Manganese activates the mitochondrial apoptotic pathway in rat astrocytes by modulating the expression of proteins of the Bcl-2 family

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Manganese (Mn) is a trace element known to be essential for brain development as well as for normal biochemical and cellular functions in the central nervous system (CNS) (for reviews see Aschner and Dorman, 2006; Aschner et al., 1999; Takeda, 2003). Mn serves as a cofactor for numerous enzymes essential for metabolic brain homeostasis, including glutamine synthetase, mitochondrial superoxide dismutase (Mn-SOD), arginase, calmodulin dependent phosphatase and phosphoenolpyruvate carboxykinase, and can substitute for magnesium in many kinases (Baly et al., 1985; Takeda and Avila, 1986; Wedler and Denman, 1984). However, chronic exposure to high levels of Mn can lead to Mn accumulation in the brain and to produce manganism, a brain disorder whose symptoms (hypokinesis, rigidity and tremor) resemble idiopathic Parkinson's disease (Pal et al., 1999). Excessive Mn concentration has also been implicated in the mechanism of hepatic encephalopathy, associated with chronic liver failure (Krieger et al., 1995). In addition, in patients under long-term parenteral nutrition, some CNS disorders have been attributed to Mn toxicity (Takeda, 2003). Nowadays, the widespread use of the Mn derivative methyl cyclopentadienyl Mn tricarbonyl (MMT) as an antiknock gasoline agent has evolved as a major environmental issue (Abbott, 1987; Cooper, 1984; Kaiser, 2003; Frumkin and Solomon, 1997), creating a potential health risk due to increased atmospheric Mn levels.

Several lines of evidence implicate astrocytes as an early target of Mn, as they are the principal Mn store in the CNS. These cells have been reported to accumulate brain Mn with concentrations 10–50-fold higher than in neurons (50–70 μM), and to possess a high affinity Mn uptake system (Aschner et al., 1992; Wedler and Denman, 1984). Once it has been transported into the cell, Mn is sequestered by mitochondria through the Ca2+ uniporter (for references, see Gunter et al., 2006).

Astrocytes are dynamic and metabolically active cells, which participate in crucial processes in brain metabolism and cell–cell communications in the CNS. Growing evidence support the role of astrocytes in ion homeostasis, regulation of extracellular glutamate concentration, synthesis of neurotrophic factors, modulation of inflammatory/immune responses and involvement in the physiological antioxidant defense of the brain (Hertz and Zielke, 2004; Nedergaard et al., 2003; Fitsanakis et al., 2006). The function of astrocytes in regulating cerebral blood flow and maintaining synaptic function is crucial for keeping neuronal environment normally active (Newman, 2003; Slezak and Pfrieger, 2003).

Activated astrocytes, however, can secrete inflammatory mediators to exert neurotoxic effects after CNS injury.
involved in the pathogenesis of neurodegenerative diseases and neurological disorders (Aschner, 1998; Maragakis and Rothstein, 2006; Nagai et al., 2007; Seifert et al., 2006; Suzumura et al., 2006). In this regard, a correlation between activated astrocytes and apoptosis has been reported (Sharma et al., 2007; Suk et al., 2001).

Apoptosis is a physiological cell death characterized by unique distinguishing features, including cytoplasmic shrinkage, nuclear fragmentation, intranucleosomal DNA cleavage, plasma membrane blebbing with the formation of apoptotic bodies (Wyllie et al., 1980) and selective cleavage of key proteins by a family of highly specific proteases called caspases (Benn and Woolf, 2004; Cohen, 1997; Earnshaw et al., 1999; Hengartner, 2000; Shi, 2002). Apoptosis has been found to be induced by Mn in human B cells (El Mchichi et al., 2007; Schrantz et al., 1999), HeLa cells (Oubrahim et al., 2001), rat pheochromocytoma (PC12) cells (Hirata, 2002; Ito et al., 2006; Kitazawa et al., 2005; Liu et al., 2005; Roth et al., 2000), NIH3T3 cells (Oubrahim et al., 2002), neural stem cells (Tamm et al., 2008) and rat astrocytes (Kotler et al., 2005; Yin et al., 2008). The mechanism by which Mn promotes apoptosis remains controversial, although oxidative stress, energy failure and mitochondrial dysfunction may be the primary routes of action (for review, see Takuma et al., 2004).

Since astrocytes play a central role in maintaining neuronal viability both under normal conditions and during different insults, the study of astrocytic response to insults or injury is essential to understand many types of brain pathologies. In this work, we provide evidence that Mn induces the mitochondrial apoptotic pathway in rat cortical astrocytes by modulating the expression of Bcl-2 family proteins.

1. Experimental procedures

1.1. Reagents

 Dulbecco’s modified Eagle’s medium (DMEM), amphotericin B, GSH (reduced form), manganese chloride, NAC, 2,7-dichlorodihydrofluorescein diacetate (DCDHF-DA), Hoechst 33258 fluorochrome and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from BIO-NOS (Buenos Aires, Argentina), N-2-hydroxyethylpiperazine-N’-2-ethane-sulfonic acid (HEPES) was from ICN Biomedicals (Irvine, CA, USA), penicillin/streptomycin and glutamine were supplied by HyClone (Logan, UT, USA). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The mitochondria-specific red fluorescent probe Mitotracker Red CMXROS was from Molecular Probes (Eugene, OR). The caspase colorimetric substrates acetyl-Asp-Glu-Val-p-nitroanilide (Ac-DEVD-pNA) and acetyl-Val-Glu-Asp-p-nitroanilide (Ac-VEID-pNA) were from Calbiochem (La Jolla, CA, USA). Caspase substrates were dissolved in dimethyl sulfoxide (DMSO). Final concentration of DMSO did not exceed 0.1%. DMSO added to the samples did not affect cell viability, morphology or other parameters tested in this study. All other chemicals used were of the highest purity commercially available.

1.2. Animal handling

For all experimental procedures, animals were handled in accordance with a protocol under the International Council for Care and Use of Laboratory Animals.

1.3. Cell culture

Cortical astrocytes were isolated from postnatal day 2–4 Sprague–Dawley rat pups as previously described (Juknat et al., 2003). Briefly, the cortical tissue was dissected and transferred to DMEM supplemented with 10% heat-inactivated FBS, 2.0 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B. Tissue was mechanically dissociated through a 18 g 6.5 cm needle and the resulting cell suspension was plated on 60 mm plastic dishes. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO2–95% air and the medium was renewed twice a week. Oligodendrocytes and microglia were detached from the dishes by shaking and removed by changing the medium. Under these conditions, confluent cultures were obtained after 3 weeks (1.5–1.6 × 10^6 cells/dish) and the cells were 95–97% positive for glial fibrillary acidic protein (GFAP) as determined by immunostaining. For all experiments, confluent attached astrocytes were removed from Petri dishes with 0.25% trypsin, diluted with DMEM/10% FBS and replated into 12-well plates (2 ml; 1.4 × 10^5 cells/well). After 7–8 days the cells became confluent and ready for use. Cultures were exposed to different treatments in serum-containing medium and in serum-free medium as detailed in Section 2.

1.4. Viability assay

MTT assay was routinely used to assess metabolic activity, by determining the reduction of the tetrazolium salt into the blue formazan product, by the mitochondrial dehydrogenases, as previously described (Juknat et al., 2005). Briefly, MTT was dissolved in PBS and filtered through a Millipore filter (pore size, 0.22 μm). After treatments, the medium was removed and the cells were washed twice with Earle solution. MTT solution was added to the culture at a final concentration of 0.5 mg/ml. After 90 min incubation at 37 °C, the solution was removed and the produced formazan was solubilized in 1 ml of DMSO. Absorbance was measured at 570 nm with background subtraction at 650 nm, in a Bio-RAD Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA) and the MTT reduction activity was expressed as a percentage of the control.

1.5. Phase-contrast microscopy

In order to analyse the effect of Mn on the cellular morphology, astrocytes were incubated with 1 mM MnCl2 for different times, in the absence or presence of FBS as indicated in Section 2. Morphological changes were observed using a phase-contrast microscope (Olympus CK2, UK Ltd.) equipped with an Olympus SC55 camera.

1.6. Detection of apoptotic cells by fluorescence microscopy

Morphological determination of apoptosis was performed by cell staining with the nuclear fluorochrome Hoechst 33258 and visualized by fluorescence microscopy, as previously described (Juknat et al., 2005). Briefly, astrocytes (1.4 × 10^6 cells/well) were cultured on poly-1-lysine-coated glass coverslips and kept for 7 days as indicated above. After treatments, cells were washed twice with PBS and fixed in glacial acetic acid:methanol (1:3, v/v) for 10 min at room temperature. Fixed astrocytes were washed twice with PBS, stained with Hoechst 33258 (1 μg/ml in PBS) for 10 min at room temperature and then examined by fluorescence microscopy (Eclipse E600, Nikon; Nikon Instech Co., Ltd., Karagawa, Japan), using filters for DAPI (excitation: 330–380 nm; emission: 435–485 nm). The images were captured with a CoolPix5000 digital camera (Nikon; Nikon Instech Co., Ltd., Karagawa, Japan). Digital pictures were analysed on a Pentium PC computer and assembled using Adobe Photoshop 7.0 software. Apoptotic cells were scored on the basis of the presence of highly condensed or fragmented nuclei.

1.7. Caspase 3/7 and caspase 6 activity assays

Caspase activity was measured according to Juknat et al. (2003). In brief, treated cells were lysed in lysis buffer (50 mM Tris–HCl pH 7.4 containing 1 mM EDTA, 10 mM EGTA, 10 μM digitonin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin and 10 μg/ml benzamidine), for 30 min at 37 °C. Cell lysates were clarified by centrifugation and 150 μl of the resultant supernatant (90–100 μg protein) was incubated with 146 μl of incubation buffer (100 mM HEPS pH 7.5, 10% glycerol, 1.0 mM EDTA and 10 mM DTT) and 4 μl of the substrate Ac-DEVD-pNA (100 μM) for caspase 3/7 (EC 3.4.22.56/EC 3.4.22.60) or Ac-VEID-pNA (100 μM) for caspase 6 (EC 3.4.22.59), at 37 °C for 1 h. Blanks were also run, containing either the substrate or the cell lysate alone, to deduce in each case. Caspase-catalyzed release of the chromophore pNA from the substrate was measured at 405 nm in a Bio-RAD Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA) and cleavage activity was expressed as pNA absorbance units per mg protein. Protein concentration was determined by Bradford’s method (1976) using bovine serum albumin as a standard.

1.8. Analysis of mitochondrial transmembrane potential

Astrocytes (1.4 × 10^6 cells/well) were grown on poly-1-lysine coated glass coverslips in 12-well plates. After treatments, cells were washed twice with PBS and incubated with the cell-permeant mitochondria-specific red fluorescent probe Mitotracker Red CMXROS at a final concentration of 100 nM in culture media for 30 min at 37 °C. Coverslips were rinsed with PBS, followed by fixation with cold methanol for 20 min at –20 °C, washed twice with PBS and then mounted on glass microscope slides. Samples were examined under a Nikon fluorescence microscope Eclipse E600 (Nikon Instech Co., Ltd., Karagawa, Japan) using TRITC filters (excitation: 528–553 nm; emission: 600–660 nm) or stored covered at –20 °C until used. The images were captured with a CoolPix5000 digital camera (Nikon; Nikon Instech Co., Ltd., Karagawa, Japan) and analysed on a Pentium PC computer. Images were assembled using Adobe Photoshop 7.0 software.
1.5. Determination of cytochrome c location by immunofluorescence

Astrocytes (1.4 x 10^5 cells/well) were cultured on poly-lysine coated glass coverslips in 12-well plates as described above. After fixation with cold methanol for 20 min at -20°C, the cells were washed twice with PBS, permeabilized with 0.1% SDS in PBS for 5 min at room temperature, washed twice with PBS and incubated for 30 min in a 3% bovine serum albumin (BSA)-PBS blocking solution. Cytochrome c was detected with a rabbit anti-rat polyclonal antibody, followed by a goat anti-rabbit fluorescein-conjugated secondary antibody. Immunofluorescence staining was assessed using a fluorescence microscope (Eclipse E600, Nikon Instech Co., Ltd., Karagawa, Japan) equipped with image acquisition system. Images were captured with a Nikon CoolPix5000 digital camera (Nikon Instech Co., Ltd., Karagawa, Japan) and analysed on a Pentium PC computer. Images were assembled using Adobe Photoshop 7.0 software.

1.10. Isolation of mitochondria

All procedures were carried out on ice and all solutions were ice-cooled prior to use. Cell lysates were prepared in mitochondrial lysis buffer (62.5 mM Tris–HCl buffer pH 6.8, 6 M urea, 2% SDS, 0.5% NP-40, 10 mM EDTA) to obtain mitochondrial integrity. Lysates were centrifuged at 900 g for 15 min at 4°C to remove nuclei and large cellular debris. The supernatants were recovered and centrifuged at 12,000 g for 15 min at 4°C to obtain the crude mitochondrial pellet, which was resuspended in electrophoresis lysis buffer, as described below.

1.11. Western blots

Western blots were performed according to standard procedures. Cells were resuspended in electrophoresis lysis buffer (50 mM HEPEs buffer pH 7.0, 1% NP-40, 0.5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml benzamidine and 10 μg/ml pepstatin). Ice-cold sucrose buffer (2.5 mM) containing 600 mM sucrose, 150 mM mannitol, 50 mM Tris–HCl pH 6.8 and 5 mM EDTA was added to maintain mitochondrial integrity. Lysates were centrifuged at 900 g for 15 min at 4°C, to remove nuclei and large cellular debris. The supernatants were recovered and centrifuged at 12,000 g for 15 min at 4°C, yielding a mitochondrial rich pellet. Mitochondria were carefully resuspended in the supernatant buffer (1×) described above and centrifuged at 9000 g for 10 min at 4°C. The supernatant was further centrifuged at 12,000 g for 15 min at 4°C to obtain the crude mitochondrial pellet, which was resuspended in electrophoresis lysis buffer, as described above.

1.12. Determination of Mn uptake

Cells (2 x 10^4) were transferred to borosilicate tubes and acidified with concentrated analytical grade nitric acid to pH < 2. All the glassware was pretreated with 5% nitric acid for 24 h, thoroughly rinsed with double distilled water several times and dried before use. Manganese concentrations were measured by flame atomic absorption spectrophotometry (Atomic Absorption Spectrophotometer Varian 575 AA(λ = 279.5 nm), using a deuterium lamp for background correction. To check the accuracy of the analyses, standard addition methods were used to overcome matrix effects. Blanks values were negligible.

1.13. Statistical analysis

Results shown are representative of at least three independent experiments performed in triplicate. Data are presented as mean± S.E.M. values. Experimental differences between treatments were analysed by one-way ANOVA followed by Student–Newman–Keuls post hoc test, using the software GraphPad Instat 3.06. Values of p < 0.05 were taken as being statistically significant.

2. Results

2.1. Rat astrocytes undergo cell death after serum deprivation and manganese treatment

Rat astrocytes were incubated as detailed in Section 1, under both serum deprivation and 10% PBS conditions. Results showed in Fig. 1 demonstrate that under serum-free medium (SF), cell viability decreases by 30% (p < 0.001), and 24 h exposure to 1.0 mM Mn in SF produces 61% cell survival (p < 0.001). Under SF conditions, 0.72% Mn (p < 0.01) was incorporated and accumulated cells (0.72% in 24 h; p < 0.01), reaching levels in the order reported for astroglial cells from chick embryo cortex cultured in SF grown medium (50–70 μM) (Tholey et al., 1988). Therefore, we used 1.0 mM Mn in subsequent experiments to evaluate the cellular mechanisms of cell death in rat cortical astrocytes.

In order to analyse the effect of Mn on cellular morphology, monolayers of rat cortical astrocytes were incubated with or without 1.0 mM Mn in the presence of 10% PBS or under SF conditions. Phase-contrast microscopy images of astrocytes are shown in Fig. 2A. After 5 days in culture, astrocytes exhibited typical flattened, fibroblast-like morphology, showing cellular processes. Cells under serum deprivation underwent slight morphological changes, becoming rounded and shrunken. However, phase-contrast microscopy revealed a dramatic disruption of the cell monolayer with retraction of astrocytic bodies and cellular processes after 24 h treatment with 1.0 mM Mn in SF-medium.

In dying cells, nuclear morphology and chromatin structure can change drastically during the death process. Moreover, different nuclear events can be associated with apoptotic or necrotic cell death. To further investigate the type of death induced by Mn in rat astrocytes, nuclear shape and chromatin integrity were visualized by fluorescence microscopy after staining nuclear DNA with Hoechst 33258 dye. In control cultures, nuclei exhibited normal shape and uniform stained chromatin (Fig. 2B). In the presence of Mn, 23 ± 2% (p < 0.001) of condensed nuclei appeared which increased to 39 ± 4% (p < 0.001) in the absence of serum, the latter with condensed and fragmented nuclear morphology, typical for apoptotic cell death. Apoptotic nuclei were hyperfluorescent, condensed and smaller as compared to normal nuclei. Serum deprivation itself did not produce any observable alterations. These results suggest that Mn-induced cell death proceeds, at least in part, via an apoptotic mechanism.

Fig. 1. Mn and serum deprivation decrease cell viability. Cortical rat astrocytes were exposed to 1 mM Mn for 24 h in either the absence (SF) or presence (S) of serum and the extent of cell injury was evaluated using MTT assay as described in Section 1. Statistically significant differences between the controls and experimental groups are indicated by *p < 0.001 vs. S control; **p < 0.001 vs. SF control. Other experimental details are given in the text.
2.3. Mn affects mitochondrial function and releases pro-apoptotic cytochrome c

One of the rate-limiting steps in apoptotic cell death is the increase in the permeability of the inner and/or outer mitochondrial membrane, accompanied by the release of pro-apoptotic factors (e.g., cytochrome c), normally confined to the intermembrane space. In many models of apoptosis, the mitochondrial transmembrane potential \( (\Delta \Psi_m) \) has been reported to be decreased. As \( \Delta \Psi_m \) decrease is an early event, 1.0 mM Mn treatments were carried out for 6 h. Using the fluorescent dye MitoTracker Red, which accumulates in active mitochondria, we observed a characteristic punctate distribution of mitochondria in control cells (Fig. 3). In the absence of serum or after the addition of 1.0 mM Mn to FBS-containing medium, mitochondrial dysfunction can be observed. Cells showed changes in mitochondrial morphology and dye accumulation in the cytoplasm. Moreover, addition of 1.0 mM Mn for 6 h under SF-medium conditions, caused a dramatic reduction in cellular volume and a collapse of the mitochondrial membrane potential, demonstrated by the presence of high dye fluorescence in the cytoplasm. Disruption of \( \Delta \Psi_m \) was higher after 12 and 24 h de Mn treatment (data not shown).

Cytochrome c release from the intermembrane space of mitochondria is one of the initial steps of the mitochondrial apoptotic pathway known as the intrinsic pathway (Danial and Korsmeyer, 2004). In order to gain more insights into cytochrome c subcellular location after Mn-induced apoptosis, astrocytes were fixed following Mn treatment and the distribution of cytochrome c was visualized by immunostaining using an anti-cytochrome c antibody.
antibody followed by a fluorescein-labeled secondary antibody (Fig. 3). Control cells showed that cytochrome c was distributed mainly in the mitochondria. Six hours after addition of Mn under SF conditions, astrocytes released cytochrome c as indicated by the diffuse fluorescence staining. This effect was more pronounced after 12 and 24 h of Mn treatment (data not shown).

Western blotting from mitochondrial fractions coming from Mn-treated cells under SF conditions, confirm that translocation of cytochrome c to the cytoplasm is nearly complete (84%; p < 0.01) for 24 h in comparison to the control (Fig. 4). Released cytochrome c will accumulate in the cytoplasm, where it will trigger the activation of mitochondrial downstream caspases and the onset of apoptosis.

2.4. Apoptosis triggered by Mn involves activation of effector caspases and cleavage of PARP-1

Activation of the cascade of proteolytic caspases is a known pathway of apoptosis in many biological systems. We investigated whether Mn treatment activates effector caspases (caspase 3, 6 and 7) and promotes cleavage of the enzyme poly(ADP-ribose) polymerase-1 (PARP-1), further downstream in the apoptotic pathway. Fig. 5 shows that caspase 3/7 specific activity increased by 95% (p < 0.05) in astrocytic cells after serum deprivation for 24 h. Exposure to 1.0 mM Mn under SF conditions enhanced caspase 3/7 and caspase 6 specific activity by 5.2-fold (p < 0.001) and 1.8-fold (p < 0.05), respectively, above basal levels. Caspase 6 activity did not change after serum deprivation or after the addition of Mn to serum-containing medium. On the other hand, increased caspase 3 activity in cells treated with 1.0 mM Mn in serum-containing medium, correlates with the presence of apoptotic nuclei shown in Fig. 2. These results suggest that Mn induces apoptosis through a caspase-dependent pathway.

Cleavage of PARP-1 by effector caspases is one of the markers of cells undergoing apoptosis. Cleavage can be detected either by reduction of full length PARP-1 (116 kDa) and/or by increase of the 89 kDa C-terminal apoptotic fragment. PARP-1 can be cleaved in vitro by almost all caspases, while in vivo it is the target of caspase 3 and 7 (Soldani and Scovassi, 2002). We examined the cleavage of PARP-1 in Mn-treated astrocytes by Western blotting using an anti-PARP polyclonal antibody, which recognizes both the 116- and 89-kDa polypeptides (Fig. 6). PARP-1 breakdown, with complete disappearance of the 116 kDa form was observed after 24 h treatment with 1.0 mM Mn under SF conditions (p < 0.001). A partial PARP-1 cleavage (56%; p < 0.001) was detected under serum deprivation, but no breakdown could be observed in Mn-treated cells in serum-containing medium.

2.5. Bax promotes Mn-mediated cell death

One of the regulators of cytochrome c release from the mitochondria is the pro-apoptotic protein Bax, which in viable cells remains in the cytoplasm in an inactive monomeric form or loosely attached to membranes. Upon receipt of a death signal, Bax translocates and inserts into the mitochondrial outer membrane as a homo-oligomerized multimer, resulting in the loss of mitochondrial membrane potential and the release of apoptogenic factors from the mitochondria (for review see Danial and Korsmeyer, 2004).

In an attempt to gain a better understanding of the pathway underlying Mn-induced apoptosis, we analysed Bax protein expression from Mn-treated astrocytes by Western blotting (Fig. 7). Cell lysates were prepared in a lysis buffer-containing CHAPS that did not affect Bax conformation (Hsu and Youle, 1997, 1998). Exposure to Mn under SF conditions increased Bax
conditions led to a 11-fold increase (p < 0.05 vs. S control) in Bax protein (1:500). Reprobing of the same blot with β-actin antibody was performed to normalize for protein loading. Other experimental conditions are given in the text. (B) Bands were quantified with a Phosphoimager (Fuji Photo Film Co. Ltd.). Image corresponds to one representative experiment. AU: arbitrary units.

**3. Discussion**

Exposure to divalent heavy metals, such as Mn, has been linked to several neurological disorders, including Parkinson’s-like syndrome (Olanov, 2004). High concentrations of Mn can trigger a series of intracellular events that lead to apoptosis in different cell types (Schrantz et al., 1999; El Mchichi et al., 2007; Oubrahim et al., 2001; Roth et al., 2000; Kotler et al., 2005; Yin et al., 2008; Desole et al., 1997; Tamm et al., 2008) but the underlying molecular mechanism in astrocytes has not been clearly elucidated yet.

In the present study we used primary cultures of rat cortical astrocytes in order to examine the molecular signaling pathways involved in Mn-induced cell death. Exposure to Mn significantly decreased astrocytes viability (39%, Fig. 1), producing morphological changes, with cells becoming rounded, shrunken and more loosely attached to the plastic surface (Fig. 2A). These events were accompanied by a marked increase (39%) in the number of condensed and fragmented nuclei, a characteristic morphological feature of apoptosis (Fig. 2B). Since MTT assay is based on mitochondrial dehydrogenases activity (Mosmann, 1983), the viability results implicate the inhibition of mitochondria functionality by Mn, as previously suggested (Chen and Liao, 2002; Barhoumi et al., 2004; Kakulavarapu et al., 2004).

Chronic exposure of various cell types to Mn has shown to induce oxidative stress and mitochondrial energy failure, factors that are often implicated in the induction of the mitochondrial permeability transition (MPT) (Desole et al., 1997; Chen and Liao, 2002; Barhoumi et al., 2004; Kakulavarapu et al., 2004; Sun et al., 1993; Chen et al., 2006; Liao et al., 2006; Norenberg and Rao, 2007; Tretter et al., 2007). Even more, employing the oxidation-sensitive fluorescent probe DCDHF-DA and fluorescence microscopy, we detected ROS generation when astrocytes were exposed to Mn accordingly to previous results (Chen and Liao, 2002; Liao et al., 2006) (data not shown).

It has been reported that in rat cortical astrocytes, Mn caused dissipation of the mitochondrial membrane potential at concentrations (10–100 μM) that do not produce morphological alterations (Kakulavarapu et al., 2004). Similar effects were found by Barhoumi et al. (2004) in C6 glioma cells. In this report mitochondrial depolarization was evaluated employing Mito-Tracker Red (Fig. 3). A significant decrease in the MitoTracker Red

![Fig. 7. Mn increases Bax levels.](image)

![Fig. 8. Mn increases the Bcl-Xs/Bcl-Xl ratio.](image)
fluorescence was observed in mitochondria after exposure to Mn 1.0 mM for 6 h in SF, suggesting substantial loss of the mitochondrial membrane potential. At this point, cell death, as assessed by MTT assay, was not evident (data not shown), as previously reported by Kakulavarapu et al. (2004). Loss of \( \Delta \Psi_m \) was more pronounced in apoptotic cells (12 and 24 h treatment, data not shown). Mn treatment in the presence of serum also dissipated the \( \Delta \Psi_m \), but the effect was smaller (Fig. 3).

During apoptosis, cytochrome c translocates from mitochondria to cytosol, where it triggers a cascade of events involving caspases activation which, in turn, cleave multiple cytoplasmic and nuclear substrates. Exposure of cells to Mn in SF induced cytochrome c translocation into the cytoplasm as demonstrated by subcellular fractionation and Western blot, and immunofluorescence (Figs. 3 and 4). Translocation of cytochrome c to the cytoplasm is nearly complete at 24 h treatment (Fig. 4). Although our results confirm previous reports indicating that Mn induces loss of \( \Delta \Psi_m \), we go deeper by showing that this metal also produces the release of cytochrome c from mitochondria to cytosol. Lower release of cytochrome c was also observed in the presence of serum. Taking together these results and those obtained with Hoechst staining suggest that the time lapse appearance of apoptotic events in Mn-treated astrocytes is longer in the presence of serum compared to SF.

The mitochondrial release of cytochrome c is modulated by the Bcl-2 family of proteins (for review, see Antonssohn, 2001). Although many details remain unclear at present, in general, the ratio between anti-apoptotic proteins such as Bcl-2 relative to pro-cell death proteins such as Bax determines the ultimate sensitivity of cells to various apoptotic stimuli. Therefore, we examined the influence of Mn on the expression level of anti-apoptotic members Bcl-2 and Bcl-XL and pro-apoptotic proteins Bax and Bcl-Xa. Immunoblotting analysis revealed that Bcl-2 protein in astrocytes is hardly detectable, and no changes in its abundance could be observed (data not shown). In contrast, Mn in SF conditions, led to an increase in the pro-apoptotic Bax protein (2.3-fold) (Fig. 7). Furthermore, the Bcl-Xa/Bcl-Xl ratio was also increased (Fig. 8). The ratio of anti to pro-apoptotic molecules such as Bcl-2/Bax and Bcl-Xa/Bcl-Xl constitutes a rheostat that sets the threshold of susceptibility to apoptosis in the intrinsic pathway (Korsmeyer, 2002). Although Bax translocation to mitochondria was not demonstrated in astrocytes, it is possible that enhanced Bax levels could increase the probability of homo- or heterodimerization that seems necessary for its translocation (Gross et al., 1998). Therefore, results presented here suggest that Mn shifts the balance of cell death/survival effectors to favor the apoptotic demise of astrocytes.

Historically, release of cytochrome c from mitochondria had been associated with activation of caspases (Green and Reed, 1998). To determine if the loss of mitochondrial cytochrome c would activate effector caspases, we assayed caspases 3/7 and 6 activities. As shown in Fig. 5, caspases 3/7 and 6 activities were significantly activated by Mn treatment in SF. Despite caspase 3 activity being similar both under serum deprivation as well as in incubations with Mn in the presence of serum, PARP-1 cleavage was not detected in this last condition. These differences suggest that the mechanisms underlying manganese-induced apoptotic events could differ from those related with serum withdrawal-associated apoptosis in rat cortical astrocytes as suggested by Hirata (2002) in PC12 cells.

The cleavage of PARP-1 by caspases is recognized as a hallmark of apoptosis (Tong et al., 2001). In the present study we observed a complete disappearance of the 116 kDa form after treatment with Mn under SF conditions (Fig. 6). The lack of 89 kDa band is consistent with the results obtained by Alexandre et al. (2000), reporting the degradation of PARP-1 in apoptotic SHE cells, probably mediated by proteases different to caspase 3.

Translocation of cytochrome c, together with caspase 3/7 and caspase 6 activation and PARP cleavage, provide evidence suggesting that mitochondria-mediated apoptosis involving the caspases cascade is clearly induced by Mn treatment in rat cortical astrocytes. Several lines of evidence indicate that manganese cell treatment induces different cellular changes, depending on the cell types studied. This suggests that Mn-activated transduction pathways may differ according to the targeted cell. The central nervous system, and the basal ganglia in particular, are an important target in manganese neurotoxicity. In this regard, it has been reported that neurotoxic concentrations of Mn induce mitochondrial depolarization in cultured astrocytes, while in cultured neurons such effect is delayed and less severe (Norenberg and Rao, 2007). This differential effect could be due to the higher capacity of astrocytes to accumulate Mn as compared to neurons. Due to this characteristic, it has been postulated that astrocytes represent an initial site of Mn-induced damage. As mentioned above, astrocytes are essential for normal neuronal activity. Thus, disruption of their functions may contribute to neuronal injury in manganism. Further elucidation of molecular signaling mechanisms underlying Mn-induced apoptosis will shed light on the effective molecular intervention targets in manganism. Our model system, using cortical rat astrocytes treated with Mn, could serve as a good tool for investigations aimed to elucidate the role of apoptosis in manganism and perhaps in other neurodegenerative diseases.

Acknowledgments

This work was supported by grants from the University of Buenos Aires (UBACYT Tx-36 and UBACYT 007) to A.A.J. and from the Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET (PIP2000/02631) to A.A.J. and (PEI No. 6398 and PIP2005-2006/5406) to M.L.K. The authors are very grateful to Dr. Elba Vazquez for the critical reading of the manuscript. We acknowledge the contribution of Dr. Dante Paz for providing the goat anti-rabbit fluorescein-conjugated secondary antibody.

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