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Attenuation of Magnesium Sulfate on $CoCl_2$-Induced Cell Death by Activating ERK1/2/MAPK and Inhibiting HIF-1α *via* Mitochondrial Apoptotic Signaling Suppression in a Neuronal Cell Line

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Abstract

Magnesium sulfate ($MgSO_4$) ameliorates hypoxia/ischemia-induced neuronal apoptosis in a rat model. This study aimed to investigate the mechanisms governing the anti-apoptotic effect of $MgSO_4$ on cobalt chloride ($CoCl_2$)-exposed NB41A3 mouse neuroblastoma cells. $MgSO_4$ increased the viability of NB41A3 cells treated with $CoCl_2$ in a dose-dependent manner. $MgSO_4$ treatment was shown to lead to an increase in the anti-apoptotic Bcl-2 family proteins, with a concomitant decrease in the pro-apoptotic proteins. $MgSO_4$ also attenuated the $CoCl_2$-induced disruption of mitochondrial membrane potential ($\Delta\Psi_m$) and reduced the release of cytochrome *c* form the mitochondria to the cytosol. Furthermore, exposure to $CoCl_2$ caused activation of the hypoxia-inducible factor 1α (HIF-1α). On the other hand, $MgSO_4$ markedly reduced $CoCl_2$-induced HIF-1α activation and suppressed HIF-1α downstream protein BNIP3. $MgSO_4$ treatment induced ERK1/2 activation and attenuated $CoCl_2$-induced activation of p38 and JNK. Addition of the ERK1/2 inhibitor U0126 significantly reduced the ability of $MgSO_4$ to protect neurons from $CoCl_2$-induced mitochondrial apoptotic events. However, incubation of cultures with the p38 and JNK inhibitors did not significantly affect $MgSO_4$-mediated neuroprotection. $MgSO_4$ appears to suppress $CoCl_2$-induced NB41A3 cell death by activating ERK1/2/ MAPK pathways, which further modulates the role of Bcl-2 family proteins and mitochondria in NB41A3 cells. Our data suggest that $MgSO_4$ may act as a survival factor that preserves mitochondrial integrity and inhibits apoptotic pathways.

Key Words: apoptosis, Bcl-2 family, $CoCl_2$, ERK1/2, $MgSO_4$, mitochondria

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Introduction

Numerous studies in a variety of animal models have shown that magnesium has a protective effect against neurodegenerative diseases (6, 24, 39). The mechanisms underlying $MgSO_4$-mediated protection are complex, including anti-excitotoxic effects through inhibition of glutamate release (20, 31), antagonizing calcium entry *via* NMDA and voltage gated ion channels (22), lipid peroxidation inhibition (31) and increasing neuronal expression of glucose transporter 3 (13). However, the precise cellular mechanisms of $MgSO_4$-induced neuroprotection are not fully elucidated. Previous studies have suggested that magnesium pre-treatment reduces neuronal apoptosis in newborn rats with hypoxia-ischemia (39).

Apoptosis plays a crucial role in the pathogenesis of neuronal diseases (21). Mitochondria are the central executioners of apoptosis (25). The anti-apoptotic proteins such as Bcl-2 and Bcl-xL enhance cell survival by maintaining mitochondrial membrane integrity and preventing cytochrome *c* release from the mitochondria into the cytosol (5, 19). However, Bad, Bid and BNIP3 function as the pro-apoptotic proteins by opening the permeability transition pore to enhance cytochrome *c* release. Bad and Bid have been reported to play a critical role in the regulation of cell death in cerebral ischemia (25, 42). Besides Bcl-2 family proteins, hypoxia-inducible factor 1α (HIF-1α) and HIF-1α target genes have recently been shown to protect or destroy hypoxic neurons under hypoxic/ischemic stress (34). In rodents and human cells, HIF-1α activates BNIP3 (6, 21), contributing to cell death under hypoxia.

In both *in vitro* and *in vivo* ischemia models, mitogen-activated protein kinases (MAPK) signaling plays an important role in regulating cell death and survival following ischemia (14). The three major classes characterized in mammals are: the extracellular signal-regulated kinase (ERK1/2), the c-Jun N-terminal kinase (JNK) and p38 reactivating kinase (p38 MAPK). ERK1/2 is thought to be involved in cell survival, whereas p38 and JNK are associated with cell stress and death (2, 14, 44). The ERK1/2 signaling pathway can activate and phosphorylate several nuclear transcription factors such as cAMP-response element binding protein (CREB) and then upregulate Bcl-2 (17, 18).

Cerebral hypoxic/ischemic insults trigger multiple cellular events resulting in neuronal apoptosis. Studies have reported that cobalt chloride ($CoCl_2$) could mimic hypoxic/ischemic conditions (19). In this study, $CoCl_2$-treated NB41A3 neuroblastoma cells were used as a hypoxic model to explore whether $MgSO_4$ protected against $CoCl_2$-induced cytotoxicity, and found that $MgSO_4$ inhibited $CoCl_2$-induced loss of cell viability. We also investigated whether such neuroprotection by $MgSO_4$ could be related to apoptotic pathway *via* the mitochondria.

Materials and Methods

Materials

A NB41A3 mouse neuroblastoma cell line was obtained from the American Tissue Culture Collection (ATCC). Ham's F10 medium, fetal bovine serum (FBS), horse serum, penicillin and streptomycin were obtained from Gibco (Grand Island, NY, USA). $MgSO_4$, $CoCl_2$ and 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (Sigma Chemical Co., St. Louis, Missouri, USA). U0126 (MEK1/2 inhibitor), SB203680 (p38 MAPK inhibitor) and SP600125 (JNK inhibitor) were purchased from TOCRIS (Ellisville, Missouri, USA). 5,58,6,68-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) was purchased from BioVision (Palo Alto, CA, USA).

Cell Cultures

NB41A3 cells were cultured in Ham's F10 medium supplemented with 2.5% fetal bovine serum (FBS), 15% horse serum and 1% penicillin/streptomycin. All cells were grown in a 5% CO_2 incubator at 37°C. $CoCl_2$ and $MgSO_4$ were dissolved in distilled H_2O and sterilized through a 0.2 μm filter before use. Cells were pretreated with $MgSO_4$ for 1 h, and then treated with $CoCl_2$ for 24 h. To examine the effects of $CoCl_2$ and $MgSO_4$ on MAPKs activities, cells were incubated with the the pharmacological inhibitors U0126 (0.5 μM), SB203680 (0.5 μM) or SP600125 (0.5 μM) in the medium for 1 h prior to treatment with $MgSO_4$.

Cytotoxicity Assay

NB41A3 cells were plated onto new plates and allowed to attach for 5 h before being exposed to 500 μM $CoCl_2$ alone, or pretreated with $MgSO_4$ (1 or 2 mM) for 1 h. MTT was added to the culture medium at a final concentration of 500 μg/ml and incubated at 37°C for 4 h. The MTT reaction product was extracted in dimethyl sulfoxide (DMSO) and optical density was then measured in an enzyme-linked immunosorbent assay plate reader at 570 nm with DMSO as the blank.

Western Blot Analysis

NB41A3 cells were scraped and washed once with phosphate buffer saline (PBS). Cell samples were lysed for 30 min in lysis buffer (50 mM Tris,

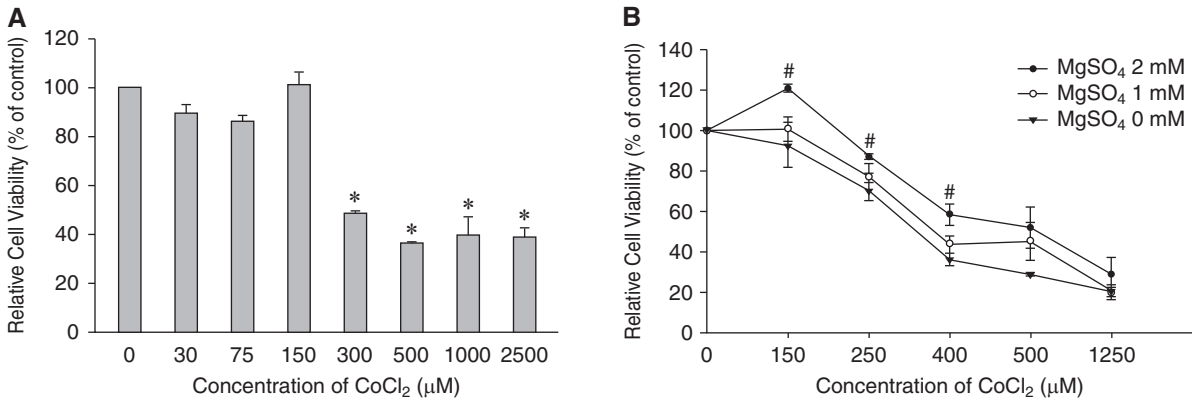

Fig. 1. $MgSO_4$ protects NB41A3 cells from $CoCl_2$-induced cytotoxicity. (A) The cells were cultured in medium containing $CoCl_2$ at the indicated doses for 12 h. Cell viability was estimated using the MTT assay. (B) Cell viability in the cells preincubated with the indicated concentrations of $MgSO_4$ for 1 h prior to $CoCl_2$ treatment at different doses for 24 h. The data are represented as mean ± SEM from three independent experiments. $^*P < 0.05$, significant differences from the control group. $^{\#}P < 0.05$, significant differences from the $MgSO_4$-treated group.

0.5 M NaCl, 1.0 mM EDTA, 1% glycerol, 1 mM β-mercaptoethanol, 1% NP40, and proteinase inhibitor cocktail tablet) and spun down at 12,000 *g* for 10 min. Cell samples were homogenized with ice-cold PBS and subjected to lysis in a solution containing 20 mM Tris, 2 mM EDTA and 1% glycerol. Supernatants were collected after centrifugation at 12,000 *g* for 40 min, and the protein concentration was determined by the Bradford method (Bio-Rad Protein Assay, Hercules, CA, USA). After boiling, protein homogenates were separated in 12% gradient SDS-PAGE with a constant voltage of 75 V for 2.5 h. After the electrophoresis, the proteins were blotted onto a PVDF membrane (Millipore, Belford, MA, USA) with a transfer apparatus at a constant voltage of 100 V for 2 h. Membranes were blocked with blocking buffer (5% non-fat milk, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween-20) for 1 h at room temperature and then probed with specific primary antibodies. Primary antibodies directed against Bcl-xL, Bcl-2, Bad, Bid, cytochrome *c*, pMEK1/2, pERK1/2, CREB, pCREB, p-p38, pJNK, HIF-1α, Bnip3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and α-tubulin (Neo Markers, Fremont, CA, USA) were diluted 1:500 in the blocking solution at 4°C overnight. The blots were washed 3 times in Tris-buffer saline (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), diluted in TBS buffer. The membranes were washed 3 times for 10 min each in TBS buffer. The immunoblotted proteins were visualized using an ECL western blotting luminal reagent and results were quantified using a Fujifilm LAS-3000 chemiluminescence detection system (Tokyo, Japan).

Measurement of Mitochondrial Membrane Potential ($\Delta\Psi_m$)

Apoptotic cells were detected using a mitochondrial membrane potential detection kit. 5,58,6,68-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Sigma-Aldrich) is a lipophilic fluorescent cation. JC-1 exists either as a green fluorescent monomer at a depolarized mitochondrial membrane potential or as a red fluorescent J-aggregate at a hyperpolarized mitochondrial membrane potential. JC-1 exhibits potential-dependent accumulation in mitochondria as indicated by the fluorescence emission shift from 530 to 590 nm. After a treatment of $CoCl_2$ (500 μM) for 24 h alone, or pretreatment with $MgSO_4$ at 1 or 2 mM, cells were washed with PBS, and JC-1 was loaded. After 20 min incubation at 37°C, cells were examined under an Olympus CKX41 fluorescence microscope (Olympus, Hamburg, Germany).

Statistical Analysis

Each experiment was duplicated at least three times. Results were presented as mean ± SEM, and statistical comparisons were analyzed by Student's *t*-test. Statistical significance was defined as $P < 0.05$.

Results

$MgSO_4$ Inhibited $CoCl_2$-Induced Cell Death on NB41A3 Neuroblastoma Cells

To investigate whether $MgSO_4$ may act as a survival factor for cells, we assayed the $MgSO_4$ effect on cell death induced by $CoCl_2$-induced insult, which causes apoptotic cell death in neurons *in vitro* (19, 45). After 12 h exposure of cells to 300 μm $CoCl_2$ insult, 50% of the cells were not viable as determined by the MTT assay (Fig. 1A). Treatment

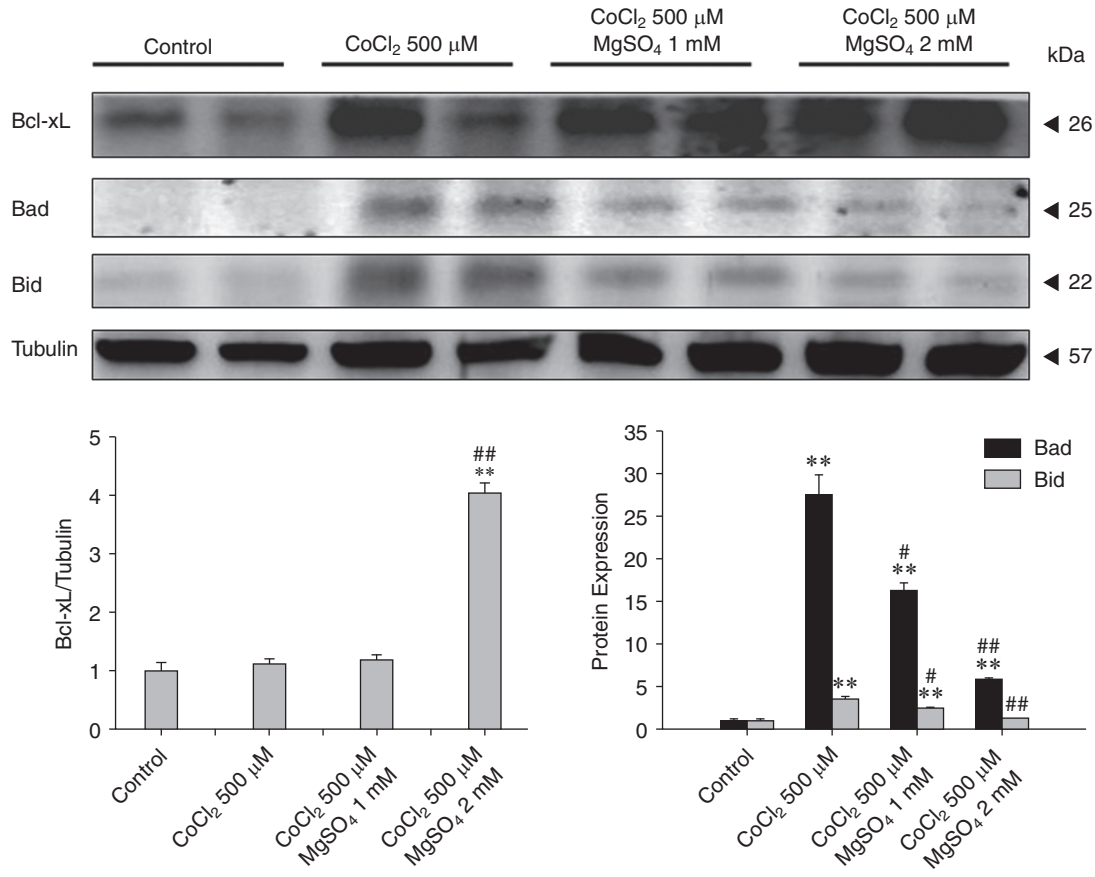

Fig. 2. Effects of $MgSO_4$ upon the expression of Bcl-2 family proteins. The cells were pretreated with vehicle or 1 mM or 2 mM $MgSO_4$ for 1 h. Cells were then exposed to vehicle or $CoCl_2$ for 24 h. Anti-apoptotic and pro-apoptotic proteins activation were assayed by immunoblotted with specific anti-Bcl-xL, anti-Bad and anti-Bid antibodies. The band intensities were normalized to that of the tubulin band and were expressed as relative band intensity. The mean ± SEM data are shown, and n = 3. $^{**}P < 0.01$, relative to the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, relative to the $CoCl_2$-treated group.

with $CoCl_2$ at different doses (0, 150, 250, 400, 500, 1250 μM) for 24 h caused loss of cell viability; however, $MgSO_4$ pretreatment at 1 or 2 mM for 1 h significantly prevented the loss of cell viability (Fig. 1B).

Effects of $MgSO_4$ on Bcl-2 Family Proteins

To elucidate the $MgSO_4$ effects on Bcl-2 family proteins in NB41A3 cells undergoing $CoCl_2$-induced apoptosis, the Bcl-xL, Bad and Bid proteins were determined by western blotting. Treatment with 500 μM $CoCl_2$ for 24 h significantly increased the expression of Bad and Bid (Fig. 2). Pretreatment of cells with $MgSO_4$ at 1 or 2 mM prior to $CoCl_2$ exposure markedly blocked these effects on the cells in a dose-dependent manner. In addition, 2 mM $MgSO_4$ significantly increased the levels of the Bcl-xL protein in NB41A3 cells exposed to $CoCl_2$ (Fig. 2).

Effects of $MgSO_4$ on Mitochondrial Transmembrane Permeability Transition

To examine whether inhibition of mitochondrial disruption may account for the anti-apoptotic effects of $MgSO_4$, the effects of $CoCl_2$ on mitochondrial membrane permeability were tested. When cells were exposed to 500 μM $CoCl_2$, $\Delta\Psi_m$ was depolarized, as shown by the increase in green fluorescence (Fig. 3). Pretreatment with $MgSO_4$ at 1 or 2 mM reduced the change in $\Delta\Psi_m$ as indicated by repression of green fluorescence and restoration of red fluorescence. It is, thus, concluded that pretreatment with $MgSO_4$ caused a marked inhibition of $CoCl_2$-induced apoptosis in a dose-dependent manner.

ERK1/2 Signaling Pathway Played an Important Role in $MgSO_4$–Mediated Neuroprotection

Because activation of MAPKs pathways (ERK1/2, p38 and JNK) are important in regulating cell death and survival after ischemia (14), effects of $MgSO_4$ on this process was next investigated. Compared to the control cells, cells treated with 500 μM

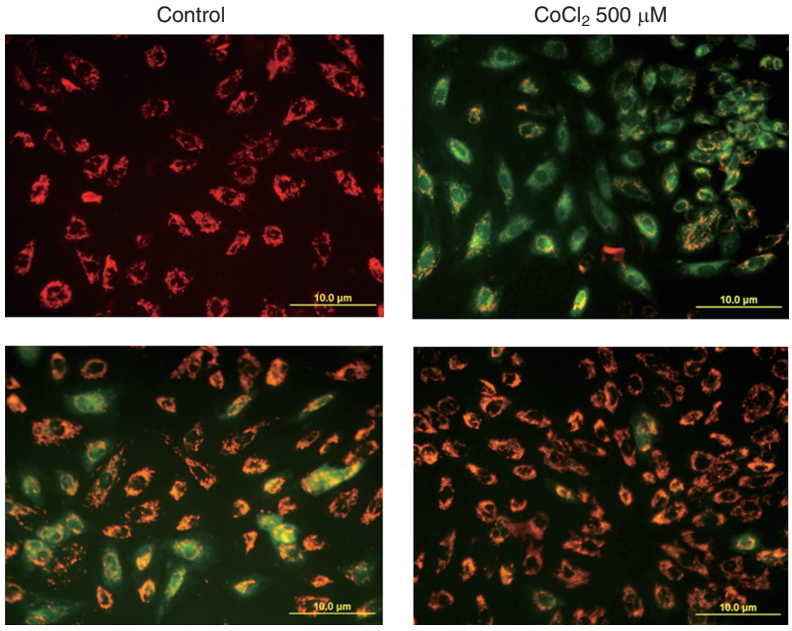

Fig. 3. $MgSO_4$ stabilizes the mitochondrial transmembrane potential ($\Delta\Psi_m$). The cells were pretreated with vehicle or 1 mM or 2 mM $MgSO_4$ for 1 h. Cells were then exposed to vehicle or $CoCl_2$ for 24 h. $\Delta\Psi_m$ was assessed *via* a signal from the monomeric and J-aggregate JC-1 fluorescence as described in Materials and Methods.

$CoCl_2$ for 24 h did not change the levels of pMEK1/2 (upstream of ERK1/2), pERK1/2 and pCREB (Fig. 4A). Pretreatment of cells with $MgSO_4$ prior to exposure of cells to $CoCl_2$ significantly increased the phosphorylation of ERK1/2 and CREB (Fig. 4A). The expression levels of the anti-apoptotic protein Bcl-2, a target transcription factor of CREB, was parallel with the pCREB response to these agents (Fig. 4C). The p38 and JNK MAPK are known to be linked to neuronal apoptosis during cerebral ischemia. As shown in Fig. 4B, $CoCl_2$ treatment increased expression of p-p38 and pJNK. Pretreatment with $MgSO_4$ prior to exposure of cells to $CoCl_2$ markedly suppressed overexpression of p-p38 and pJNK induced by $CoCl_2$ treatment. Furthermore, to further verify the role of MAPKs in $MgSO_4$-mediated neuroprotection, the NB41A3 cells were pretreated with various inhibitors to block these MAPK pathways following the administration of $MgSO_4$ and $CoCl_2$. NB41A3 cells were preincubated with 0.5 μM U0126, a MEK1/2 inhibitor, 0.5 μM SB203680, a p38 MAPK inhibitor or 0.5 μM SP600125, a JNK inhibitor, for 1 h and followed by 1 mM $MgSO_4$ administration for 1 h. Cells were then exposed to 500 μM $CoCl_2$ for 24 h. $CoCl_2$ treatment significantly increased the release of cytochrome *c* and the expression of Bad (Fig. 4C). The expression of Bcl-2 in NB41A3 cells decreased compared with that of the control cells. However, $MgSO_4$ pretreatment markedly blocked these effects. In addition, U0126 effectively prevented the $MgSO_4$-induced upregulation of Bcl-2 and reversed the $MgSO_4$-induced down-regulation of Bad and cytochrome *c* (Fig. 4C). However, p38 and JNK inhibitors showed no suppressive influences on these effects by $MgSO_4$. This finding suggested that $MgSO_4$-mediated neuroprotection was associated with the inhibition of $CoCl_2$-induced altered expression of apoptosis-related proteins and activation of the ERK1/2 signaling pathway.

$MgSO_4$ Suppressed $CoCl_2$-Induced Activation of HIF-1α and BNIP3

HIF is a master regulator of cellular response to hypoxia (34). The pro-apoptotic protein BNIP3 is one of the HIF-1 target genes. The effects of $MgSO_4$ on HIF-1α and BNIP3 activation were next examined. As shown in Fig. 5, exposure of cells to 500 μM $CoCl_2$ for 24 h significantly increased the expression levels of HIF-1α and BNIP3. Pretreatment with 1 or 2 mM $MgSO_4$ prior to $CoCl_2$ also obviously decreased these effects in a dose-dependent manner.

Discussion

In both *in vitro* (31) and *in vivo* experiments (20), magnesium reduced the neuronal damage elicited by ischemia. Here, our results indicate that $MgSO_4$ possesses neuroprotective and anti-apoptotic properties under the hypoxic/ischemic conditions induced by $CoCl_2$. Hypoxia-induced cell death is known to play

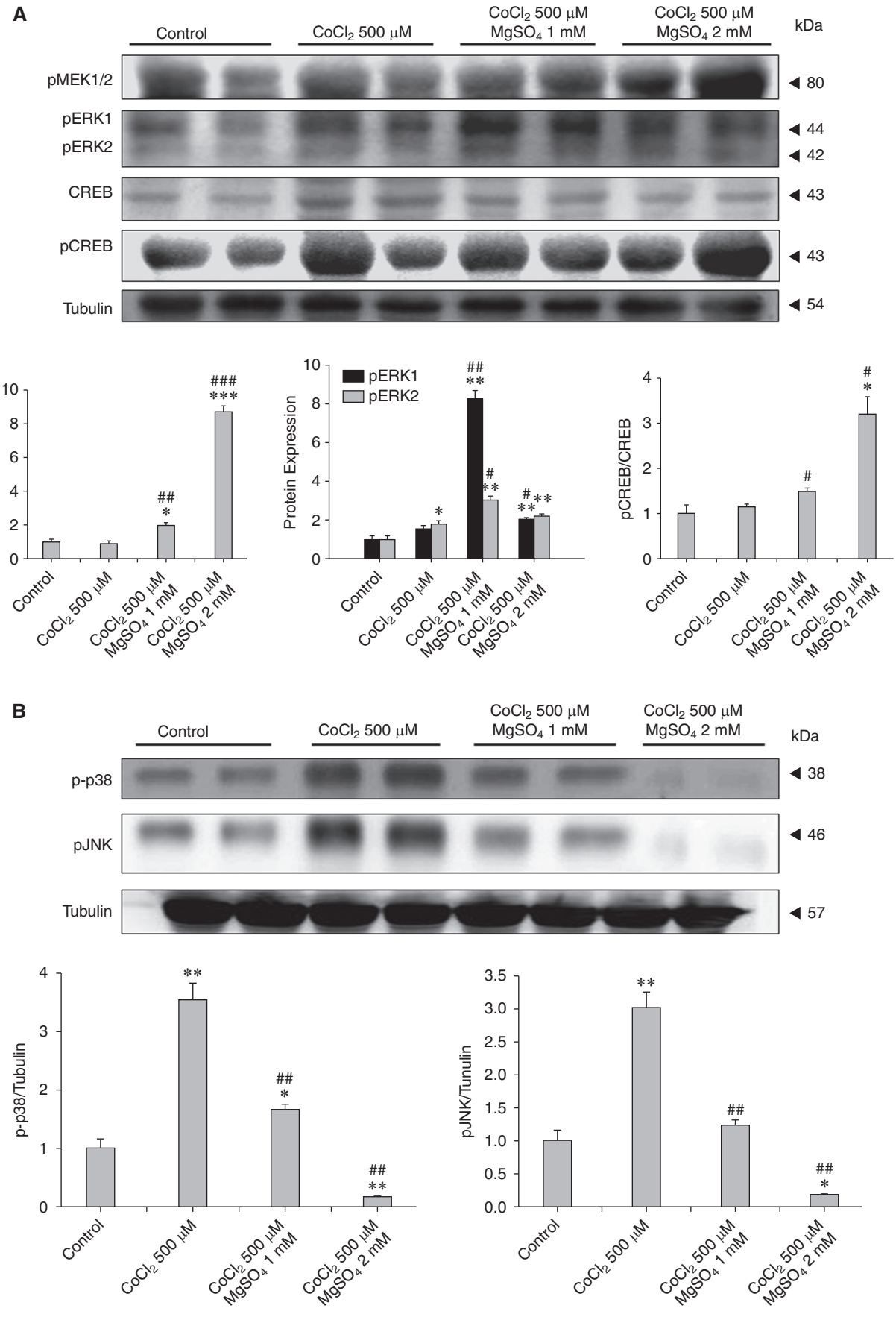

Fig. 4. (Continued)

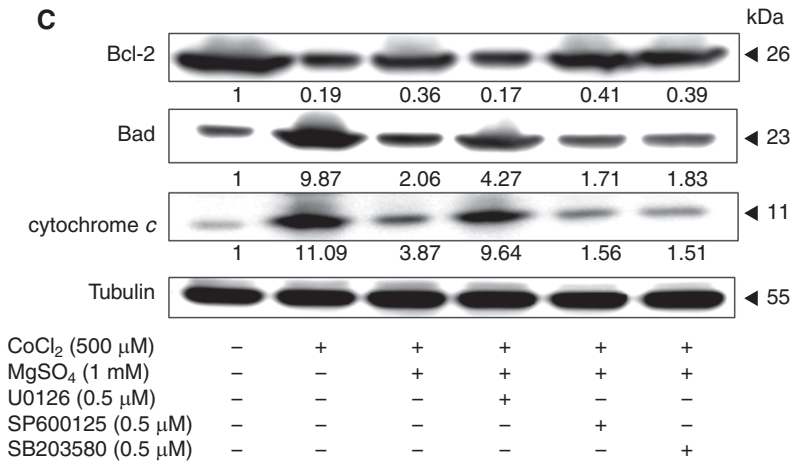

Fig. 4. Role of MAPK pathway in $MgSO_4$ inhibition of the $CoCl_2$-induced activation of the pro-apoptotic proteins Bad and cytochrome *c*. (A) $MgSO_4$ activates ERK1/2 and CREB phosphorylation in NB41A3 cells. (B) $MgSO_4$ attenuates $CoCl_2$-induced activation of p38 and JNK. The cells were pretreated with vehicle or 1 mM or 2 mM $MgSO_4$ for 1 h. Cells were then exposed to vehicle or $CoCl_2$ for 24 h. (C) $MgSO_4$-mediated neuroprotection was dependent on the ERK1/2 pathway but independent of the p38 and JNK pathways. NB41A3 cells were pretreated with vehicle, U0126, SP600125 or SB203580 for 1 h, and followed by administration of 1 mM $MgSO_4$ for 1 h. Cells were then exposed to $CoCl_2$ for 24 h. Anti-apoptotic protein and pro-apoptotic proteins expression were assayed by immunoblotted with specific anti-Bcl-2, anti-Bad and anti-cytochrome *c* antibodies. Proteins in total cell extracts were separated in 12% SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies against proteins as indicated. The band intensities were normalized to that of the tubulin band and were expressed as relative band intensity. The mean ± SEM data are shown, and n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, relative to control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, relative to $CoCl_2$-treated group.

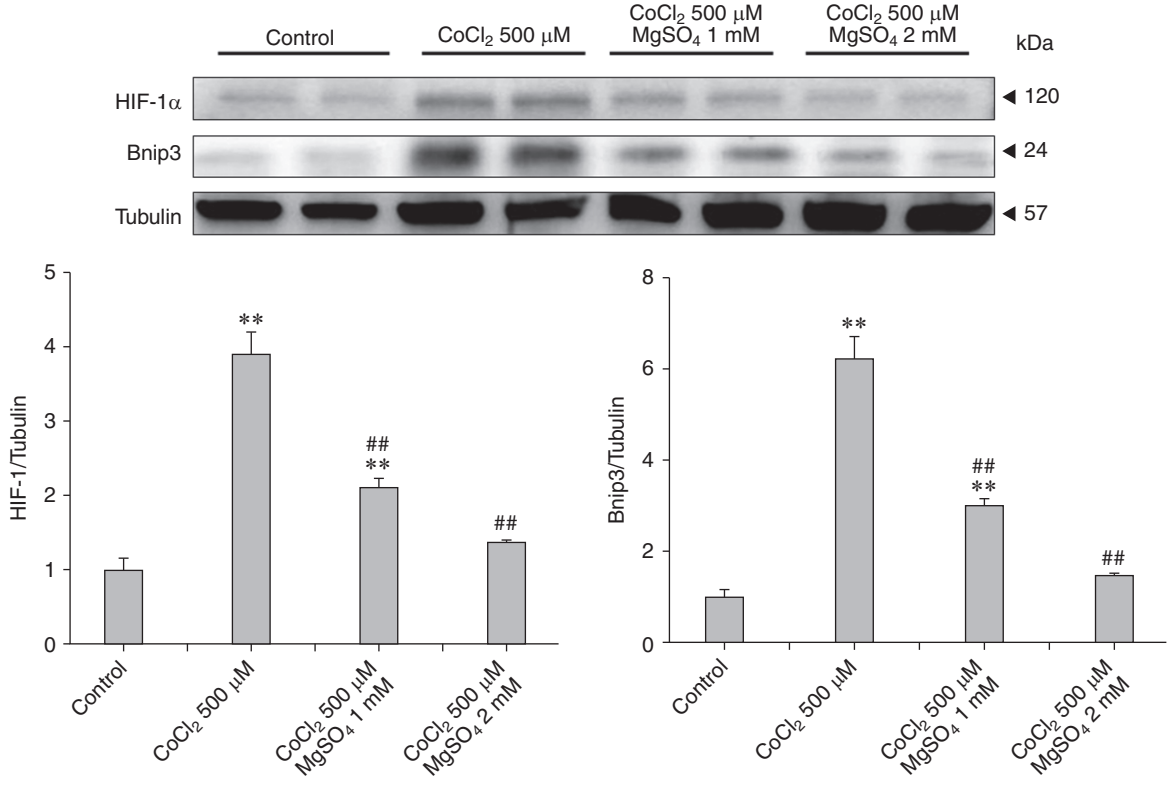

Fig. 5. $MgSO_4$ inhibits $CoCl_2$-induced activation of HIF-1α and the pro-apoptotic protein BNIP3. The cells were pretreated with vehicle or 1 mM or 2 mM $MgSO_4$ for 1 h. Cells were then exposed to vehicle or $CoCl_2$ for 24 h. HIF-1α and the pro-apoptotic proteins activation were assayed using immunoblotting with specific anti-HIF-1α and anti-BNIP3 antibodies. The band intensities were normalized to that of the tubulin band and expressed as relative band intensity. The mean ± SEM data are shown, and n = 3. $^{**}P < 0.01$, relative to control group. $^{\#\#}P < 0.01$, relative to $CoCl_2$-treated group.

an important role in various clinical entities such as ischemia and organ transplantation. In our previous *in vivo* study, we found that $MgSO_4$ treatment attenuates ischemia-induced activation of proapoptotic proteins Bax, Bad and caspase 3 while increasing the antiapoptotic Bcl-2 proteins (12). Solaroglu *et al*. reported that $MgSO_4$ treatment decreases caspase 3 activity after experimental spinal cord injury (36). $MgSO_4$ exerts antiapoptotic effects through caspase 3 inhibition in newborn hypoxia-ischemia (32). In the current study, $CoCl_2$ shifted the balance of Bcl-2 family toward pro-apoptotic effects in NB41A3 cells. We also observed that $CoCl_2$ disrupted the mitochondrial membrane potential and caused the release of cytochrome *c*. $MgSO_4$ markedly decreased Bad and Bid levels but increased Bcl-xL and Bcl-2 levels in NB41A3 cells exposed to $CoCl_2$ insult, suggesting that $MgSO_4$ affected the status of anti-apoptotic and pro-apoptotic Bcl-2 family proteins, a change that was opposed to the initiation of the apoptotic cascade. In addition, $MgSO_4$ significantly prevented the $CoCl_2$-induced mitochondrial membrane instability and decreased the release of cytochrome *c*. Taken together, the anti-apoptotic effects of $MgSO_4$ are hypothesized to be resulted from its effects on shifting the balance of Bcl-2 family toward anti-apoptotic effects and stabilizing mitochondrial membrane potential, thereby preventing the release of mitochondrial protein cytochrome *c*, a molecular species required for the activation of caspase 3 that executes the cell death program (Fig. 6).

Mitogen-activated protein kinases (MAPKs) are involved in many cellular processes. These kinases are activated by phosphorylation of both threonine and tyrosine residues, and subsequently MAPKs phosphorylate intracellular enzymes and transcription factors (14). The ERK MAPK is known to be involved in cell survival, whereas p38 and JNK MAPK are associated with cell stress and death. Previous studies have abundantly described that pharmacological selective activation of the ERK1/2 signaling pathway provides neuroprotective effects through inhibition of neuronal apoptosis during cerebral ischemia (5, 37, 41, 43). Zhang and Chen found that leptin protects against delayed ischemic neuronal death in the hippocampal CA1 by maintaining the pro-survival status of Akt and ERK1/2 MAPK signaling pathways (43). Our previous study showed that $MgSO_4$ activated the ERK1/2 signaling pathway in gerbil focal cerebral ischemia models (12). In this study, we used the MEK1/2 inhibitor U0126 to show that $MgSO_4$ induces the neuroprotective effects through the ERK1/2 signaling pathway and activating its target CREB induced Bcl-2 expression in focal ischemia (12). Consistent with these results, this study also found that ERK1/2, CREB and

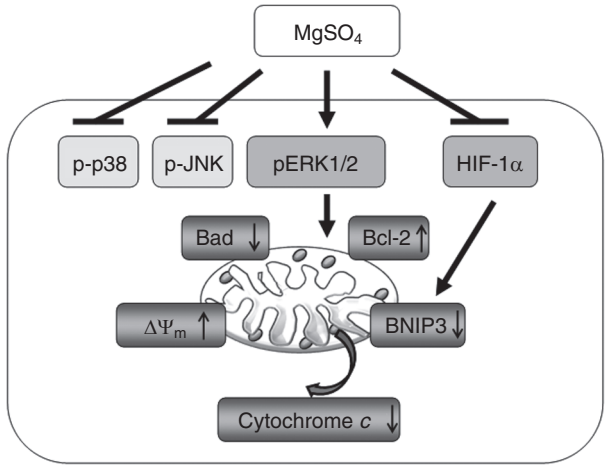

Fig. 6. Proposed signaling pathway related to anti-apoptotic effects of $MgSO_4$ on $CoCl_2$-induced damage of neuronal cells. $CoCl_2$ insult slightly activates ERK1/2, while markedly activates JNK and p38. $MgSO_4$ treatment significantly activates ERK1/2, ERK1/2 then shifting the balance of Bcl-2 family members toward anti-apoptotic effects and stabilizing the mitochondrial membrane potential, thereby preventing the release of cytochrome *c*. $MgSO_4$ treatment also inhibits the HIF-1α/BNIP3 signaling pathway.

Bcl-2 were activated by $MgSO_4$. The selective inhibitor of ERK1/2, U0126, abolished $MgSO_4$-mediated up-regulation of Bcl-2 and reversed the $MgSO_4$-mediated down-regulation of Bad and cytochrome *c*. Our results suggested that $MgSO_4$ elicits neuroprotective action against mitochondrial-mediated neuronal apoptosis through activation of the ERK1/2 MAPK signaling pathway under the hypoxic/ischemic conditions induced by $CoCl_2$. Furthermore, we showed that $CoCl_2$-induced activation of p38 and JNK was attenuated by $MgSO_4$ treatment. P38 MAPK can be activated by ischemia, and inhibition of p38 MAPK activity has been shown to be neuroprotective (2, 14). Insulin promotes fetal neurons survival by inhibiting p38 MAPK (10). The JNK MAPK signaling pathway is also activated in the brain after cerebral ischemia (9, 26). JNK MAPK phosphorylation directly enhances the pro-apoptotic activity of p53, but reverses the anti-apoptotic function of Bcl-2 and Bcl-xL (8). However, in the current study, pretreatment with a p38 MAPK inhibitor (SB203680), or a JNK inhibitor (SP600125), had no effects on $MgSO_4$-mediated upregulation of Bcl-2 and downregulation of Bad and cytochrome *c*. The results suggest that p38 and JNK may not be implicated in $MgSO_4$-mediated signaling through mitochondria in NB41A3 cells under the hypoxic/ischemic conditions induced by $CoCl_2$. Indeed, activation of p38 and JNK phosphorylates a variety of downstream effectors of progression, including pro-apoptotic, inflammatory and cytoskeletal mediators, tau neuro-

filaments as well as numerous transcription factors (28). The exact downstream targets of p-p38 and pJNK MAPK that are inhibited by $MgSO_4$ require further clarification. Thus, another unknown protective mechanism of $MgSO_4$ on NB41A3 cells may occur through the inhibition of p38 and JNK MAPK phosphorylation.

In the brain, HIF-1α protein expression has been detected under hypoxic condition (40) or subjected to global and focal ischemia (3, 15). Increased HIF-1α regulates different target genes that mediate glycolysis, angiogenesis, erythropoiesis, and cell death (29, 33). The role of HIF-1α in hypoxic insults is controversial (1). The neuroprotective role of HIF-1α has been examined in hypoxic preconditioning models (16, 35). However, accumulating evidences suggest that cerebral ischemia HIF-1 mediates hypoxia-induced neuronal cell death either by enhancing the expression of BNIP3, a proapoptotic member of the Bcl-2 family, or through a p53-mediated apoptotic cascade (27). Helton *et al*. demonstrated that brain-specific HIF-1α knock-out reduces hypoxic-ischemic damage supporting that HIF-1α is pro-death in the brain after ischemia (11). In the present study, we observed that $CoCl_2$ induced an increase in HIF-1α and BNIP3 levels. $MgSO_4$ treatment resulted in a significant decrease in HIF-1α and BNIP3 levels in neuronal cells. The results imply that the neuroprotective effects of $MgSO_4$ may partly be the result of blocking the HIF-1α signaling pathway involved in neuronal apoptosis. It has been reported that the induction of HIF-1α by $CoCl_2$ is dependent on the generation of reactive oxygen species (ROS) (4, 38). An earlier study has shown that magnesium inhibits lipid peroxidation at the high concentrations of 1 and 3 mM in murine cortical cells (31). Similar findings were observed in non-neuronal systems in which magnesium deficiency promoted cellular oxidative injury (23, 30). The anti-apoptotic protein Bcl-2 is a plausible target for the putative antioxidant capacity of $MgSO_4$ because it has been shown that Bcl-2 can protect cells from apoptosis by preventing ROS accumulation (7). Therefore, inhibition of HIF-1α expression by $MgSO_4$ is probably secondary to the antioxidant action of magnesium. It remains to be determined whether the ROS level and HIF-1α activity are involved in the anti-apoptotic mechanism of $MgSO_4$.

Acknowledgments

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