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Cobalt Chloride-induced Apoptosis and Extracellular Signal-regulated Protein Kinase 1/2 Activation in Rat C6 Glioma Cells

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Brain ischemia brings about hypoxic insults. Hypoxia is one of the major pathological factors inducing neuronal injury and central nervous system infection. We studied the involvement of mitogen-activated protein (MAP) kinase in hypoxia-induced apoptosis using cobalt chloride in C6 glioma cells. *In vitro* cytotoxicity of cobalt chloride was tested by MTT assay. Its IC_{50} value was 400 μM. The DNA fragment became evident after incubation of the cells with 300 μM cobalt chloride for 24 h. We also evidenced nuclear cleavage with morphological changes of the cells undergoing apoptosis with electron microscopy. Next, we examined the signal pathway of cobalt chloride-induced apoptosis in C6 cells. The activation of extracellular signal-regulated protein kinase 1/2 (ERK 1/2) started to increase at 1 h and was activated further at 6 h after treatment of 400 M cobalt chloride. In addition, pretreatment of PD98059 inhibited cobalt chloride-induced apoptotic cell morphology in Electron Microscopy. These results suggest that cobalt chloride is able to induce the apoptotic activity in C6 glioma cells, and its apoptotic mechanism may be associated with signal transduction via MAP kinase (ERK 1/2).

Keywords: Apoptosis, Cobalt chloride, Cytotoxicity, ERK 1/2, Hypoxia

Introduction

The central nervous system consists of neurons and glial cells. Neurons constitute about half the volume of the CNS, and glial cells make up the remainder. Glial cells not only provide support and protection for neurons, but also supply oxygen to neurons. Neuronal dysfunction and loss contribute to a variety of acute as well as chronic diseases of the brain. Understanding the mechanisms underlying neuronal cell death and the means by which it can be prevented may lead to better treatments.

Hypoxia is one of the major pathological factors that induce neuronal injury. Adaptive responses to hypoxia are achieved by the coordinated expression of a number of genes. These include erythropoietin (EPO), vascular endothelial growth factor (VEGF), glycolytic enzymes, glucose transporter (Glut), inducible NO synthase (i-NOS) and many others (Guillemin and Krasnow, 1997). Induction of these genes is mediated by the hypoxia inducible factor 1 (HIF-1), a transcription factor known as a global regulator of hypoxic gene expression (Guillemin and Krasnow, 1997; Chandel *et al.*, 1998). HIF-1 consists of 120-kDa HIF-1α and 91- to 94-kDa HIF-1β subunits (Wang and Semenza, 1995). HIF-1α is the subunit regulated by hypoxia (Minet *et al.*, 2000). Cobalt chloride has been suggested as cobaltous ions that substitute for ferrous ions in heme, causing a conformational change in a heme protein O_2 sensor (Goldberg *et al.*, 1988; Chandel *et al.*, 1998). Recent work reported that the presence of cobalt chloride not only induces hypoxia, but also induces DNA damage and activates the cellular DNA damage response (Wang *et al.*, 2000).

Apoptosis is a regulated cell death process that is characterized by cytoplasmic shrinkage, nuclear condensation and DNA fragmentation (Kerr *et al.*, 1972). Apoptosis begins with condensation of nuclear chromatin at the nuclear periphery, followed by blebbing of the nuclear and cytoplasmic membranes. Apoptosis culminates in the fragmentation of the residual nuclear structure into discrete membrane-bounded apoptotic bodies (Allen, 1987; Kerr *et al.*, 1987). The morphological alterations of apoptosis are accompanied by a variety of biochemical changes. Elevations in cytosolic free calcium (McConkey *et al.*, 1990) and

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cytoplasmic hydrogen ion (Barry *et al.*, 1992) are followed by internucleosomal DNA degradation (Lockshin *et al.*, 1990; Arends *et al.*, 1991) and sharp decreases in cellular NAD levels (Denisenko *et al.*, 1989). Reportedly the exposure of cells to hypoxia induced DNA fragmentation that is characteristic of apoptosis and ultimately led to apoptosis (Yao *et al.*, 1995; Bae *et al.*, 1998).

Recently, mitogen-activated protein (MAP) kinase cascades, which are well known for cell proliferation and differentiation-inducing pathway, were reported to be associated with the apoptotic pathway. MAP kinase families include extracellular regulated protein kinase (ERK 1/ERK 2), p38 and stress-activated protein kinase/c-jun N-terminal kinase (SAPK/JNK) (Davis, 1993). Well-studied members of the MAP kinase family are ERK 1/2, which can be triggered by growth factors and phorbol esters through Ras-dependent activation of the Raf-MEK-ERK pathway (Davis, 1993; Stokoe *et al.*, 1994). The JNK cascade is operated by a parallel signaling module consisting of the MEKK-1/ SEK 1/ JNK cascade (Kyriakis *et al.*, 1994; Coso *et al.*, 1995). Unlike ERK, JNK is strongly activated by environmental stress such as inflammatory cytokine (Martin *et al.*, 1997), ultraviolet C (Kyriakis *et al.*, 1994), gamma irradiation (Yu *et al.*, 1996), and DNA-damaging drug (Jimenez *et al.*, 1997). In addition, the phosphorylated MAP kinase in cytosol is translocated in the nucleus (Chen *et al.*, 1992) and is involved in the regulation of transcription factors including c-Jun, c-fos and c-Myc, which have been shown to influence apoptosis (Cavigelli *et al.*, 1995; van Dam *et al.*, 1995; Baek *et al.*, 1996). Recently, it was reported that hypoxia activated MAP kinase, Raf (Muller *et al.*, 1997; Conrad *et al.*, 1999; Seimiya *et al.*, 1999), phosphatidylinositol 3-kinase(PI 3-kinase) (Mazure *et al.*, 1997), PKC, c-fos (Yao *et al.*, 1994; Muller *et al.*, 1997; Bae *et al.*, 1998; Seimiya *et al.*, 1999) and c-Jun (Yao *et al.*, 1994). However, the molecular mechanism of hypoxia-induced apoptosis has not been clearly elucidated.

In this study, we investigated the involvement of MAP kinase to hypoxia-induced apoptosis using cobalt chloride in C6 rat glioma cells, a cloned rat astrocytoma that is commonly used as a glial cell model. We examined the cytotoxicity, DNA fragmentation and apoptotic effect of cobalt chloride in C6 glioma cells with electron microscopy. In particular, potent activation of ERK 1/2 was observed after the cobalt chloride treatment. We confirmed this effect with a MEK inhibitor, PD 98059.

Materials and Methods

Materials The cobalt chloride was purchased from the Sigma Chemical Co. (St. Louis, USA). Anti-HIF-1 antibodies were purchased from BD Transduction Laboratories (USA). Anti-ERK 1/2, anti-phospho-ERK 1/2 and MEK 1 inhibitor (PD98059) were purchased from New England Biolabs (Beverly, USA). All of the other chemicals and reagents were the highest grade commercially available.

Cell culture and i*n vitro* cytotoxicity assay C6 rat glioma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) that was supplemented with 10% fetal bovine serum with 100 units/ml of penicillin, 100 µg/ml of streptomycin. All of the cells were grown at 37ºC in a humidified atmosphere of 5% CO_2. Cytotoxicity was measured by the microculture tetrazolium (MTT) method. Exponentially growing cells were inoculated to 5×10^3 cells/well using a 96 well microplate that was supplemented with 100 µl DMEM. For experiments that studied cells in plateau phase of growth, the cells were permitted to grow for a minimum of 72 h before they were exposed to drugs. The cells were exposed to various concentrations of cobalt chloride. Distilled water was used to dissolve cobalt chloride. After the treated cells were incubated for 24 h, 50 µl MTT (1 mg/ml, Sigma) was added and the plates were incubated at 37ºC for 4 h. To dissolve formazan, 100 µl DMSO was added and the plates were measured at 540 nm by spectrometer. The IC_{50} value was determined by plotting the drug concentration versus the survival ratio of the treated cells.

DNA extraction and electrophoresis The 5×10^6 cells, which were treated with cobalt chloride for 24 h, were washed twice in a solution of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl and were lysed with 500 µl lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 20 mM EDTA). Lysates were harvested by $1{,}000 \times g$ for 10 min and the supernatants were incubated for 4 h at 37ºC with 50 µg/ml RNase A, 120 µg/ml proteinase K. Then phenol/chloroform/isoamylalcohol (25 : 24 : 1, Sigma) extracted the DNA. After precipitation, the pellets were resuspended in 30 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Each DNA sample was electrophoresed through 1.8% agarose gel contained with ethidium bromide. The gel was visualized by UV fluorescence.

Morphological features of apoptosis We confirmed the morphological change after treatment with cobalt chloride using an electron microscope. The cells that were treated with 400 µM cobalt chloride for 24 h and 48 h were centrifuged at $400 \times g$, fixed with 2% glutaraldehyde in PBS for 24 h, washed in 0.1 M Caocodylate, pH 7.4, and fixed with 0.1% OsO_4 in 0.1 M Caocodylate for 1 h 30 min. After fixation, the cells were washed with in 0.1 M Caocodylate, pH 7.4 and then dehydrated in graded ethanol. Next, the cells were impregnated with prophylene oxide and embedded in Polybed 812 (Polyscience, Inc., Warrington, USA). After a 60ºC incubation, the cells were cut and stained with uranyl acetate and lead citrate. To analyze the effects of PD98059 on the cobalt chloride-treated cells, the starved cells were exposed to 5 µM PD98059 for 1 h and then treated with 400 µM cobalt chloride for 24 h. The harvested cells were treated in the same manner.

Preparation of cytosolic and nuclear fractions Exponentially growing cells were starved for 24 h and then exposed to 400 µM cobalt chloride for 1, 2, 3 and 6 h. The treated cells were washed twice and collected into 1 ml hypotonic lysis buffer (1 mM EGTA, 1 mM EDTA, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 40 µg of PMSF per ml and 10 mg of both pepstain and leupeptin per ml, pH 7.5). The cellular suspensions were homogenized and harvested by $600 \times g$ for 5 min. To prepare the cytosolic fraction, the supernatant was obtained and

centrifuged at 12,000 × *g* for 20 min. The pellets were suspended by 0.25 M sucrose in 1.5% citric acid and passed three times through a 26 gauge needle and loaded onto 1 ml of 0.88 M sucrose cushion in 1.5% citric acid. To get the nuclear fraction following centrifugation at 900 × *g* for 10 min, the pellets were dissolved with a hypotonic lysis buffer containing 0.5% Igepal CA-630, 0.1% deoxycholate, 0.1% Brij-35, and then centrifuged at 10,000 × *g* for 10 min. All of the fractional procedures were completed on ice.

The purity of the nuclear fraction was determined by measuring the lactate dehydrogenase activity as the cytosol marker. Then, the nuclear fractions were confirmed by a Western blot analysis.

Immunoblot analysis We first examined the protein concentrations of the prepared cytosolic and nuclear extracts with a Bradford assay solution (100 mg/L Coomassie Brilliant Blue G-250, 50 ml/L 95% ethanol, 100 ml/L 85% phosphoric acid). The same concentrations of protein samples were SDS-PAGE on 4% stacking gel and 10% running gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes, blocked in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.15% Tween-20) that contained 10% skim milk. The membranes were then incubated with anti-HIF-1, anti-phospho-ERK 1/2, anti-phospho-c-Jun antibodies at adequate dilutions in TBS for 1 h at room temperature. After the washing, the blots were incubated with horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies at a 1 : 1,000 dilution for 1 h at room temperature, washed 3 times in TBST, and detected with the enhanced chemiluminescence detection method by immersing the blots for 1 min in a 1 : 1 mixture of chemiluminescence reagents A and B (Amersham, Piscataway, USA). They were then exposed to Kodak film for a few minutes.

Results and Discussions

HIF-1 expression To demonstrate the induction of hypoxia by cobalt chloride treatment, we measured the HIF-1 expression through immunoblotting. C6 glioma cells were exposed with 400 μM cobalt chloride for 1, 2, 3 and 6 h. As a result, cobalt chloride increased the HIF-1 expression 1h after the treatment (Fig. 1). During hypoxia, HIF-1 is expressed, binds to DNA, and induces the transcriptional activation of VEGF, known as a major mediator of vasculogenesis and angiogenesis (Forsythe *et al.*, 1996; Risau, 1997; Shweiki *et al.*, 1997). Furthermore, *in vivo*, it is known that HIF-1 triggers not only the acceleration of angiogenesis but also the induction of p53-dependent apoptosis in solid tumors that are exposed to hypoxia.

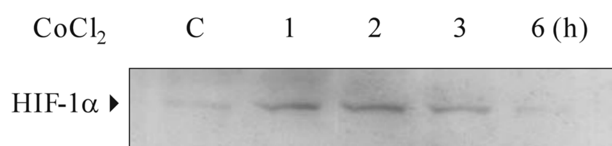

Fig. 1. HIF-1 activation by cobalt chloride. C6 glioma cells were treated with 400 μM $CoCl_2$ for 1, 2, 3 and 6 h (lane 2-5), or untreated (lane 1).

***In vitro* cytotoxic effects** Exponentially growing cells were exposed to various concentrations of cobalt chloride. After 24 h of exposure to cobalt chloride, the cytotoxic activity was evaluated by the MTT method against C6 glioma cells. The minimal concentration of cobalt chloride to inhibit the growth of C6 glioma cells by 50% (IC_{50}) was determined. The cobalt chloride showed that the concentration-dependent cytotoxicity and IC_{50} value of cobalt chloride was 400 μM (Fig. 2).

DNA fragmentation In order to determine the apoptotic effect of cobalt chloride, we examined the apoptotic response as judged by the appearance of a DNA ladder by 1.8% gel electrophoresis at various concentrations of cobalt chloride.

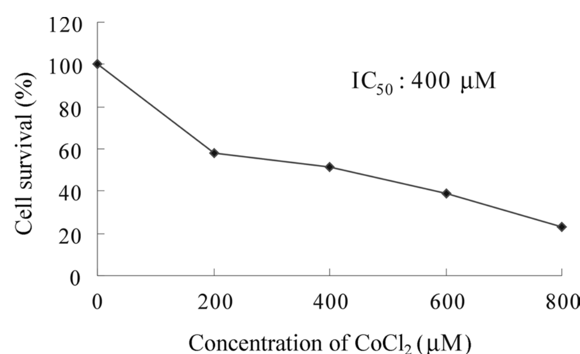

Fig. 2. Cytotoxic effect of cobalt chloride in C6 glioma cells. The cells were incubated with various concentrations of $CoCl_2$ for 24 h. Cytotoxicity was measured by an MTT assay. The results are presented as mean value for triplicate.

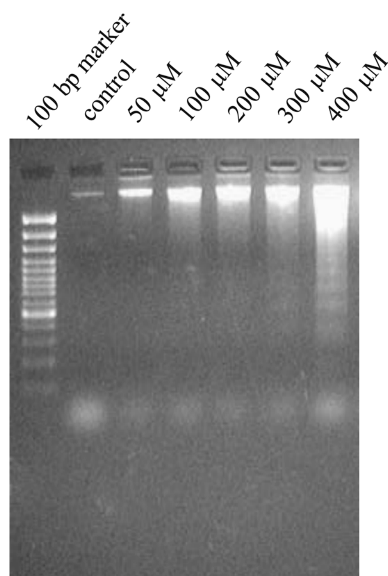

Fig. 3. DNA fragmentation extracted from C6 glioma cells treated with cobalt chloride for 24 h. C6 glioma cells were treated with various concentrations of $CoCl_2$ for 24 h. Extracted DNA was fractionated by electrophoresis and stained by ethidium bromide.

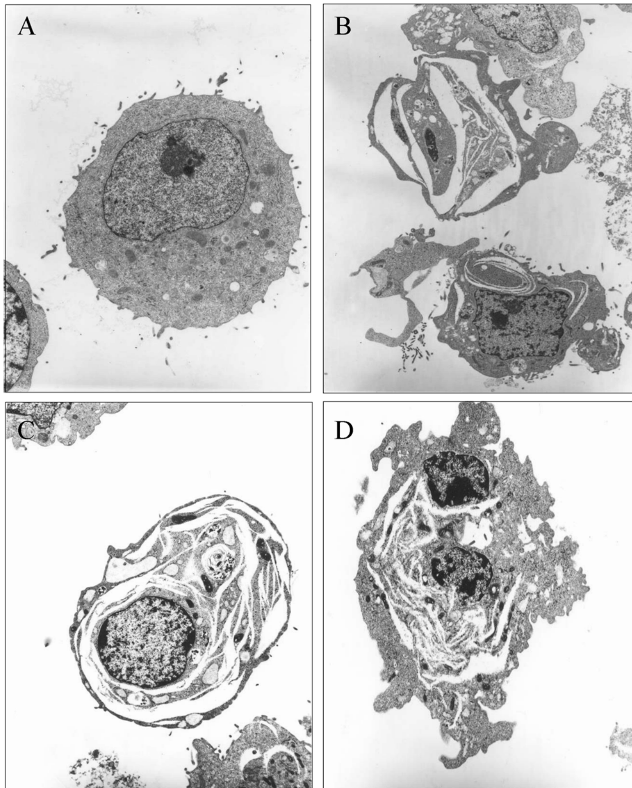

Fig. 4. Effect of $CoCl_2$ treatment on the morphology of C6 glioma cells. The cells were treated with the control vehicle (A), 400 μM cobalt chloride for 24 h (B), 48 h (C and D), stained with uranyl acetate and lead citrate, then analyzed under an electron microscope.

The DNA ladder patterns with cobalt chloride appeared at concentrations more than 300 μM (Fig. 3). Thus, cobalt chloride exhibited DNA fragmentations in a broader range of concentrations in C6 glioma cells. Many studies reported that hypoxia triggered DNA fragmentation in various cell lines (Yao *et al.*, 1995; Bae *et al.*, 1998); however, there has been no research about how cobalt chloride-induced hypoxia provokes apoptosis in glioma cells.

Electron microphotography When the condensed nuclei were fixed with glutaraldehyde and examined by thin section EM, treatment of the C6 glioma cells with cobalt chloride resulted in morphological changes that were consistent with the process of apoptosis. Initially in the nucleus, a rim of heterochromatin appeared at the nuclear periphery and the nucleolus simultaneously disappeared after 24 h (Fig. 4B). In this case, mitochondria and the plasma membrane remained intact throughout the course of these morphological changes. Consequently, 50% of the cells continued to exclude trypan blue. Then nuclei subsequently fragmented (Fig. 4C). Finally, packaging of the nuclear fragments into multiple membrane-enclosed apoptotic bodies was found after 48 h (Fig. 4D).

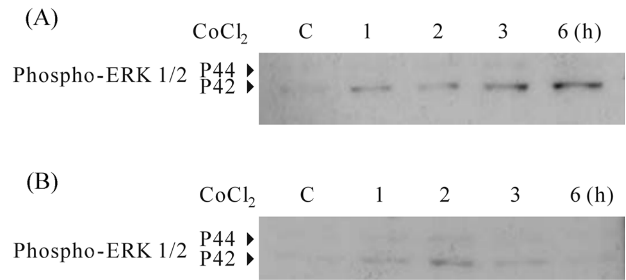

Fig. 5. Phospho-ERK 1/2 activation and ERK 1/2 translocation from to cytosol nucleus by cobalt chloride. (A) C6 glioma cells were treated with 400 μM $CoCl_2$ for 1, 2, 3 and 6 h (lane 2-5) or untreated (lane 1). (B) C6 glioma cells were treated with 400 μM $CoCl_2$ for 1, 2, 3 and 6 h (lane 2-5) or untreated (lane1).

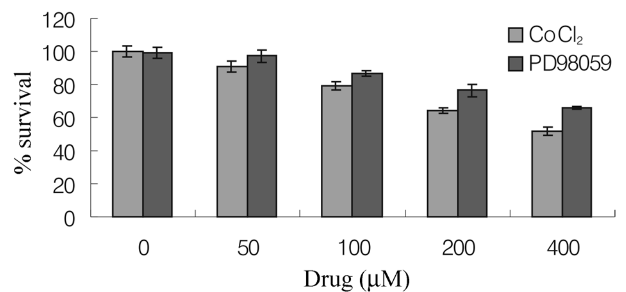

Fig. 6. Effect of MEK inhibitor, PD98059, on cytotoxicity in $CoCl_2$ treated C6 glioma cells. The cells were treated with 5 μM PD98059 for 1 h and then with the indicated concentrations of $CoCl_2$ for 24 h.

Activation and translocation into nucleus of ERK 1/2 The MAP kinase pathway is involved in apoptotic signal transduction (Wang *et al.*, 1998). To determine if ERK 1/2, one member of MAP kinase family, was activated by cobalt chloride stimulation by inducing an apoptotic response, we examined the phosphorylation of ERK 1/2 by immunoblot analysis. C6 glioma cells were exposed with 400 μM cobalt chloride for 1, 2, 3 and 6 h. As a result, cobalt chloride started to increase after 1 h and was activated further 6 h after treatment of 400 μM cobalt chloride (Fig. 5A). It has been proposed that prolonged activation of MAP kinase is accompanied by the translocation of the enzyme to the nucleus (Chen *et al.*, 1992), with subsequent alterations in the gene expression (Marshall *et al.*, 1995). To confirm the nuclear translocation of ERK 1/2, we prepared the separation of nuclei from the cells that were exposed to 400 μM of cobalt chloride for 1, 2, 3 and 6 h. These were examined by an immunoblot analysis. As shown in Fig. 5B, phospho-ERK 1/2 (44/42 kDa) was translocated into the nucleus by cobalt chloride at the same condition of inducing apoptosis. Accordingly, ERK 1/2 may be involved in the signal transduction of cobalt chloride-induced apoptosis in glioma cells.

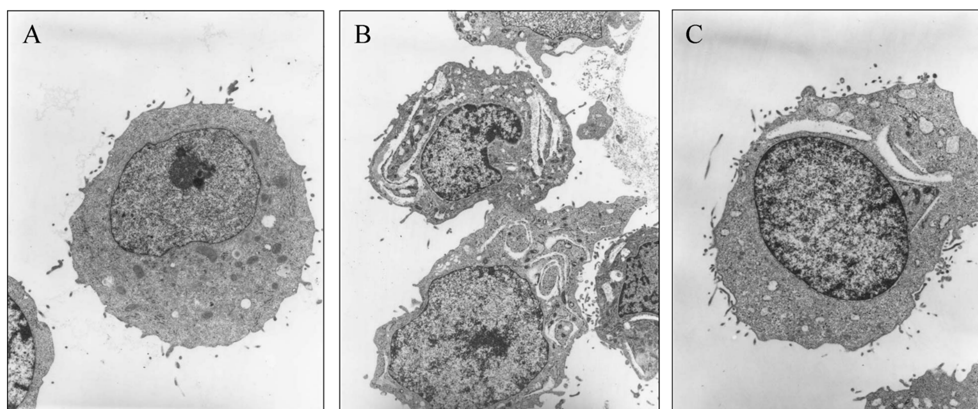

Fig. 7. The inhibition of cobalt chloride induced apoptotic nuclei concentration and cleavage by PD98059. C6 glioma cells were treated with 400 μM $CoCl_2$ for 24 h (B) and treated 5 μM PD98059 for 1 h, then they were treated with 400 μM $CoCl_2$ for 24 h (C) or untreated (A).

ERK MAP kinases are phosphorylated in the hippocampus in response to global brain ischemia (Campos-Gonzalez *et al.*, 1991; Kindy, 1993). The use of general tyrosine kinase inhibitors, such as genistein, decreases ERK2 phosphorylation in this model and is associated with protection against neuronal cell damage (Campos-Gonzalez *et al.*, 1991). The inhibition of MEK1 protects hippocampal neurons in a cell-culture model of seizure (Murray *et al.*, 1998). In this study, we have shown that ERK is important for hypoxia by cobalt chloride in the glial cell system. Inhibition of the ERK1/2 pathway after focal cerebral ischemia may also lead to the transcriptional and/or translational stability of gene products such as c-fos, resulting in protection against damage (Alessandrini *et al.*, 1999). But we observed no phosphorylation of c-jun or c-fos. The enhanced neuronal c-fos expression has been associated with cell survival in brain ischemia models (Uremuar *et al.*, 1991). It has been demonstrated in some systems that the MEK/ERK pathway may have anti-apoptotic effects that appose the pro-apoptotic effects that are associated with the activation of the JNK and p38 MAP kinases (Xia *et al.*, 1995). However, we confirmed that the inhibition of MAP kinase by PD98059 attenuated the formation of the apoptotic body in Electron Microscopy and the cytotoxicity of $CoCl_2$ in MTT assays.

In conclusion, cobalt chloride has an cytotoxic effect against C6 Rat glioma cells and induces the apoptotic response. Also, our results imply that the activation and translocation into nucleus of ERK 1/2 may be related to apoptotic signal transduction of cobalt chloride.

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