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 IC50 value was 400 \( \mu M \). The DNA fragment became evident after incubation of the cells with 300 \( \mu M \) 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-1\( \alpha \) and 91- to 94-kDa HIF-1\( \beta \) subunits (Wang and Semenza, 1995). HIF-1\( \alpha \) 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 O2 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...
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 (Kyiakas 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; Back 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 in 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 37°C in a humidified atmosphere of 5% CO2. Cytotoxicity was measured by the microculture tetrazolium (MTT) method. Exponentially growing cells were inoculated to 5 × 10^4 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 37°C for 4 h. To dissolve formazan, 100 µl DMSO was added and the plates were measured at 540 nm by spectrophotometer. The IC50 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 × g for 10 min and the supernatants were incubated for 4 h at 37°C 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 × g, fixed with 2% glutaraldehyde in PBS for 24 h, washed in 0.1 M Caocodylate, pH 7.4, and fixed with 0.1% OsO4 in 0.1 M Caocodylate for 1 h 30 min. After fixation, the cells were washed with 0.1 M Caocodylate, pH 7.4 and then dehydrated in graded ethanol. Next, the cells were impregnated with propyline oxide and embedded in Polybed 812 (Polyscience, Inc., Warrington, USA). After a 60°C 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 Na3VO4, 2 mM MgCl2, 10 mM KCl, 1 mM DTT, 40 µg of PMSF per ml and 10 µg of both pepstatin and leupeptin per ml, pH 7.5). The cellular suspensions were homogenized and harvested by 600 × g for 5 min. To prepare the cytosolic fraction, the supernatant was obtained and
centrifuged at 12,000 \times 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 \times 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 \times 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 antibody 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.

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% (IC50) was determined. The cobalt chloride showed that the concentration-dependent cytotoxicity and IC50 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.
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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).

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 cobalt chloride for 1, 2, 3 and 6 h. 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.
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 (Uremuur 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 CoCl2 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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References


