Chinese Journal of Physiology 53(5): 299-309, 2010
DOI: 10.4077/CJP.2010.AMK063

Role of ERK Signaling in the Neuroprotective Efficacy of Magnesium Sulfate Treatment During Focal Cerebral Ischemia in the Gerbil Cortex

Chih-Yang Huang[1, 2], Yi-Fan Liou[3], Shu-Ying Chung[4], Wen-Yuan Lin[5], Gwo-Ping Jong[6], Chia-Hua Kuo[7], Fuu-Jen Tsai[8], Yi-Chang Cheng[9], Fu-Chou Cheng[10], and Jing-Ying Lin[11]

[1]*Graduate Institute of Basic Medical Science, China Medical University, Taichung*
[2]*Department of Health and Nutrition Biotechnology, Asia University, Taichung*
[3]*Institute of Life Sciences*
[4]*Department of Nursing, Central Taiwan University of Science and Technology, Taichung*
[5]*Department of Family Medicine, China Medical University and Hospital, Taichung*
[6]*Division of Cardiology, Armed Force Taichung General Hospital, Taichung*
[7]*Laboratory of Exercise Biochemistry, Taipei Physical Education College, Taipei*
[8]*Graduate Institute of Chinese Medical Science, China Medical University, Taichung*
[9]*Emergency Department*
[10]*Department of Medical Research, Taichung Veterans General Hospital, Taichung*
and
[11]*Department of Medical Imaging and Radiological Science, Central Taiwan University of Science and Technology, Taichung, Taiwan, Republic of China*

Abstract

Magnesium sulfate ($MgSO_4$) ameliorates focal ischemia-induced neuronal death in the rat and gerbil models. However, the molecular mechanisms for this neuroprotection are not known. Focal cerebral ischemia was produced by unilateral occlusion of the right common carotid artery and the right middle cerebral artery (CCAO + MCAO) for 30 min or 60 min. Treatment with $MgSO_4$ significantly increased the level of mitogen-activated protein kinase/extra-cellular signal-regulated kinase kinase 1/2 (MEK1/2), extra-cellular signal-regulated kinase 1/2 (ERK1/2), cyclic-AMP response element binding protein (CREB) phosphorylation and the anti-apoptotic protein Bcl-2 both in the non-ischemic (contralateral) and ischemic (ipsilateral) cortex. However, these effects were reversed by administration of U0126, a MEK kinase inhibitor. In the ipsilateral cortex, a significant increase in the level of the pro-apoptotic proteins Bax, Bad, BNIP3 and activated caspase 3 were detected at the end of focal ischemia compared to the non-ischemic cortex. Treatment of $MgSO_4$ prevented these ischemia-induced activations of the death cascade. Collectively, these data indicate that the ERK-CREB-Bcl-2 signaling pathway might be involved in $MgSO_4$-induced neuroprotection following focal ischemia. Moreover, $MgSO_4$ treatment also resulted in a reduction in pro-apoptotic proteins. These results enhance our understanding on the role of $MgSO_4$ in treating cerebral ischemia.

Key Words: magnesium sulfate, ERK, CREB, Bcl-2 family, mitochondria, focal cerebral ischemia

Corresponding author: Dr. Jing-Ying Lin, Department of Medical Imaging and Radiological Science, Central Taiwan University of Science and Technology, Taichung 40601, Taiwan, Republic of China. Tel: +886-4-22391647-7119, Fax: +886-4-22396762, E-mail: jylin@ctust.edu.tw
Received: October 2, 2009; Revised: January 14, 2010; Accepted: February 5, 2010.
 ISSN : 0304-4920. http://www.cps.org.tw

Introduction

Magnesium (Mg^{2+}) is widely recognized for its importance in a wide variety of critical cellular processes including oxidative phosphorylation, glycolysis, cellular respiration and protein synthesis (6). The serum level of ionized Mg^{2+} was lower than the normal level in patients shortly after a stroke (3). Ischemia brain injury also decreased Mg^{2+} concentration in the rat brain (36). Moreover, treatment with exogenous Mg^{2+} has been shown to reduce the infarction volume (17), regional cerebral edema, injury-related motor and cognitive dysfunction (22). $MgSO_4$ treatment improved early functional scores after mechanical injury to the spinal cord (8). However, the molecular mechanisms by which $MgSO_4$ intervenes in focal ischemia-induced apoptotic cell death are unclear. In the vascular smooth muscle cells, Mg^{2+} activates ERK/MAPK (34), a well-characterized intracellular signaling cascade implicated in cellular growth and survival (37).

In both *in vivo* and *in vitro* ischemia models, extra-cellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinase (MAPKs) family, has been demonstrated to be involved in regulating brain cell death and survival after ischemia (11, 24). Activation of ERK signaling pathway was linked to neuroprotection in ischemic tolerance and in estradiol protection of CA1 neurons in global ischemia (12, 32). The ERK signaling pathway activates and phosphorylates nuclear transcription factors such as the cAMP-response element binding protein (CREB) which regulates target genes important in neuronal survival and protection. CREB regulates genes that mediate neuronal survival including the brain-derived neurotrophic factor and the anti-apoptotic protein Bcl-2 (13).

Activation of the mitochondria-dependent apoptotic signaling pathway plays a crucial role in ischemic neuronal cell death (20). Since the Bcl-2 family proteins act upstream of the cellular damage and focus their effects at the mitochondria, Bcl-2 family proteins is likely to play an essential role (23). Commitment to apoptosis is typically governed by opposing factions of the Bcl-2 family including pro-apoptotic as opposed to anti-apoptotic family members (20). Pro-apoptotic and anti-apoptotic Bcl-2 family members can homo- or heterodimerize and neutralize each other so that the relative balance of these effectors strongly influences cell fate (23). Bcl-2, an anti-apoptotic protein, prevents cytochrome c release, whereas the pro-apoptotic proteins Bax, Bad and BNIP3 enhance cytochrome c release from the mitochondria. Bad has been reported to play a critical role in the regulation of cell death in cerebral ischemia (23, 40). When cytochrome c is released from the mitochondria into the cytosol, it is responsible for activating caspase-9 which further activates caspase-3, a principal effector caspase of apoptosis, and executes the apoptotic program (23).

Many models have been developed to study focal cerebral ischemia in different animal species. Increasing interests have been shown for developing a reliable focal ischemia model in Mongolian gerbils (4). This animal species lacks the connection between carotid and vertebrobasilar circulation which makes the circle of Willis incomplete resulting in less collateral blood supply to the gerbil's brain. Each hemisphere seems to have an independent blood supply. In this study, unilateral occlusion of the right common carotid artery (CCAO) and the right middle cerebral artery (MCAO) was used to induce focal cerebral ischemia in the right cortex of the gerbils. The results obtained on the ipsilateral (ischemic) and contralateral (non-ischemic) sides from one gerbil could be compared and contrasted (17). Changes in the ERK signaling cascade and mitochondrial apoptogenic proteins in the ipsilateral and contralateral cortexes of these gerbils were measured simultaneously during ischemia, with or without $MgSO_4$ pre-treatment. This study showed that pre-treatment with the MEK inhibitor U0126 reduced Bcl-2 upregulation observed in response to $MgSO_4$ treatment in the ipsilateral cortex 60 min after the onset of ischemia. Moreover, pro-apoptotic proteins were decreased in $MgSO_4$ treatment groups.

Materials and Methods

Induction of Focal Cerebral Ischemia

Male gerbils (65-85 g) were obtained from the Laboratory Animal Center at Taichung Veterans General Hospital (Taichung, Taiwan). These animals were allowed to acclimate to their environmentally controlled quarters (25°C and 12 h : 12 h light-dark cycle) before the experiments. All protocols were approved by the Institutional Animal Care and Use Committee of Central Taiwan University of Science and Technology, Taichung, Taiwan, and the principles of laboratory animal care (NIH publications) were followed.

The gerbil was anesthetized with chlorohydrate (400 mg/kg, i.p.) and its body temperature was maintained at 37°C with a heating pad (CMA/150). A midline neck incision was made and the right carotid artery was exposed and separated from the vago-sympathetic trunks. The right carotid artery was loosely encircled with a 4-O suture for later occlusion. The gerbil's head was placed in a stereotaxic frame (David Kopf, CA, USA) with the nose bar positioned 4.0 mm below the horizontal line. Following a midline

incision, the skull was partially removed to expose the right middle cerebral artery. The middle cerebral artery was loosely encircled with an 8-O suture for later occlusion. A focal cerebral ischemia was induced by occlusion of the right common carotid artery and the right cerebral artery (CCAO + MCAO) for 30 or 60 min. The animals were deeply anesthetized with chlorohydrate and killed at the end of occlusion. Animals were treated with either a single injection of saline or $MgSO_4$ (90 mg/kg, i.p.) at 30 min prior to the occlusion. Isolated cerebral hemispheres and incomplete cerebral Willie's circle are unique anatomical features of gerbils. The ischemia (ipsilateral) side can be compared with the non-ischemia (contralateral) side which, being almost intact, can serve as a control (17). In addition, we have shown previously (17) that gerbils pretreated with $MgSO_4$ have significantly smaller brain infarct volumes than gerbils without $MgSO_4$ pretreatment after the 60 min CCAO + MCAO. The non-ischemia sides of the brain did not have infarct areas after 60 min right CCAO + MCAO. We found that the ipsilateral infarction volume was reduced by 38% by $MgSO_4$ (90 mg/kg, i.p., 10.6 ± 2.3 mm^3) compared with saline treatment (17.1 ± 2 mm^3).

U0126 Intracerebroventricular (icv) Administration

Chlorohydrate-anesthetized gerbils were injected with the MEK inhibitor U0I26 (5 µl 100 µM, in 3% dimethyl sulfoxide) or vehicle (3% dimethyl sulfoxide) at a flow rate of 5 µl/min into the right lateral ventricle 45 min before the onset of CCAO + MCAO. The gerbil's head was placed in a stereotaxic frame with the nose bar positioned 4.0 mm below the horizontal line. A stainless steel cannula (26 gauge) was stereotaxically implanted into the right lateral ventricle (AP-1.3 mm, ML + 2.0 mm, and DL-2.5 mm from bregma).

Western Blotting

Cortex or hippocampus was homogenized in ice-cold lysis buffer for 1 min. The homogenates were centrifuged at 12,000 g for 40 min twice. The supernatant was collected and stored at –70°C for further experiments. Protein concentration of tissue extract was determined using the Bradford method (Bio-Rad Protein Assay, Hercules, CA, USA). Protein homogenates were separated on a 10% SDS-PAGE with a constant voltage of 75 V. Electrophoresed proteins were transferred to 0.45 µm polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) with a transfer apparatus (Bio-Rad, Hercules, CA, USA). The PVDF membranes were incubated in 5% non-fat milk in TBS buffer at room temperature for 1 h. Primary antibodies against pMEK1/2, pERK1, pERK2, CREB, pCREB, Bcl-2, Bax, Bad, BNIP3, caspase 3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and α-tubulin (Neo Markers, Fremont, CA, USA) were diluted in an antibody binding buffer overnight at 4°C. The blots were washed 3 times in TBS buffer for 10 min and then immersed in the second antibody solution containing goat anti-mouse IgG-HRP or goat anti-rabbit IgG-HRP (Santa Cruz) antibodies for 2 h and diluted in TBS buffer. The membranes were washed 3 times for 10 min in TBS buffer. The immunoblotted proteins were visualized using an ECL Western blotting luminal reagent and quantified using a Fujifilm LAS-3000 chemiluminescence detection system (Tokyo, Japan).

Statistical Analysis

The protein levels were compared using one-way ANOVA with preplanned contrast comparison. In all cases, $P < 0.05$ was considered significant.

Results

$MgSO_4$-induced ERK Phosphorylation During Focal Ischemia in Gerbil Cortex

To investigate the effects of ischemia and $MgSO_4$ on ERK activity, we subjected gerbils that had been treated with $MgSO_4$ or saline 30 min before CCAO + MCAO occlusion to examination of pMEK1/2, pERK1 and pERK2 protein levels in the ipsilateral and contralateral cortex at the end of ischemia. Focal cerebral ischemia increased the pMEK1/2 and pERK1 levels in the ischemic (ipsilateral) cortex as compared with non-ischemic (contralateral) cortex at the end of ischemia (Figs. 1a and 1b). Using these focal ischemic models by 30 or 60 min CCAO + MCAO, $MgSO_4$ significantly increased MEK1/2, ERK1/2 phosphorylation both in non-ischemic cortex (60 min ischemia: pMEK1/2 increased to 2.2-fold, $P < 0.01$; pERK1 increased to 33-fold, $P < 0.01$; pERK2 increased to 6.5-fold, Fig. 1b) and in ischemic cortex (60 min ischemia: pMEK1/2 increased to 3.3-fold, $P < 0.01$; pERK1 increased to 8.5-fold, $P < 0.01$; pERK2 increased to 9.2-fold $P < 0.01$, Fig. 1b), as compared with saline-treated gerbils. These findings indicated that focal ischemia induced MEK and ERK1/2 phosphorylation during the ischemic period in the gerbil cortex. $MgSO_4$ administration dramatically induced a marked increases the phosphorylation of MEK1/2 and ERK1/2 in the gerbil cortex in the presence or absence of ischemia.

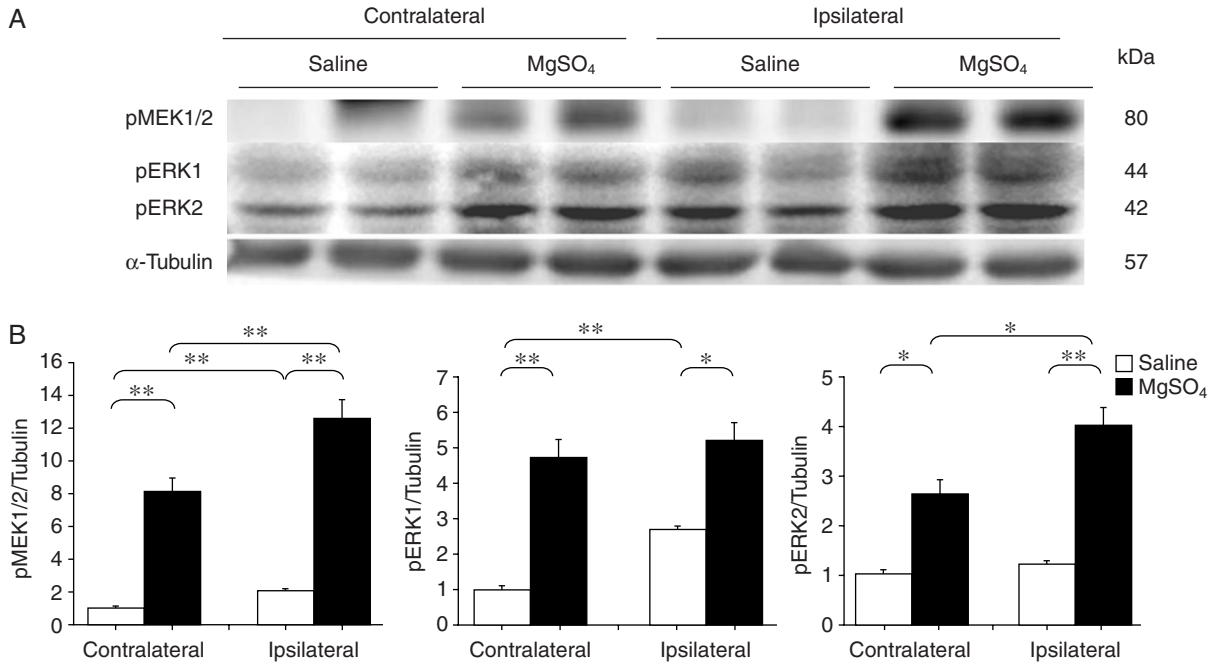

Fig. 1a. $MgSO_4$ increases ERK phosphorylation at the end of 30 min focal ischemia in gerbil cortex. A: The protein products of pMEK1/2, pERK1 and pERK2 extracted from contralateral and ipsilateral cortex at the end of 30 min right CCAO + MCAO in the 2 saline-treated and 2 $MgSO_4$-treated gerbils were measured by Western blots. B: Bars represent the relative proteins quantification of pMEK1/2, pERK1 and pERK2 on the basis of α-tubulin and the mean values ± SD (n = 6 in each group). *$P < 0.05$; **$P < 0.01$.

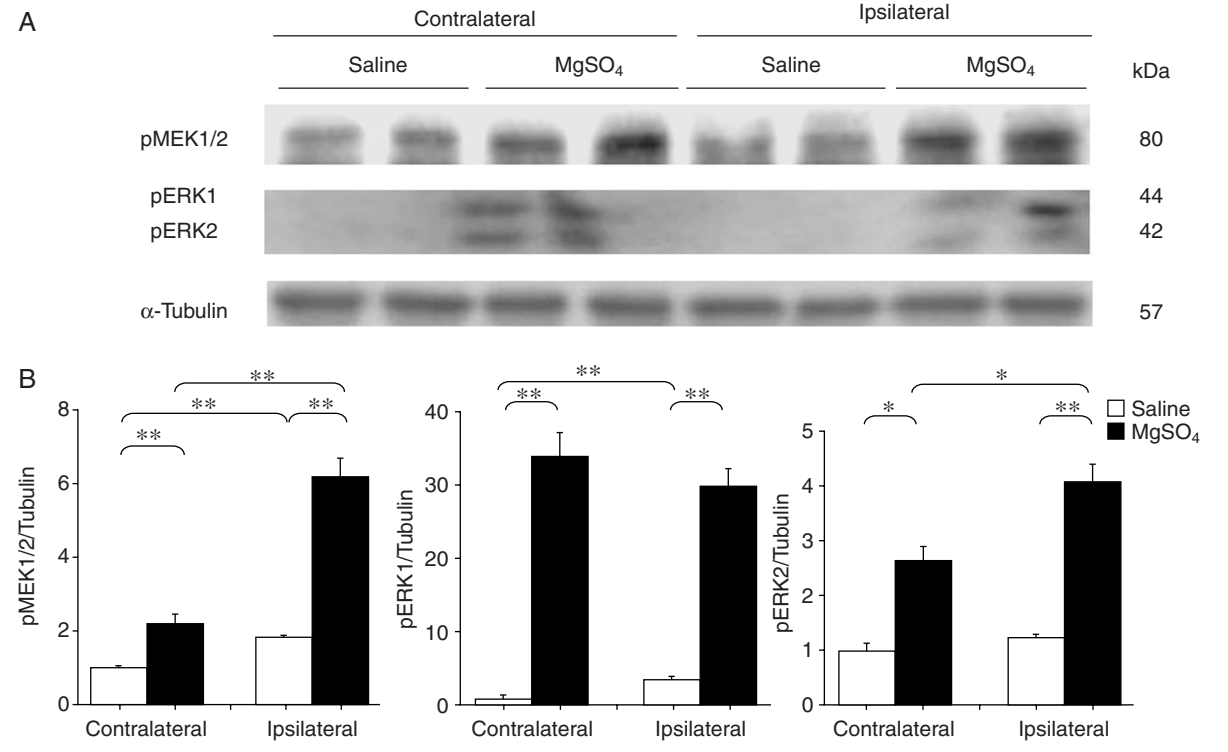

Fig. 1b. $MgSO_4$ increases ERK phosphorylation at the end of 60 min focal ischemia in gerbil cortex. A: The protein products of pMEK1/2, pERK1 and pERK2 extracted from contralateral and ipsilateral cortex at the end of 60 min right CCAO + MCAO in the 2 saline-treated and 2 $MgSO_4$-treated gerbils were measured by Western blots. B: Bars represent the relative proteins quantification of pMEK1/2, pERK1 and pERK2 on the basis of α-tubulin and indicate mean values ± SD (n = 6 in each group). *$P < 0.05$; **$P < 0.01$.

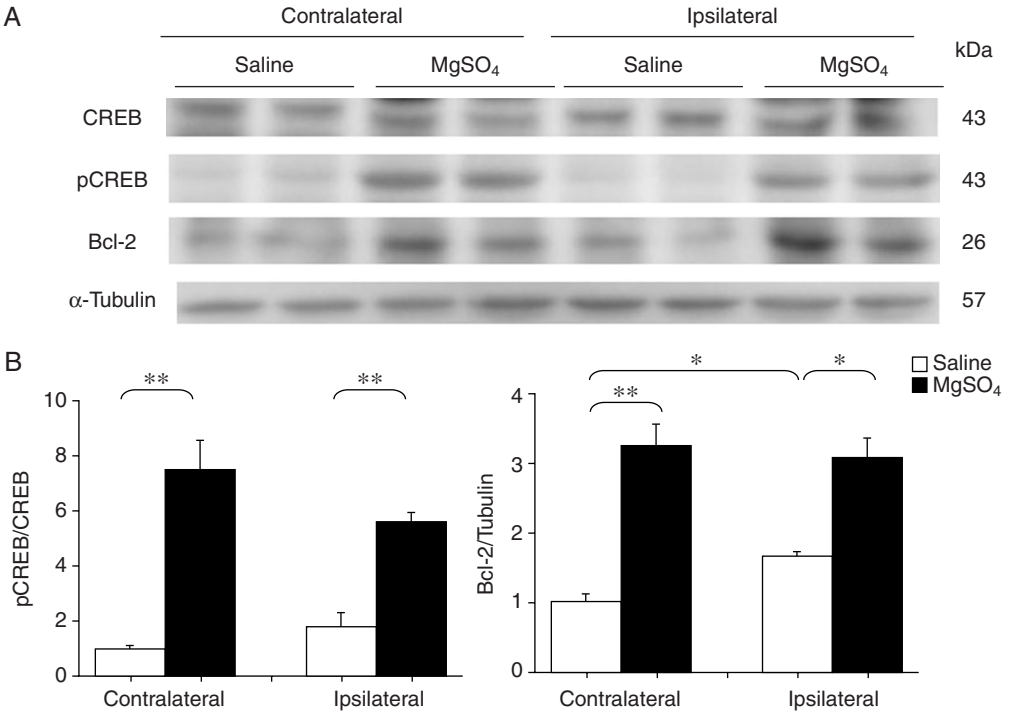

Fig. 2a. $MgSO_4$ increases CREB phosphorylation and Bcl-2 expression at the end of 30 min focal ischemia in gerbil cortex. A: The protein products of CREB, pCREB and Bcl-2 extracted from contralateral and ipsilateral cortex at the end of 30 min right CCAO + MCAO in the 2 saline-treated and 2 $MgSO_4$-treated gerbils were measured by Western blots. B: Bars represent the relative proteins quantification of Bcl-2 on the basis of α-tubulin and indicate mean values ± SD (n = 6 in each group). CREB was used to normalize the densities of pCREB. $*P < 0.05$; $**P < 0.01$.

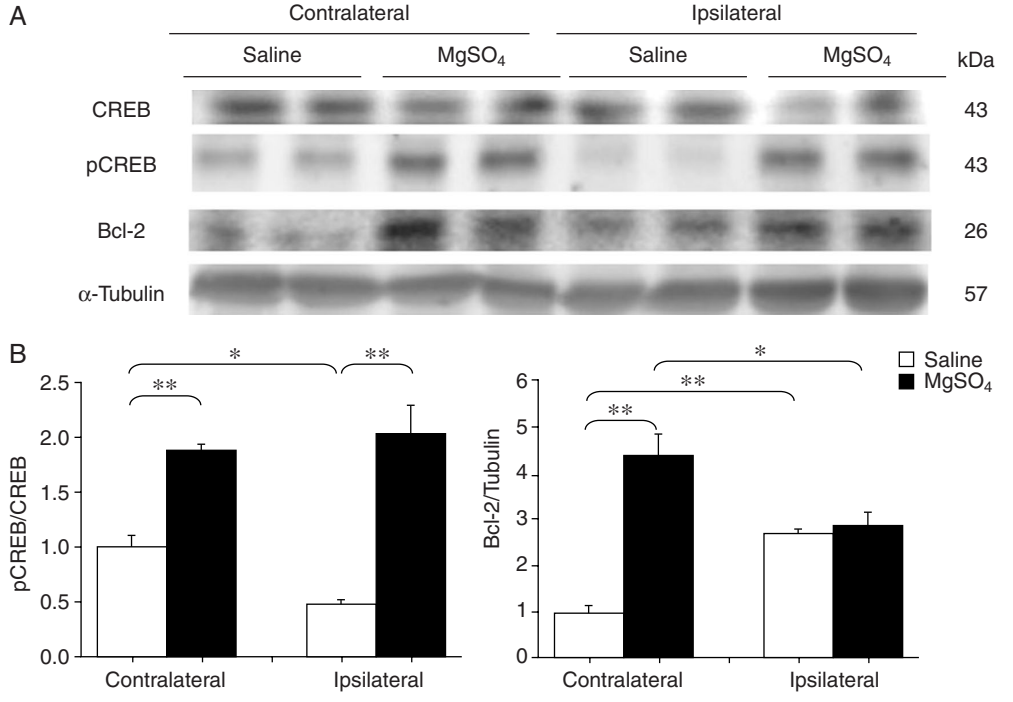

Fig. 2b. $MgSO_4$ increases CREB phosphorylation and Bcl-2 expression at the end of 60 min focal ischemia in gerbil cortex. A: The CREB, pCREB and Bcl-2 protein products extracted from the contralateral and ipsilateral cortex at the end of 60 min right CCAO + MCAO in the 2 saline-treated and 2 $MgSO_4$-treated gerbils were measured by Western blots. B: Bars represent the relative proteins quantification of Bcl-2 on the basis of α-tubulin and indicate mean values ± SD (n = 6 in each group). $*P < 0.05$; $**P < 0.01$.

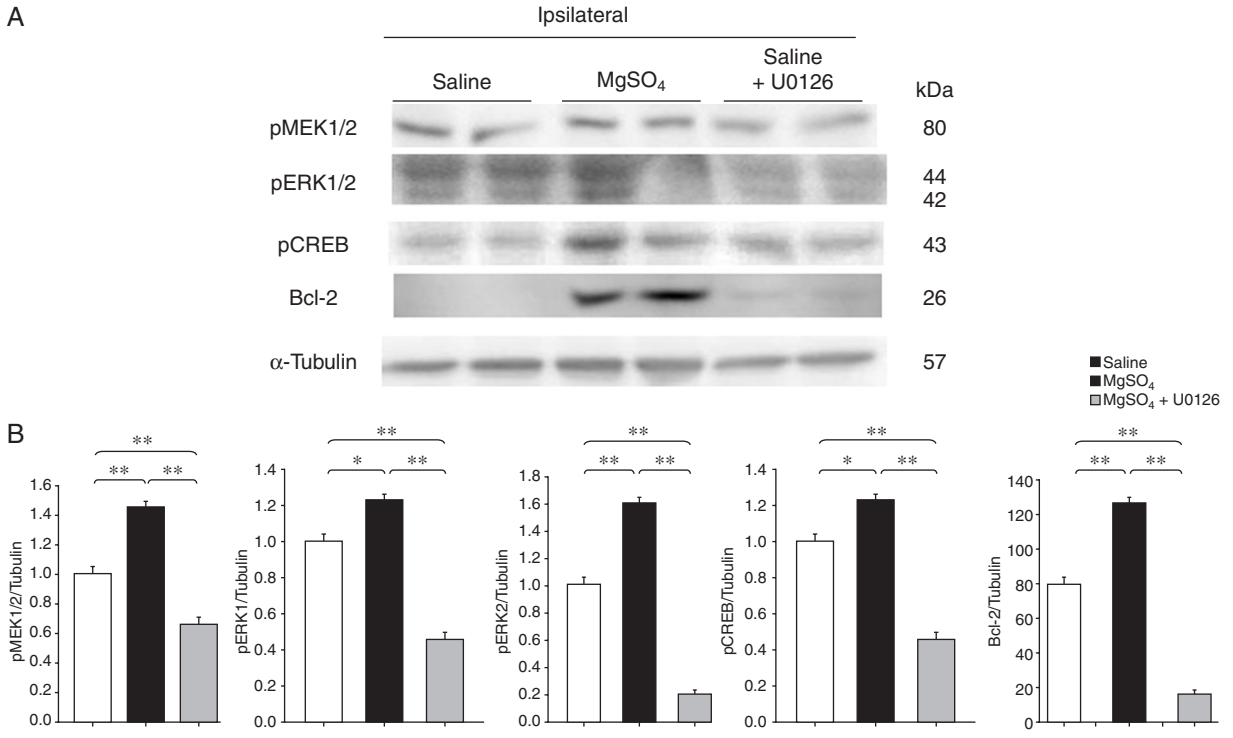

Fig. 3. $MgSO_4$ acts *via* MAPK signaling to induce ERK phosphorylation, CREB phosphorylation and Bcl-2 protein expression. A: The pMEK1/2, pERK1, pERK2, pCREB and Bcl-2 protein products extracted from the ipsilateral cortex at the end of 60 min right CCAO + MCAO in the 2 saline-treated, 2 magnesium sulfate-treated couple with received vehicle icv, and 2 magnesium sulfate-treated couple with received U0126 icv gerbils were measured by Western blots. B: Bars represent the relative proteins quantification of pMEK1/2, pERK1, pERK2, pCREB and Bcl-2 on the basis of α-tubulin and indicate mean values ± SD (n = 6 in each group). **P* < 0.05; ***P* < 0.01.

$MgSO_4$-induced CREB Phosphorylation and Bcl-2 Protein Expression During Focal Ischemia in Gerbil Cortex

A well-characterized downstream target of MAPK/ERK is the transcription factor CREB which promotes transcription of several genes. *Bcl-2* gene is one of the downstream targets of CREB, and Bcl-2 is an antiapoptotic factor implicated in the preservation of integrity of the mitochondrial outer membrane. To examine whether $MgSO_4$ regulates CREB phosphorylation and Bcl-2 protein expression, we subjected $MgSO_4$ or saline-treated gerbils 30 min before CCAO + MCAO to examination of changes in CREB, pCREB and Bcl-2 in ipsilateral and contralateral cortex at the end of ischemia. Thirty minutes of CCAO + MCAO did not affect CREB phosphorylation. Sixty minutes of CCAO + MCAO induced a decrease in pCREB (reduction to 40%, $P < 0.05$) at the end of ischemia as compared with non-ischemic cortex, respectively (Fig. 2b). $MgSO_4$ exposure significantly increased CREB phosphorylation in non-ischemic (increased to 1.8-fold, $P < 0.01$) and ischemic cortex (increased to 5-fold, $P < 0.01$). Incidentally, $MgSO_4$ prevented the 60 min ischemia-induced CREB dephosphorylation (Fig. 2b). Focal ischemia induced an increase in Bcl-2 expression in gerbil cortex at the end of ischemia (60 min ischemia, increased to 2.6-fold, $P < 0.01$, Fig. 2b). $MgSO_4$ exposure significantly increased the expression of Bcl-2 proteins in non-ischemic (60 min ischemia, increased to 4.4-fold, $P < 0.01$, Fig. 2b) and ischemic cortex (30 min ischemia, increased to 1.8-fold, $P < 0.05$; 60 min ischemia, magnesium sulfate maintained the high level of Bcl-2) as compared with saline-treated gerbils.

ERK Signaling Is Critical to $MgSO_4$-Induced CREB Phosphorylation and Bcl-2 Protein Expression

To examine if the MEK/ERK signaling pathway is involved in the $MgSO_4$ ability to maintain CREB phosphorylation and Bcl-2 protein expression, a MEK inhibitor U0126, or vehicle, was administered into the right lateral ventricle 45 min before ischemia. As shown in Fig. 3, U0126 administration blocked the $MgSO_4$-induced phosphorylation increase in MEK1/2, ERK1/2 and CREB and the expression of Bcl-2 proteins in the ischemic (ipsilateral) cortex of gerbils. These results demonstrated that U0126 effectively infused into the right ventricles and inhibited its

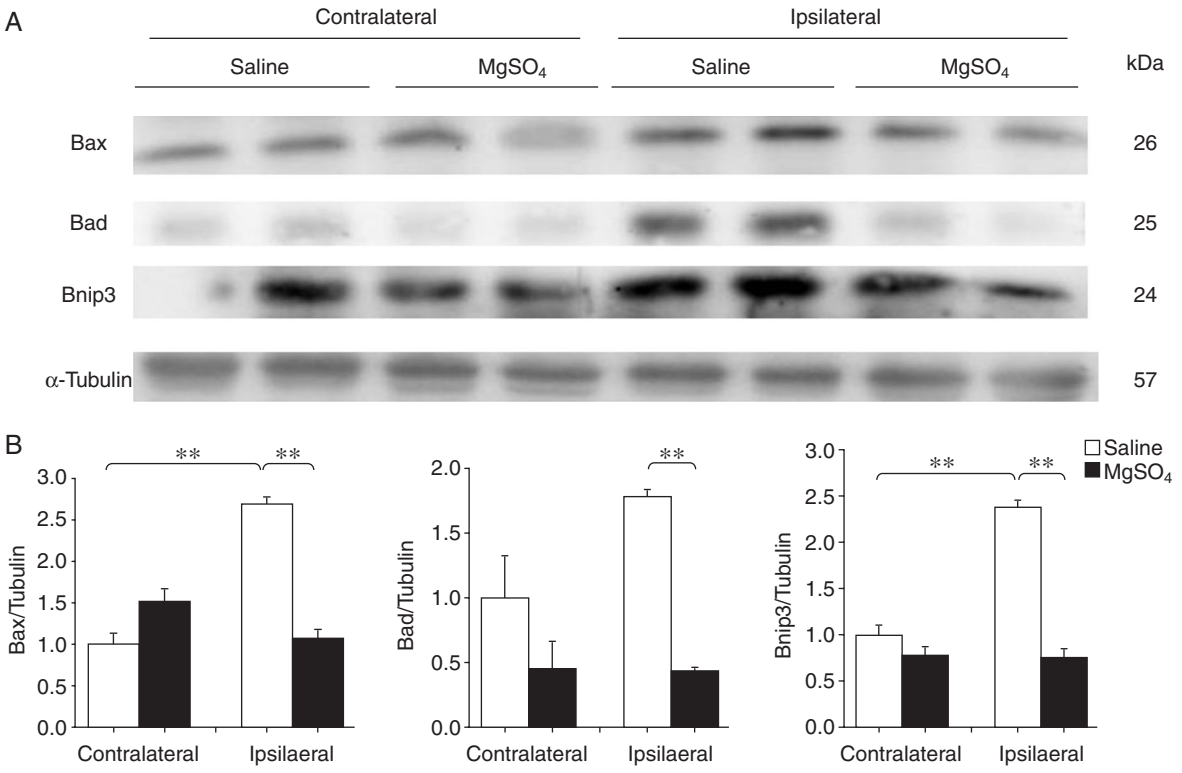

Fig. 4. $MgSO_4$ prevents focal ischemia-induced Bax, Bad and BNIP3 proteins in the ischemic (ipsilateral) cortex of gerbil. A: The Bax, Bad and BNIP3 protein products extracted from contralateral and ipsilateral cortex at the end of 60 min right CCAO + MCAO in the 2 saline-treated and 2 $MgSO_4$-treated gerbils were measured by Western blots. B: Bars represent the relative proteins quantification of Bax, Bad and BNIP3 on the basis of α-tubulin and indicate mean values ± SD (n = 6 in each group). $*P < 0.05$; $**P < 0.01$.

target MEK1/2. These findings suggest a critical role for ERK signaling in the ability of $MgSO_4$ to maintain CREB phosphorylation and to activate Bcl-2 protein expression.

$MgSO_4$ Prevents the Expression of Apoptotic Protein Signaling at the Focal Ischemia in Gerbil Cortex

The proapoptotic proteins Bax, Bad and BNIP3 promote ischemic cell death by inducing the release of apoptogenic factors (1, 10, 40). In this study, we examined the effect of $MgSO_4$ exposure effect on the expression of the Bax, Bad and BNIP3 proteins that could potentially contribute to mitochondrial apoptosis signaling after ischemia. We subjected $MgSO_4$ or saline-treated gerbils for 30 min before CCAO + MCAO occlusion to analysis of the Bax, Bad and BNIP3 proteins in the ipsilateral and contralateral cortex at the end of ischemia. As shown in Fig. 4, 60 min ischemia significantly upregulated the expression of Bax, Bad and BNIP3 proteins in the ischemic (ipsilateral) cortex when compared with non-ischemic (contralateral) cortex. $MgSO_4$ exposure significantly prevented ischemia-induced Bax, Bad and BNIP3 proteins in the ischemic cortex. In contrast, $MgSO_4$ exposure did not affect the expression of Bax and Bad proteins in the non-ischemic cortex. $MgSO_4$ also induced Bcl-2 protein expression in the ischemic cortex. These results suggest that $MgSO_4$ administration in the ischemic cortex could result in a shift in the balance between anti- and pro-apoptotic proteins in favor of promoting cell survival.

$MgSO_4$ Prevents the Activation of Caspase-3 Proteins During Focal Ischemia in Gerbil Cortex

The upregulated pro-apoptotic proteins Bax and Bad disrupt mitochondrial membrane integrity during ischemia leading to the release of cytochrome C and the activation of caspase-3, a terminator caspase implicated in the execution step of apoptosis (1, 10, 40). Sixty-minute focal ischemia induced a 5-fold increase in the activated forms of the caspase 3 protein when compared with non-ischemic (contralateral) cortex. Consistent with the results from other pro-apoptotic proteins, the active caspase 3 (17 kDa) in the ischemic cortex was also significantly reduced by $MgSO_4$ exposure (Fig. 5).

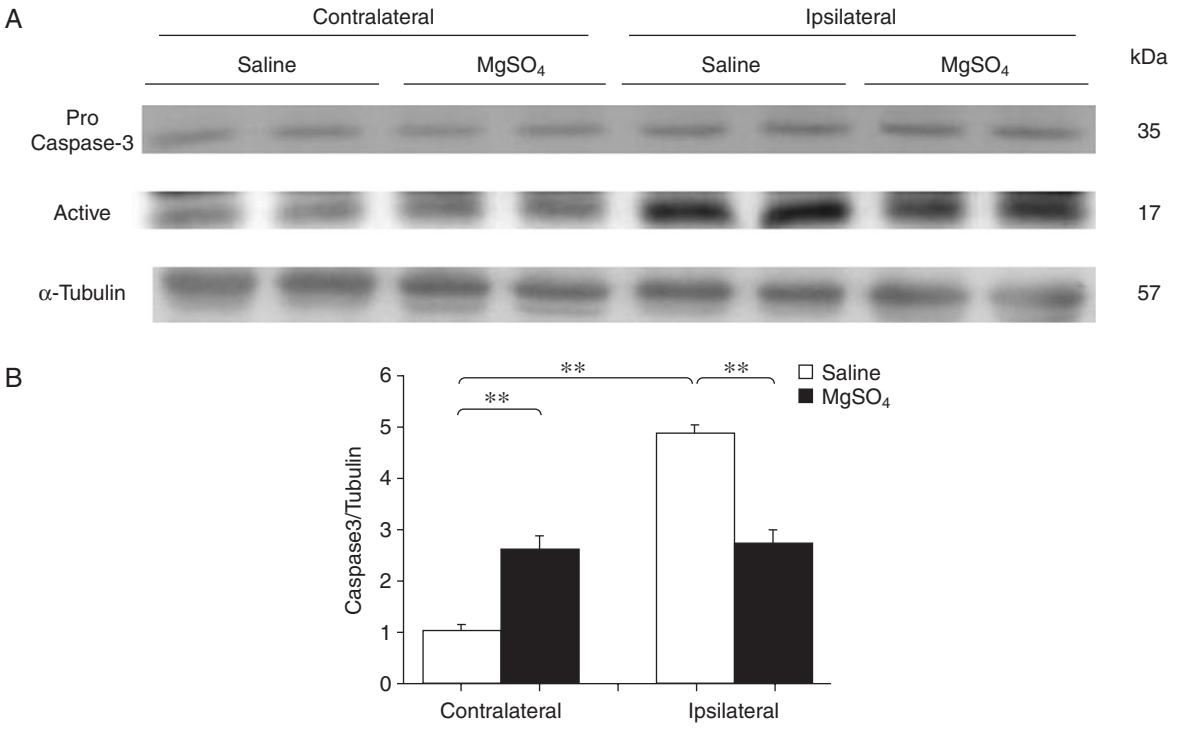

Fig. 5. $MgSO_4$ prevents focal ischemia-induced activation of caspase 3 in the ischemic (ipsilateral) cortex of gerbil. A: The activated caspase 3 protein products extracted from contralateral and ipsilateral cortex at the end of 60 min right CCAO + MCAO in the 2 saline-treated and 2 $MgSO_4$-treated gerbils were measured by Western blots. B: Bars represent the relative proteins quantification of activated caspase 3 on the basis of α-tubulin and indicate mean values ± SD (n = 6 in each group). $*P < 0.05$; $**P < 0.01$.

Discussion

It has been shown that $MgSO_4$ treatment has a neuroprotective effect on reversing focal ischemia in the gerbil model (17). Here we demonstrated that ERK-dependent CREB phosphorylation and CREB-mediated Bcl-2 expression occurred in $MgSO_4$-treated gerbils when subjected to focal ischemia. Furthermore, we found that administration of $MgSO_4$ could prevent ischemia-induced the pro-apoptotic proteins Bax, Bad, BNIP3 and caspase 3 activation.

The role of phosphorylated ERK in neuronal survival or death is controversial. Our study showed that after 30 min or 60 min focal cerebral ischemia in gerbil, the pMEK1/2 and pERK1/2 levels were slightly increased within the cortex. Administration of $MgSO_4$ significantly upregulated the pERK1/2 signaling pathway during focal ischemia. Furthermore, we found that ERK1/2 activation caused by ischemia alone, or ischemia with $MgSO_4$ treatment, differentially activated downstream-signaling pathways. ERK1/2 was activated caused by ischemia with $MgSO_4$ treatment coupled to CREB phosphorylation. In contrast, slight ERK1/2 activation caused by ischemia alone was unable to link with CREB phosphorylation, and might even have reduced the level of CREB phosphorylation in ischemia alone suggesting that other downstream cascades other than CREB phosphorylation might be involved subsequent to ERK activation.

Slight ERK1/2 activation caused by ischemia alone may mediate injury-induced tissue damages. ERK-mediated cell death has been reported in numerous animal models (41). In a mouse model of stroke induced by transient occlusion of the middle cerebral artery, an increase in phosphorylated ERK in the cortical cells was detected, and pretreatment with the MEK inhibitor PD98059 blocked ERK phosphorylation resulting in a 55% decrease in focal infarct volume at 22 h (2). In a forebrain gerbil model of ischemia induced by bilateral carotid artery occlusion, Namura *et al*. obtained similar results using another MEK inhibitor U0126 (24). What are the mechanisms for slight ERK1/2 activation caused by ischemia alone that induced tissue damage? Wang *et al*. found that ERK1/2 acted upstream of caspase-3 in the brain after ischemia/reperfusion (39). ERK1/2 may act on mitochondria through Bax and p53. In osteoblastic cells, Bax expression is increased after treatment with H_2O_2, and inhibition of the ERK pathway decreased Bax expression (26). The mechanism by which ERK mediates cell death is mediated *via* activation of

caspase 3, Bax and p53 (41). In our study, we found that ischemia alone induced expression of Bax, Bad and the activation of caspase 3 but not the expression of Bcl-2 and CREB phosphorylation suggesting the presence of caspase death cascades and the absence of the endogenous survival and repair mechanisms. Slight ERK1/2 activation may participate in these signaling pathways but other intracellular signal transduction pathways may also be involved.

In contrast, many studies have shown that ERK activation is a neuroprotective signal (9, 12, 28, 32). In ischemic animal models, neuroprotective exposures such as hypothermia and preconditioning have been linked to ERK activation (9, 32). Cho *et al*. showed that N-acetyl-O-methyldopamine (NAMDA) activated ERK1/2 and protected hippocampal neurons after exposure to *in vitro* and *in vivo* ischemia (28). Furthermore, estradiol, the primary estrogen secreted by the ovary, has been shown to afford neuroprotection by activating ERK in models of global ischemia (12), quinolinic acid (15) and glutamate excitotoxicity (31), oxidative stress (21) and β-amyloid toxicity (7). In addition, NAMDA- and estrogen-induced neuroprotection *via* ERK activation also leads to CREB phosphorylation. In this study, we observed that $MgSO_4$ maintained CREB phosphorylation in the face of ischemia and that U0126 reversed magnesium sulfate-dependent CREB phosphorylation indicating a causal relation between ERK MAPK signaling and CREB activation. Therefore, $MgSO_4$-induced neuroprotection *via* ERK activation leading to CREB phosphorylation may be a key step toward transcription of a CRE-mediated gene that is necessary for survival.

CREB phosphorylation has been linked to neuroprotection in experimental animal stroke models (18, 38) and inhibition of ERK by U0126 reversed the protective effect of CREB following transient global ischemia (28). Phosphorylation of CREB influences the expression of subsequent CRE-mediated genes including BDNF, *c-fos* and the antiapoptotic protein Bcl-2. Accumulating evidence indicates that increased Bcl-2 expression provides protection against apoptosis and ischemic insults (14, 19). Thus, the protective effect of CREB phosphorylation against ischemic neuronal death may be attributable to increased Bcl-2 expression. In the present study, we observed increased Bcl-2 expression 90 min after exposure to $MgSO_4$ both in ischemic (ipsilateral) cortex and nonischemic (contralateral) cortex sides, the former being inhibited by pretreatment with U0126, suggesting that Bcl-2 expression was induced by ERK MAPK-mediated CREB phosphorylation and might be involved in the neuroprotective role of $MgSO_4$ after exposure to ischemia.

Many studies have suggested that cerebral ischemia disrupt the functional integrity of the outer mitochondrial membrane releasing cytochrome c and Smac/DIABLO which subsequently activates the caspase death cascades (25, 33). The Bcl-2 family of proteins includes both pro-apoptotic and anti-apoptotic members and the relative balance of each other strongly influences cell fate (20). The anti-apoptotic proteins, such as Bcl-2, enhance cell survival after ischemia by preserving mitochondrial membrane integrity (19). Our findings suggest that $MgSO_4$ promotes neuronal survival, at least in part, by increasing expression levels of Bcl-2; our data are consistent with the observation that Bcl-2 mediates magnesium neuroprotection in traumatic brain injury models (16). In contrast, the pro-apoptotic proteins Bax, Bad and Bim promote ischemic cell death by inducing the release of apoptogenic factors from the mitochondria and by activating the caspase death cascades (5, 40). In the current study, the expression of Bax, Bad and BNIP3 was found to be significantly increased in the ischemic cortex. $MgSO_4$ treatment significantly decreased these pro-apoptotic protein expression lelves in the ischemic cortex. Our results indicate that $MgSO_4$ treatment upregulated the antiapoptotic Bcl-2 family proteins (Bcl-2) as well as downregulated the proapoptotic Bcl-2 family proteins (Bax, Bad and BNIP3) in ischemic cortex of gerbils resulting in a shift in the balance between antiapoptotic and proapoptotic effects toward the former. In other words, our findings suggest that $MgSO_4$ treatment shifted the balance of Bcl-2 family toward antiapoptotic effects in ischemic cortex of gerbils. Similar findings were observed in cortical neurons of newborn piglets in which hypoxia decreased the Bcl-2/Bax ratio while $MgSO_4$ treatment increased it (29).

Shifting the balance of the Bcl-2 family proteins toward antiapoptotic effects blocks the release of apoptogenic factors from the mitochondria and inactivating the caspase death cascades. Consistent with this idea, our results showed that active caspase-3, a terminator caspase implicated in the execution step of apoptosis, was decreased by $MgSO_4$ treatment in the ischemic cortex. These results suggest that $MgSO_4$ treatment provides protection by shifting the Bcl-2 family balance toward antiapoptotic effects to block the activation of caspase 3. Consistent with our findings, previous studies have shown that $MgSO_4$ reduced the number of apoptotic neurons and the activation of caspase-3 in a moderate diffuse axonal injury model and in perinatal hypoxic-ischemic brain injury models (27, 30, 35).

In summary, this study showed that the ERK-CREB-Bcl-2 signaling pathway might be involved in $MgSO_4$-induced neuroprotection following focal cerebral ischemia. Additionally, $MgSO_4$ treatment also decreased the expression of proapoptotic proteins Bax, Bad, BNIP3 and the activation of caspase 3. The

findings from the present study suggest that $MgSO_4$ protects neurons from apoptotic death by regulating the activity of specific cell survival and death pathways and that interference with these signaling pathways may lead to neuroprotection.

Acknowledgments

This work was supported in part by the grant from the Central Taiwan University of Science and Technology (grant No: TCVGH-CTUST977701 and CTU96-P-20). This study was also supported in part by Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH99-TD-B-111-004) and in part by Taiwan Department of Health Cancer Research Center of Excellence (DOH99-TD-C-111-005).

References

1. Abe, T., Takagi, N., Nakano, M., Furuya, M. and Takeo, S. Altered Bad localization and interaction between Bad and Bcl-xL in the hippocampus after transient global ischemia. *Brain Res*. 1009: 159-168, 2004.
2. Alessandrini, A., Namura, S., Moskowitz, M.A. and Bonventre, J.V. MEK1 protein kinase inhibition protects against damage resulting from focal cerebral ischemia. *Proc. Natl. Acad. Sci. U.S.A.* 96: 12866-12869, 1999.
3. Altura, B.T., Memon, Z.I., Zhang, A., Cheng, T.P., Silverman, R., Cracco, R.Q. and Altura, B.M. Low levels of serum ionized magnesium are found in patients early after stroke which result in rapid elevation in cytosolic free calcium and spasm in cerebral vascular muscle cells. *Neurosci. Lett*. 230: 37-40, 1997.
4. Baskaya, M.K., Dogan, A. and Dempsey, R.J. Application of endovascular suture occlusion of middle cerebral artery in gerbils to obtain consistent infarction. *Neurol. Res*. 21: 574-578, 1999.
5. Cao, G., Minami, M., Pei, W., Yan, C., Chen, D., O'Horo, C., Graham, S.H. and Chen, J. Intracellular Bax translocation after transient cerebral ischemia: implications for a role of the mitochondrial apoptotic signaling pathway in ischemic neuronal death. *J. Cereb. Blood Flow Metab*. 21: 321-333, 2001.
6. Ebel, H. and Gunther, T. Magnesium metabolism: a review. *J. Clin. Chem. Clin. Biochem*. 18: 257-270, 1980.
7. Fitzpatrick, J.L., Mize, A.L., Wade, C.B., Harris, J.A., Shapiro, R.A. and Dorsa, D.M. Estrogen-mediated neuroprotection against beta-amyloid toxicity requires expression of estrogen receptor alpha or beta and activation of the MAPK pathway. *J. Neurochem*. 82: 674-682, 2002.
8. Gok, B., Okutan, O., Beskonakli, E. and Kilinc, K. Effects of magnesium sulphate following spinal cord injury in rats. *Chinese J. Physiol*. 50: 93-97, 2007.
9. Hicks, S.D., Parmele, K.T., DeFranco, D.B., Klann, E. and Callaway, C.W. Hypothermia differentially increases extracellular signal-regulated kinase and stress-activated protein kinase/c-Jun terminal kinase activation in the hippocampus during reperfusion after asphyxial cardiac arrest. *Neuroscience* 98: 677-685, 2000.
10. Hsu, Y.T., Wolter, K.G. and Youle, R.J. Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* 94: 3668-3672, 1997.
11. Irving, E.A. and Bamford, M. Role of mitogen- and stress-activated kinases in ischemic injury. *J. Cereb. Blood Flow Metab*. 22: 631-647, 2002.
12. Jover-Mengual, T., Zukin, R.S. and Etgen, A.M. MAPK signaling is critical to estradiol protection of CA1 neurons in global ischemia. *Endocrinology* 148: 1131-1143, 2007.
13. Kitagawa, K. CREB and cAMP response element-mediated gene expression in the ischemic brain. *FEBS J*. 274: 3210-3217, 2007.
14. Kitagawa, K., Matsumoto, M., Tsujimoto, Y., Ohtsuki, T., Kuwabara, K., Matsushita, K., Yang, G., Tanabe, H., Martinou, J.C., Hori, M. and Yanagihara, T. Amelioration of hippocampal neuronal damage after global ischemia by neuronal overexpression of BCL-2 in transgenic mice. *Stroke* 29: 2616-2621, 1998.
15. Kuroki, Y., Fukushima, K., Kanda, Y., Mizuno, K. and Watanabe, Y. Neuroprotection by estrogen *via* extracellular signal-regulated kinase against quinolinic acid-induced cell death in the rat hippocampus. *Eur. J. Neurosci*. 13: 472-476, 2001.
16. Lee, J.S., Han, Y.M., Yoo, D.S., Choi, S.J., Choi, B.H., Kim, J.H., Kim, Y.H., Huh, P.W., Ko, Y.J., Rha, H.K., Cho, K.S. and Kim, D.S. A molecular basis for the efficacy of magnesium treatment following traumatic brain injury in rats. *J. Neurotrauma* 21: 549-561, 2004.
17. Lin, J.Y., Chung, S.Y., Lin, M.C. and Cheng, F.C. Effects of magnesium sulfate on energy metabolites and glutamate in the cortex during focal cerebral ischemia and reperfusion in the gerbil monitored by a dual-probe microdialysis technique. *Life Sci*. 71: 803-811, 2002.
18. Mabuchi, T., Kitagawa, K., Kuwabara, K., Takasawa, K., Ohtsuki, T., Xia, Z., Storm, D., Yanagihara, T., Hori, M. and Matsumoto, M. Phosphorylation of cAMP response element-binding protein in hippocampal neurons as a protective response after exposure to glutamate *in vitro* and ischemia *in vivo*. *J. Neurosci*. 21: 9204-9213, 2001.
19. Martinou, J.C., Dubois-Dauphin, M., Staple, J.K., Rodriguez, I., Frankowski, H., Missotten, M., Albertini, P., Talabot, D., Catsicas, S., Pietra, C. and *et al*. Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. *Neuron* 13: 1017-1030, 1994.
20. Mehta, S.L., Manhas, N. and Raghubir, R. Molecular targets in cerebral ischemia for developing novel therapeutics. *Brain Res. Rev*. 54: 34-66, 2007.
21. Mize, A.L., Shapiro, R.A. and Dorsa, D.M. Estrogen receptor-mediated neuroprotection from oxidative stress requires activation of the mitogen-activated protein kinase pathway. *Endocrinology* 144: 306-312, 2003.
22. Muir, K.W. Magnesium in stroke treatment. *Postgrad. Med. J*. 78: 641-645, 2002.
23. Nakka, V.P., Gusain, A., Mehta, S.L. and Raghubir, R. Molecular mechanisms of apoptosis in cerebral ischemia: multiple neuroprotective opportunities. *Mol. Neurobiol*. 37: 7-38, 2008.
24. Namura, S., Iihara, K., Takami, S., Nagata, I., Kikuchi, H., Matsushita, K., Moskowitz, M.A., Bonventre, J.V. and Alessandrini, A. Intravenous administration of MEK inhibitor U0126 affords brain protection against forebrain ischemia and focal cerebral ischemia. *Proc. Natl. Acad. Sci. U.S.A.* 98: 11569-11574, 2001.
25. Ouyang, Y.B., Tan, Y., Comb, M., Liu, C.L., Martone, M.E., Siesjo, B.K. and Hu, B.R. Survival- and death-promoting events after transient cerebral ischemia: phosphorylation of Akt, release of cytochrome C and activation of caspase-like proteases. *J. Cereb. Blood Flow Metab*. 19: 1126-1135, 1999.
26. Park, B.G., Yoo, C.I., Kim, H.T., Kwon, C.H. and Kim, Y.K. Role of mitogen-activated protein kinases in hydrogen peroxide-induced cell death in osteoblastic cells. *Toxicology* 215: 115-125, 2005.
27. Park, C.O. and Hyun, D.K. Apoptotic change in response to magnesium therapy after moderate diffuse axonal injury in rats. *Yonsei. Med. J*. 45: 908-916, 2004.
28. Park, E.M., Joh, T.H., Volpe, B.T., Chu, C.K., Song, G. and Cho, S. A neuroprotective role of extracellular signal-regulated kinase in N-acetyl-O-methyldopamine-treated hippocampal neurons after exposure to *in vitro* and *in vivo* ischemia. *Neuroscience* 123: 147-154, 2004.

29. Ravishankar, S., Ashraf, Q.M., Fritz, K., Mishra, O.P. and Delivoria-Papadopoulos, M. Expression of Bax and Bcl-2 proteins during hypoxia in cerebral cortical neuronal nuclei of newborn piglets: effect of administration of magnesium sulfate. *Brain Res*. 901: 23-29, 2001.
30. Sameshima, H. and Ikenoue, T. Effect of long-term, postasphyxial administration of magnesium sulfate on immunostaining of microtubule-associated protein-2 and activated caspase-3 in 7-day-old rat brain. *J. Soc. Gynecol. Investig*. 9: 203-209, 2002.
31. Singer, C.A., Figueroa-Masot, X.A., Batchelor, R.H. and Dorsa, D.M. The mitogen-activated protein kinase pathway mediates estrogen neuroprotection after glutamate toxicity in primary cortical neurons. *J. Neurosci*. 19: 2455-2463, 1999.
32. Strohm, C., Barancik, T., Bruhl, M.L., Kilian, S.A.R. and Schaper, W. Inhibition of the ER-kinase cascade by PD98059 R. and U0126 counteracts ischemic preconditioning in pig myocardium. *J. Cardiovasc. Pharmacol*. 36: 218-229, 2000.
33. Sugawara, T., Noshita, N., Lewen, A., Gasche, Y., Ferrand-Drake, M., Fujimura, M., Morita-Fujimura, Y. and Chan, P.H. Overexpression of copper/zinc superoxide dismutase in transgenic rats protects vulnerable neurons against ischemic damage by blocking the mitochondrial pathway of caspase activation. *J. Neurosci*. 22: 209-217, 2002.
34. Touyz, R.M. and Yao, G. Modulation of vascular smooth muscle cell growth by magnesium-role of mitogen-activated protein kinases. *J. Cell Physiol*. 197: 326-335, 2003.
35. Turkyilmaz, C., Turkyilmaz, Z., Atalay, Y., Soylemezoglu, F. and Celasun, B. Magnesium pre-treatment reduces neuronal apoptosis in newborn rats in hypoxia-ischemia. *Brain Res*. 955: 133-137, 2002.
36. Vande Linde, A.M., Chopp, M., Chen, H., Helpern, J.A., Knight, R., Schultz, L. and Welch, K.M. Chronic changes in the brain Mg^{2+} concentration after forebrain ischemia in the rat. *Metab. Brain Dis*. 6: 199-206, 1991.
37. Waas, W.F. and Dalby, K.N. Physiological concentrations of divalent magnesium ion activate the serine/threonine specific protein kinase ERK2. *Biochemistry* 42: 2960-2970, 2003.
38. Walton, M.R. and Dragunow, I. Is CREB a key to neuronal survival? *Trends Neurosci*. 23: 48-53, 2000.
39. Wang, X., Wang, H., Xu, L., Rozanski, D.J., Sugawara, T., Chan, P.H., Trzaskos, J.M. and Feuerstein, G.Z. Significant neuroprotection against ischemic brain injury by inhibition of the MEK1 protein kinase in mice: exploration of potential mechanism associated with apoptosis. *J. Pharmacol. Exp. Ther*. 304: 172-178, 2003.
40. Yin, X.M., Luo, Y., Cao, G., Bai, L., Pei, W., Kuharsky, D.K. and Chen, J. Bid-mediated mitochondrial pathway is critical to ischemic neuronal apoptosis and focal cerebral ischemia. *J. Biol. Chem*. 277: 42074-42081, 2002.
41. Zhuang, S. and Schnellmann, R.G. A death-promoting role for extracellular signal-regulated kinase. *J. Pharmacol. Exp. Ther*. 319: 991-997, 2006.