher (Zheng and Zuo, 2003); 2) preconditioning of brain slices with isoflurane for 15 to 30 min was needed for the preconditioning to be maximally protective (Zheng and Zuo, 2003); and 3) one minimum alveolar concentration (the concentration to inhibit 50% of subjects from responding to surgical stimuli) of isoflurane for adult rats is about 1.4% (Mazze, 1985; Orliaguet et al., 2001) and 2% isoflurane is approximately equal to 1.4 minimum alveolar concentrations, which is often achieved in clinical practice. Our isoflurane application is much shorter than that used in the two previous studies in the literature: one used 1.4% isoflurane for 3 h (Kapinya et al., 2002) and the other used 2% isoflurane 1 h/day for 5 days (Xiong et al., 2003). We administrated the preconditioning stimulus 24 h before brain ischemia. Because isoflurane is poorly metabolized and rapidly washed out of the bodies (Carpenter et al., 1986), we expect that all isoflurane inhaled during preconditioning period had been eliminated from the rats before the brain ischemia. Thus, our animal protocol is consistent with preconditioning concept.

Because most anesthetics are neuroprotective when present during ischemia (Miura et al., 1998; Bhardwaj et al., 2001), we awakened rats immediately once the arterial occlusion was achieved to minimize the presence of anesthetics during ischemia and to maximally simulate clinical situation. Similar to the results reported before (Kapinya et al., 2002; Xiong et al., 2003), isoflurane preconditioning improved neurological outcome assessed within 4 days after the MCAO. Infarct maturation after 2 days in rat models of stroke has been described in the literature (Garcia et al., 1993; Lin et al., 1993). However, recent studies suggest that ischemic injury is a dynamic process characterized by ongoing neuronal loss for at least 14 days after ischemia (Li et al., 1995; Du et al., 1996). Consistent with these previous reports, significantly more dying cells, as detected by TUNEL staining, were found in the ipsilateral Fr1 and SVZ than in the contralateral counterparts at 14 days after the permanent MCAO in our study. Although there was no statistical difference in the numbers of dying cells between the ipsilateral and contralateral hippocampi, three of eight rats showed obviously higher numbers of dying cells in the ipsilateral hippocampi. Both Fr1 and SVZ have decreased blood flow and are therefore classified as penumbral areas after MCAO (Nagasawa and Kogure, 1989; Memezawa et al., 1992). Although the ipsilateral hippocampus can be injured by MCAO

TABLE 1

Physiological parameters during MCAO period in representative groups.

Arterial samples were taken when making the neck incision (Beginning) or 20 min after the onset of MCAO (End). Data from rats that were sacrificed 3 and 14 days after the onset of permanent MCAO are presented here, and temporalis muscle temperatures are shown. Results are the means ± S.D. (n = 8). The same physiological parameters also were monitored for rats in other groups and no significant difference was noticed among them as well.

	pH	$PaCO_2$	PaO_2	MABP	Temperature	Glucose
		mm Hg	*mm Hg*	*mm Hg*	*°C*	*mg/dl*
3-Day						
Control						
Beginning	7.45 ± 0.04	40 ± 4	104 ± 6	95 ± 4	36.7 ± 0.2	198 ± 20
End	7.44 ± 0.05	39 ± 9	116 ± 11	96 ± 8	36.8 ± 0.1	215 ± 25
Iso-pre						
Beginning	7.42 ± 0.04	42 ± 13	121 ± 13	93 ± 7	36.6 ± 0.2	189 ± 17
End	7.39 ± 0.05	40 ± 8	119 ± 13	91 ± 5	37.0 ± 0.3	214 ± 22
14-Day						
Control						
Beginning	7.44 ± 0.02	39 ± 9	96 ± 7	88 ± 11	36.6 ± 0.2	186 ± 16
End	7.39 ± 0.06	39 ± 7	108 ± 10	93 ± 10	37.0 ± 0.1	197 ± 15
Iso-pre						
Beginning	7.40 ± 0.05	42 ± 11	97 ± 8	90 ± 8	36.7 ± 0.2	203 ± 20
End	7.39 ± 0.04	41 ± 7	104 ± 11	87 ± 13	36.6 ± 0.3	211 ± 22

Iso-pre, isoflurane preconditioning; MABP, mean arterial blood pressure.

(States et al., 1996), it is often not considered an ischemic penumbral or ischemic core area. Interestingly, isoflurane preconditioning reduced this ischemia-induced delayed cell loss in the ischemic penumbra. In addition, isoflurane preconditioning also decreased the accumulated neuronal loss in the ischemic penumbra assessed 14 days after the focal brain ischemia because more neurons detected by Nissl staining were in the ipsilateral Fr1 and SVZ in rats preconditioned with isoflurane than in control animals. Thus, our results suggest that isoflurane preconditioning-induced neuroprotection transfers into not only temporary but also long-term improved neurological outcome after ischemia.

What is/are the mechanism(s) for the isoflurane preconditioning-induced neuroprotection? Mechanisms such as inhibition of cerebral metabolic rate and antagonism of glutamate receptors have been proposed for the neuroprotection when isoflurane is applied during ischemia (Yang and Zorumski, 1991; Nellagård et al., 2000; Warner, 2000). However, isoflurane preconditioning may work through different mechanisms to protect neurons against ischemia. Indeed, rats anesthetized with 1.4% isoflurane for 3 h did not have altered local cerebral glucose utilization in multiple cerebral cortex regions 24 h after the anesthesia (Kapinya et al., 2002). In our study, we found that isoflurane induced a significant increase in the active p38 MAPK. Ischemia has been demonstrated to increase active p38 MAPK and inhibition of p38 MAPK activity during ischemia has been shown to be neuroprotective (Sugino et al., 2000). Our results suggest that activation of p38 MAPK by methods such as isoflurane preconditioning before ischemia is neuroprotective because a p38 MAPK inhibitor blocks the isoflurane preconditioning-induced protection, a p38 MAPK activator induces neuroprotection, and isoflurane increases the phosphorylated p38 MAPK. Similar results from an in vitro study showed that early activation of p38 MAPK was necessary to protect cultured L929-cyt16 cells from tumor necrosis factor α-induced cytotoxicity (Roulston et al., 1998). Thus, our results suggest a novel model of p38 MAPK function in ischemia in vivo: the presentation of neuronal death or survival after p38 MAPK activation depends on the timing of the activation under a given condition.

Our study showed that isoflurane also induced a prolonged decrease in the phosphorylated ERK. The role of phosphorylated ERKs in neuronal survival or death is controversial. Hu et al. (2000), in a rat global ischemia model, found that phosphorylated ERK was increased in the cells of the dentate gyrus, which are spared in this injury model, and no activity of ERK was detected in the CA1 neurons, which are usually killed by this ischemia. These results suggest that activation of the ERK induces a "survival" pathway to prevent cells from ischemia-induced death. However, Alessandrini et al. (1999) demonstrated that treatment of mice 30 min before focal brain ischemia with an ERK kinase inhibitor, PD98059, dramatically reduced infarct volume at 22 h after transient MCAO, suggesting that ERK is involved in ischemic brain

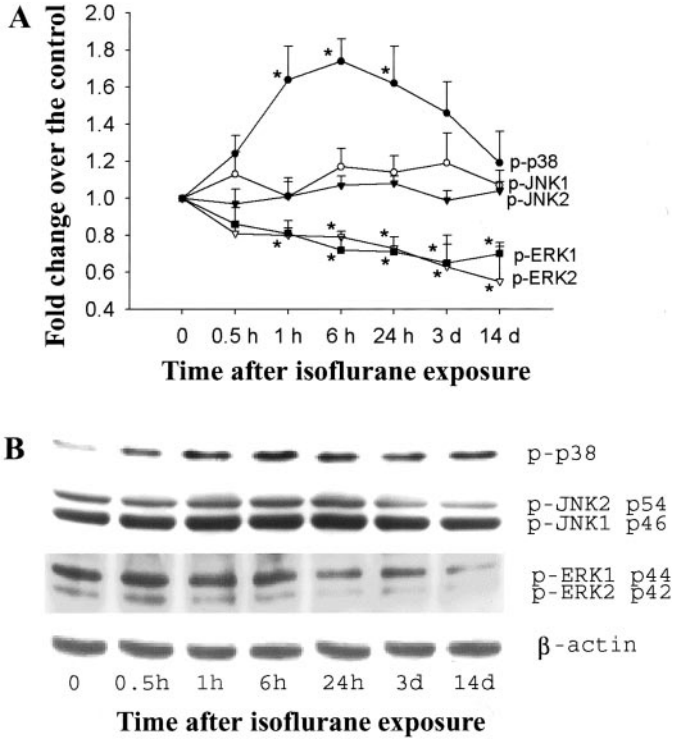

Fig. 4. Isoflurane increases the expression of the phosphorylated p38 MAPKs. Rats were exposed to 2% isoflurane for 30 min and the parietal neocortex was removed at various time points after the exposure for Western blot. A, the graphic presentation of the phosphorylated MAPK protein abundance quantified by integrating the volume of autoradiograms from four rats. Values in graphs are expressed as -fold change over the control (time 0, these rats were not exposed to isoflurane) and presented as the means ± S.D. *, $P < 0.05$ compared with the control. B, a representative Western blot. p-p38, phosphorylated p38 MAPK; p-JNK, phosphorylated JNK; p-ERK, phosphorylated ERK.

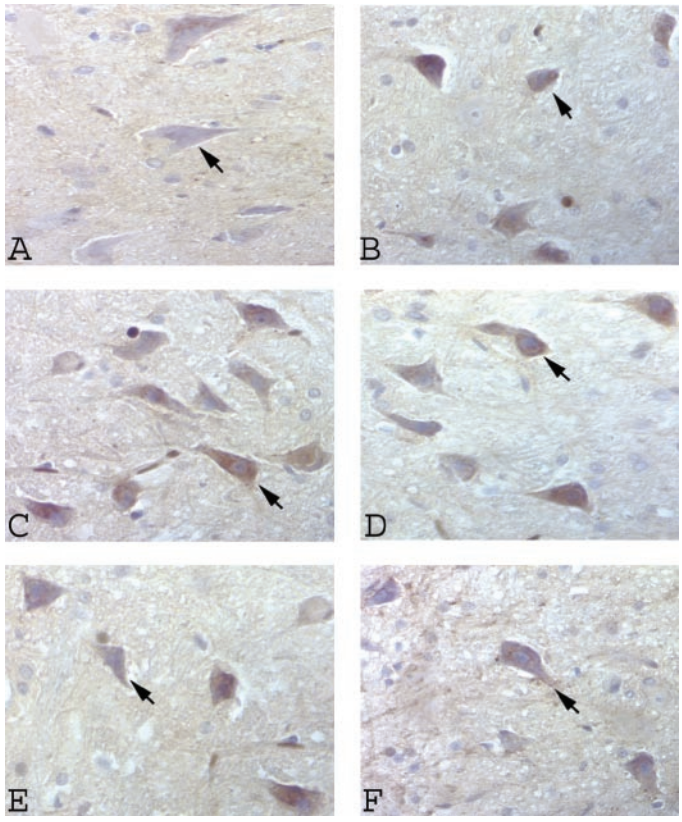

Fig. 5. The perikaryal distribution of the phosphorylated p38 mitogen-activated protein kinases (MAPK) in the presence of isoflurane. Representative sections of parietal neocortex after immunohistochemistry with an anti-phosphorylated p38 MAPK antibody are from control rats (A) or from rats at 0.5 h (B), 6 h (C), 24 h (D), 3 days (E), and 14 days (F) after the exposure to 2% isoflurane for 30 min (magnification, 400×).

injury. In a cerebellar slice model, we showed that activation of an ERK signaling pathway, which may be critical for ischemic preconditioning-induced neuroprotection (Gonzalez-Zulueta et al., 2000), might not be important for isoflurane preconditioning-induced neuroprotection (Zheng and Zuo, 2003). In our current study, we showed that SB203580, which has no effects on isoflurane-induced decrease of the phosphorylated ERK, inhibited isoflurane preconditioning-induced neuroprotection. In addition, anisomycin, which did not reduce the phosphorylated ERK, mimicked isoflurane to induce ischemic tolerance. These two lines of indirect evidence suggest that the isoflurane-induced decrease of the phosphorylated ERK may not be a major mechanism for isoflurane preconditioning-induced neuroprotection. However, further studies are needed to examine this issue in detail.

How does isoflurane activate p38 MAPK? We used SB203580 that can inhibit the p38 MAPK autophosphorylation pathway but does not usually inhibit the classic pathway via MKK3/6 to activate/phosphorylate p38 MAPK (Kumar et al., 1999; Ge et al., 2002). Our results showed that SB203580 at the doses used in this study did not change the phosphorylated MKK3/6 but decreased the phosphorylated p38 MAPK and MAPKAP2 after the isoflurane exposure. These results suggest that isoflurane may increase the phosphorylated p38 MAPK at least partly via the autophosphorylation pathway. It is not known in our study whether isoflurane directly or indirectly via other mediators activates the autophosphorylation pathway for p38 MAPK. In this regard, isoflurane has been shown to increase nitric oxide production in brains (Loeb et al., 1998; Matsuoka et al., 1999) and nitric oxide can induce p38 activation (Cheng et al., 2001).

Our study may have significant clinical and biological implications. Obviously, it is not appropriate to extrapolate our results directly to clinical situations. However, isoflurane is a commonly used volatile anesthetic in clinical neuroanesthesia, and it is tempting to speculate that isoflurane may be used to induce neuroprotection before anticipated damage to

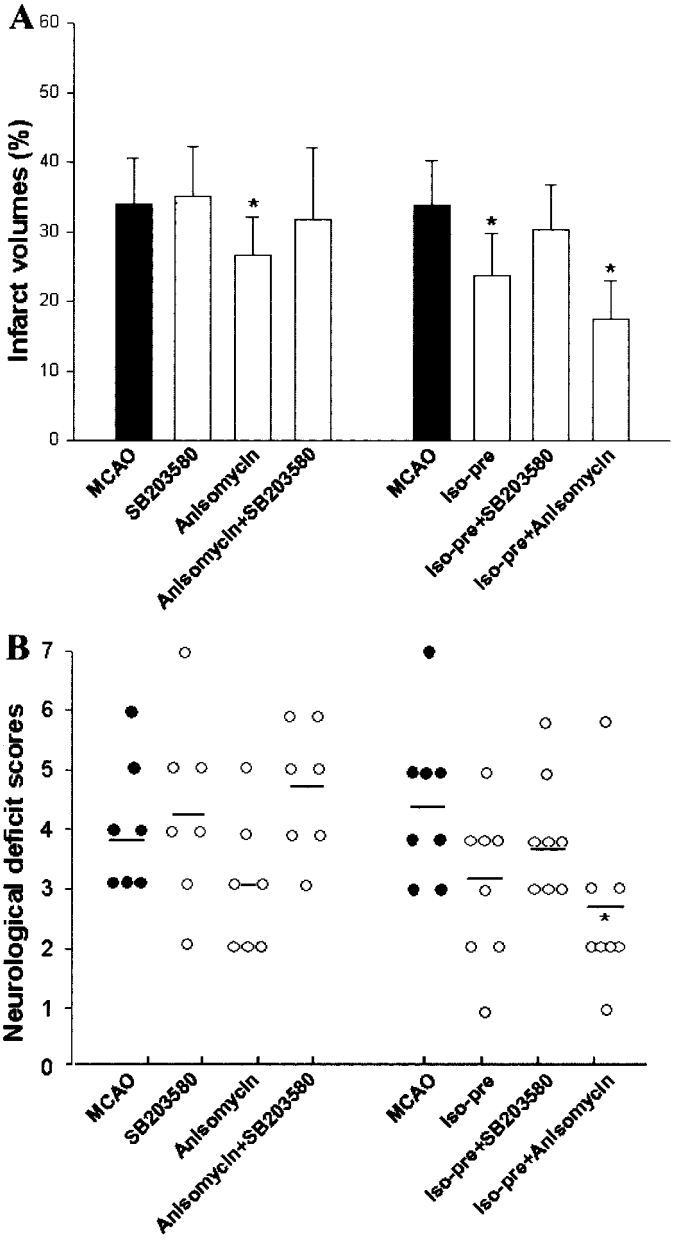

Fig. 6. Neuroprotection induced by isoflurane preconditioning is abolished by a p38 MAPK inhibitor, SB203580, and mimicked by a p38 MAPK activator, anisomycin. All groups of rats had the permanent right MCAO. SB203580 (100 μg/kg, i.p.) was injected 30 min before the isoflurane preconditioning or anisomycin administration or 24 h before the MCAO. Anisomycin (20 mg/kg, i.p.) was injected 24 h before the MCAO. Rats were evaluated 3 days after the MCAO for brain infarct sizes (A, presented as percentage of infarct volume in ipsilateral hemisphere volume) and for neurological deficit scores (B). Infarct sizes are the means ± S.D. (n = 7 - 8). Neurological deficit scores are presented in the following format: ○, the score for a single rat; horizontal bar, mean value for each group. *, $P < 0.05$ compared with the MCAO only group. Iso-pre, isoflurane preconditioning.

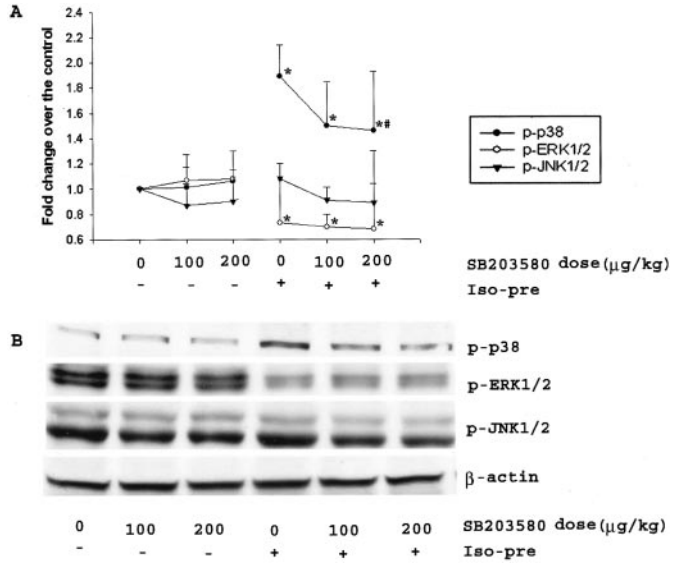

Fig. 7. SB203580 reduces isoflurane-induced increase in the phosphorylated p38 MAPKs but has no effects on the phosphorylated JNK (p-JNK) and the phosphorylated ERK (p-ERK). SB203580 was i.p. injected in rats 30 min before the isoflurane exposure (2% for 30 min). The parietal neocortex was removed at 6 h after the isoflurane exposure or at 7 h after SB203580 injection in rats without isoflurane exposure for Western analysis of the phosphorylated MAPKs. A, the graphic presentation of the phosphorylated p38 MAPK protein abundance quantified by integrating the volume of autoradiograms from four rats. Values in graphs are expressed as fold change over the control (these rats were not exposed to isoflurane or SB203580) and presented as the means ± S.D. *, $P \leq 0.05$ compared with the controll #, $P < 0.05$ compared with isoflurane exposure only rats. B, a representative Western blot. Iso-pre, isoflurane preconditioning; p-p38, phosphorylated p38 MAPK.

the CNS in clinical situations (for example, delayed vasospasm after subarachnoid hemorrhage or neurovascular surgery). In addition, prolonged CNS dysfunction, such as cognitive disorders after general anesthesia, has been recognized, and its great impact on patients' well being and our society is appreciated. Anesthetic effects on intracellular signaling molecules such as p38 and ERK MAPK may play an important role in mediating these clinical presentations. Finally, investigating and understanding the mechanisms of isoflurane preconditioning-induced neuroprotection may suggest new interventions for ischemic brain injury.

In summary, we showed that isoflurane preconditioning is neuroprotective. This neuroprotection may transfer into improved long-term neurological outcome assessed 14 days after ischemia. Our results also demonstrate that isoflurane induces a prolonged activation of p38 MAPK and that activation of p38 MAPK before ischemia is neuroprotective under in vivo conditions. Anesthetic effects on MAPK also may provide a molecular basis for the prolonged CNS dysfunction frequently observed after anesthesia.

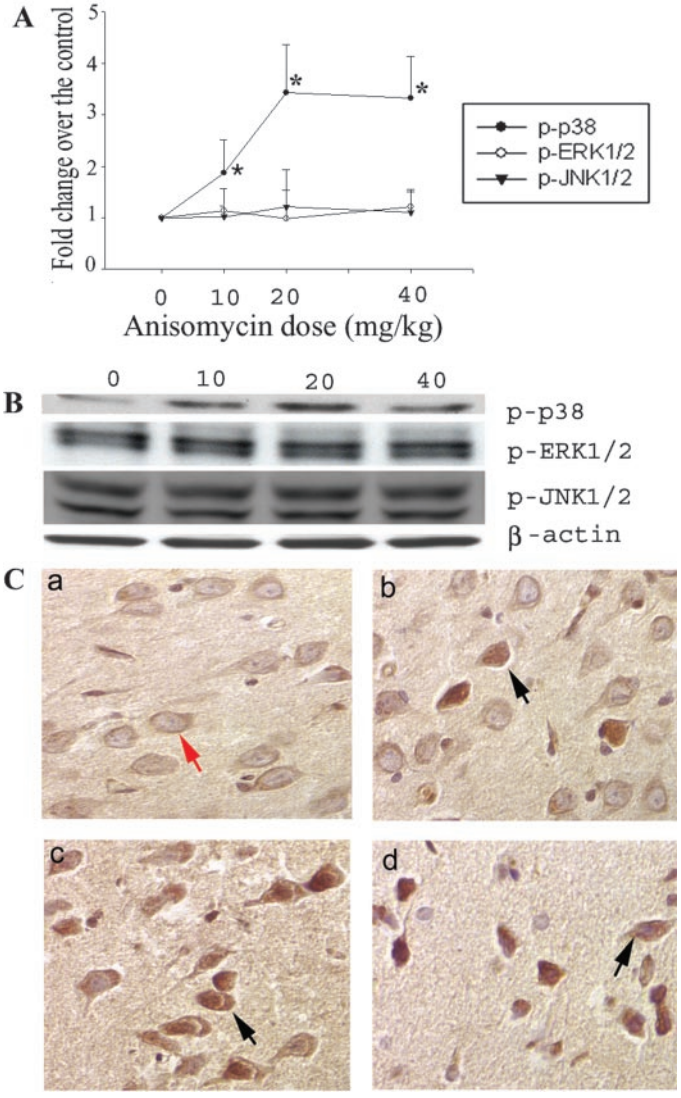

Fig. 8. Anisomycin increases the expression of the phosphorylated p38 MAPKs. Rats were injected (i.p.) with various concentrations of anisomycin, and the parietal neocortex was removed at 24 h after the injection for Western blot (A and B) of the phosphorylated p38 MAPK (p-p38), phosphorylated JNK (p-JNK) and phosphorylated ERK (p-ERK) or for immunohistochemistry (C) of the phosphorylated p38 MAPK. A representative Western blot (B) and the graphic presentation (A) of the phosphorylated p38 MAPK protein abundance quantified by integrating the volume of autoradiograms from four rats are shown. Values in graphs are expressed as -fold change over the control (at dose 0, these rats were not exposed to anisomycin) and presented as the means ± S.D. *, $P < 0.05$ compared with the control. C, representative sections are from control rats (a) or from rats 24 h after the injection of 10 (b), 20 (c), or 40 (d) mg/kg of anisomycin (magnification, 400×).

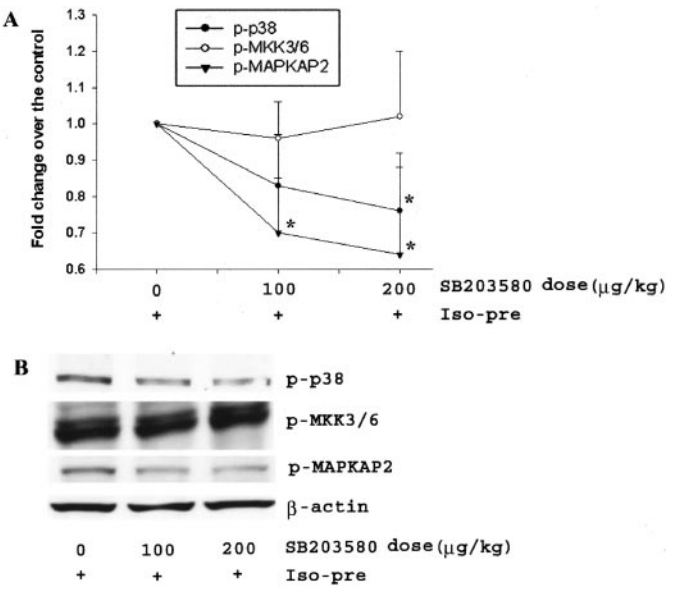

Fig. 9. SB203580 reduces the phosphorylated p38 MAPKs and the phosphorylated MAPKAP2 (p-MAPKAP2) but has no effects on the phosphorylated MKK3/6 (p-MKK3/6) after isoflurane exposure (2% for 30 min). SB203580 was i.p. injected in rats 30 min before the isoflurane exposure. The parietal neocortex was removed at 6 h after the isoflurane exposure for Western analysis of the phosphorylated proteins. A, the graphic presentation of the phosphorylated protein abundance quantified by integrating the volume of autoradiograms from four rats. Values in graphs are expressed as -fold change over the isoflurane exposure (Iso-pre) only and presented as the means ± S.D. *, $P < 0.05$ compared with isoflurane exposure only rats. B, a representative Western blot. p-p38, phosphorylated p38 MAPK.

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Address correspondence to: Dr. Zhiyi Zuo, Department of Anesthesiology, University of Virginia Health System, P.O. Box 800710, One Hospital Drive, Charlottesville, VA 22908-0710. E-mail: zz3c@virginia.edu