Mitochondria play no roles in Mn(II)-induced apoptosis in HeLa cells

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Manganese(II) has been shown to exhibit catalase-like activity under physiological conditions. In the course of studies to test the antioxidant activity of Mn(II) on HeLa cells, it was observed at high concentrations (1–2 mM) that Mn(II) also induced apoptosis, as judged by changes in cell morphology, caspase-3 activation, cleavage of poly-(ADP) ribose, and DNA condensation. However, in contrast to established mechanisms, the Mn(II)-induced apoptosis is associated with an increase rather than a decrease in mitochondrial inner-membrane potential, as monitored by the fluorescent probe tetramethylrhodamine ethyl ester. Based on immunochemical analysis, Mn(II)-induced apoptosis does not lead to the release of cytochrome *c* into the cytosol. These and other measurements show that treatment with Mn(II) leads to enhancement of the mitochondrial "membrane mass," has no effect on mitochondrial volume, and does not affect the permeability transition pore. Together, these results support the view that Mn(II)-induced apoptosis occurs by a heretofore unrecognized mechanism. In addition, it was demonstrated that Mn(II) treatment leads to an increase in the production of reactive oxygen species (peroxides) and to the induction of the manganese superoxide dismutase and catalase activities but has no effect on the Cu,Zn-superoxide dismutase level.

Manganese(II) exhibits diverse effects in cellular physiology. In trace amounts, it serves as a cofactor for the activation of several enzymes. It is an essential component of the mitochondrial superoxide dismutase (Mn-SOD). Complexes between bicarbonate, amino acids, and Mn(II) exhibit catalase-like activity (1, 2) and have been shown to protect endothelial cells from H_2O_2 toxicity and from reactive oxygen species (ROS) produced during oxidative burst of neutrophils (3, 4). However, long-term exposure to relatively high concentrations of Mn(II) leads to the intracellular accumulation of abnormally high levels (4, 5) that are toxic to a number of cell types, including neuronal cells where such accumulations can lead to the development of Parkinson-like syndromes reminiscent of the effects of mitochondrial toxins (6). Mn(II) also has been reported to induce apoptosis of PC12 and B cells (7–9). There is growing evidence that ROS derived from mitochondrial aberrations are implicated in cell-signaling pathways that lead to apoptosis. Here, we report results of studies to determine whether mitochondrial aberrations are implicated in Mn(II)-induced apoptosis.

Materials and Methods

Materials. $MnCl_2 \cdot 4H_2O$ (99.9%) was obtained from Aldrich. Materials for protein electrophoresis and poly(vinylidene difluoride) membrane (0.2 μm) were purchased from Bio-Rad. 2′,7′-Dichlorofluorescein diacetate (DCFH-DA), mitotracker green FM (MTGFM), nonyl acridine orange (NAO), tetramethylrhodamine ethyl ester (TMRE), dihydrorhodamine 123, and carbonyl cyanide *p*-(chlorophenylmethoxy)hydrazone (FCCP) were obtained from Molecular Probes. Stock solutions of these dyes were made in DMSO, except for the DCFH-DA, which was made in absolute ethanol and kept at −20°C in the dark. Amplex Red Hydrogen Peroxide Assay Kit was purchased from Molecular Probes. Hoechst 33342 and diaminobenzidine were bought from Sigma. Specific polyclonal antibodies against Mn-SOD or against Cu,Zn-SOD were obtained from Biodesign International, Kennebunkport, ME. Anti-caspase-3 antibody, caspase-3 substrate Acetyl-DEVD-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC), and monoclonal antibody raised against the poly(ADP-ribose) polymerase (PARP) clone C-2-10 that recognizes an epitope located at the carboxyl end of the DNA-binding PARP domain were purchased from PharMingen. Goat anti-mouse IgG, goat anti-rabbit, and the enhanced chemiluminescence system were purchased from Pierce.

Cell Culture. HeLa cells were grown in DMEM containing high glucose (4.5 g/liter, 25 mM) and supplemented with 10% (vol/vol) FBS without heat inactivation, 50 units/ml penicillin, and 50 mg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. The medium was changed every other day. All Mn(II) treatments were performed in the culture medium (DMEM) containing 10% (vol/vol) FBS. Immediately before treatment, the medium was removed and replaced with fresh medium.

Preparation of Cell Lysates. For each assay, HeLa cells were grown in six-well plates. When the cells reached confluence, they were treated with various concentrations of Mn(II) for 24 hr (or as otherwise indicated) at 37°C in 95% air/5% CO_2. At the end of the treatment, adherent and nonadherent cells were harvested with a rubber policeman, and the cultures were centrifuged at 500 × *g* at 4°C. The pellets containing both adherent and nonadherent cells were resuspended in 100 μl of solution consisting of 100 mM phosphate buffer (pH 7.4) composed of 1 mM PMSF, 10 mg/ml leupeptin, and 10 mg/ml aprotinin and lysed by several freezing/thawing cycles. The lysates were centrifuged, and the supernatants were frozen at −20°C until used. The protein concentrations of the supernatants were determined with the Bio-Rad protein assay with BSA as standard.

Cytotoxicity Assay. The nonradioactive assay used in this investigation was based on the measurement of lactate dehydrogenase (LDH) released from the cytosol of damaged cells. The assay was conducted according to the supplier's protocol.

Caspase-3-Like Activity. Cellular extract (10 ml) was prepared as indicated above (or buffer only for blank) and was mixed with 200 ml of ICE standard buffer (100 mM Hepes–KOH, pH 7.5/10% (wt/vol) sucrose/0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)/10 mM DTT/0.1 mg/ml ovalbumin) containing 1 mM Ac-DEVD-AFC. The fluorogenic product AFC was monitored with a Cytofluor 4000 (PerSeptive Biosystems,

Abbreviations: Mn-SOD, manganese superoxide dismutase; ROS, reactive oxygen species; DCFH-DA, 2′,7′-dichlorofluorescein diacetate reduced form; MTGFM, mitotracker green FM; NAO, nonyl acridine orange; TMRE, tetramethylrhodamine ethyl ester; FCCP, carbonyl cyanide *p*-(chlorophenylmethoxy)hydrazone; PARP, poly(ADP-ribose) polymerase; LDH, lactate dehydrogenase; AFC, 7-amino-4-trifluoromethyl coumarin; DCF, 2′,7′-dichlorofluorescein; $\Delta\psi$m, mitochondrial inner transmembrane potential; Ac-DEVD-AFC, acetyl-DEVD-7-amino-4-trifluoromethyl coumarin.

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Framingham, MA) multiwell fluorescence plate reader at 380-nm excitation and 460-nm emission. Caspase activity was normalized for protein concentration of individual extracts and compared with the caspase-3 activity in the control sample.

Western Blot Analysis. Equal amounts of total cellular protein were separated by SDS/4–20% PAGE under reducing conditions. After electrophoresis, proteins were transferred to poly(vinylidene difluoride) membranes along with prestained molecular-weight markers at 30 V for 90 min. Blots were blocked with 5% (wt/vol) dry milk in PBS containing 0.5% Tween 20 for 60 min and probed with appropriate antibodies (1 mg/ml in blocking buffer) overnight at 4°C. After washing with PBS/5% (vol/vol) Tween 20, membranes were incubated with peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies for 120 min (dilution of 1:5,000). Specific proteins were detected with the enhanced chemiluminescence system. In some cases, blots were reprobed with different antibodies after stripping for 30 min in a buffer of 62.5 mM Tris·HCl (pH 6.7), 100 mM β-mercaptoethanol, and 2% (vol/vol) SDS. Equal protein loading was controlled by amido-black staining of membranes.

Nuclear Staining with Hoechst Dye. Staining was performed as described (10). In brief, control and treated cells [0.1–2 mM Mn(II) for 24 hr at 37°C] were fixed with 3.7% (vol/vol) paraformaldehyde in PBS for 10 min at room temperature and permeated with 0.1% Triton X-100 for 10 min at room temperature. The fixed cells were stained with Hoechst 33342 (1 mg/ml of PBS) for 5 min, washed, and then examined by fluorescence microscopy (340-nm excitation and 510-nm barrier filter). Apoptotic cells were identified by the presence of highly condensed or fragmented nuclei. Although a fraction of the total cells became detached after treatment with Mn(II), only cells that remained attached to the cover slips were analyzed.

Assessment of Intracellular ROS Levels. Generation of ROS was assessed by an oxidation-sensitive fluorescent probe DCFH-DA. DCFH-DA is a nonpolar compound that readily defuses into cells, where it is cleaved by intracellular esterases to form DCFH and, thereby, is trapped inside the cells. DCFH is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) by ROS. After treatment of cells with Mn(II) (0, 0.5, and 1 mM for 24 hr), attached cells were washed with Hanks' buffer and then loaded for 60 min with 50 mg of DCFH-DA in 2 ml of culture medium. The cells were again washed three times with Hanks' buffer to remove the extracellular dye, harvested with a rubber policeman, and lysed in 0.1 M Tris containing 10% (vol/vol) SDS. The lysates were sonicated for less than 10 sec to reduce the viscosity of samples. The supernatants (100 ml) were assayed for DCF fluorescence with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The DCF fluorescence was then normalized based on the protein concentration of individual extracts. The oxidation of dihydrorhodamine to a fluorescent byproduct rhodamine by ROS in control and Mn(II)-treated cells was also monitored with fluorescence microscopy.

H_2O_2 Determination. H_2O_2 released by untreated cells (10^6 cells) or by cells treated with different concentrations of Mn(II) (0–2 mM) for 6 hr was measured with a fluorogenic probe *N*-acetyl-3,7-dihydroxyphenoxazine as described (11). After Mn(II) treatment for 6 hr, the cells were washed. Dye (2 ml) and serum-free media were added to the cells, and they were incubated for 15 min at 37°C. Supernatant (50 ml) was added to 100 ml of the reaction buffer consisting of 10 mM Amplex Red, 10 mM Tris·HCl, pH 7.5, and 1 unit/ml horseradish peroxidase and incubated at 37°C for 30 min before fluorescence measurement using a microplate reader with the excitation and emission set at 560 and 590 nm, respectively. Plots were standardized by using a known concentration of H_2O_2. The specificity of the generation and release of H_2O_2 was confirmed by the addition of catalase (4,000 units/ml).

Catalase Activity Staining. The method of catalase-activity staining was similar to that described by Clare *et al.* (12). Mn(II)-treated and control cells were disrupted by freezing and thawing, and the cell-free extracts (60 mg) were subjected to native electrophoresis on 6% polyacrylamide gels. The gels were soaked for 45 min in 100 mM phosphate buffer (pH 7) containing horseradish peroxidase (50 mg/ml). H_2O_2 was then added to a concentration of 5 mM and incubation continued for another 10 min. The gels then were rapidly rinsed once with distilled water and soaked in 0.5 mg/ml diaminobenzidine in phosphate buffer until staining was complete. Catalase activity was measured as described by Aebi (13). HeLa cells were treated with different concentrations of Mn(II) (0–1 mM) for 24 hr. Cell extracts were then prepared as described above. Cell extracts (40 μg) were added to phosphate buffer (50 mM, pH 7.8) containing 20 mM H_2O_2, and the rate of H_2O_2 consumption was monitored at 240 nm for 2 min in a spectrophotometer.

SOD Activity Measurement. SOD activity in nondenaturing 4–12% polyacrylamide gels was monitored by nitroblue tetrazolium-negative staining as described (14). Briefly, the SOD activity in Mn(II)-treated and control cells was monitored by its capacity to inhibit the reduction of nitroblue tetrazolium by superoxide radical anion.

Determination of "Mitochondrial Mass." To assess mitochondrial mass in control and Mn(II)-treated cells, MTGFM, a mitochondrion-selective probe that becomes fluorescent in the lipid environment of mitochondria, was used. Uptake of the probe is independent of mitochondrial inner transmembrane potential ($\Delta\psi$m). MTGFM contains a thiol-reactive chloromethyl moiety that is capable of forming stable peptide and protein conjugates after accumulation in mitochondria. Near confluence, cells were treated with Mn(II) (0–1 mM) for 24 hr at 37°C. After washing, the remaining cells were probed with MTGFM (dissolved in DMSO) at a concentration of 100 ng/ml in culture medium and then incubated for 30 min at 37°C. Untreated cells received 0.05% of DMSO as a vehicle, which did not induce any visible alteration of cells. After washing with Hanks' buffer to remove the unbound dye, MTGFM-treated cells then were analyzed with a fluorescence microscope and an FITC filter. In other experiments, cells growing on six-well plates were treated with Mn(II), stained with MTGFM as described above, and extracted with lysis buffer [0.1 M Tris/10% (vol/vol) SDS, pH 7.5]. The fluorescence of the extracts was measured with a Cytofluor 4000 (PerSeptive Biosystems) multiwell fluorescence plate reader with excitation and emission set at 500 $\pm$ 20 nm and 580 $\pm$ 40 nm, respectively. The fluorescence of the blank containing no cellular extracts was subtracted from the values. The results were expressed as relative fluorescence intensity per microgram of protein. Experiments with NAO to quantitate the amount of cardiolipin in mitochondria (15) were performed as with MTGFM.

Determination of $\Delta\psi$m. To evaluate $\Delta\psi$m, exponentially growing cells on cover slips were incubated with Mn(II) (0, 0.5, or 1 mM) for 24 hr at 37°C. At the end of the incubation, the medium was removed, and attached cells were labeled with TMRE (a dye that accumulates in mitochondria in response to $\Delta\psi$m) at a concentration of 100 ng/ml of culture medium for 15 min at room temperature. After washing, cells were immediately analyzed with a fluorescence microscope equipped with a 60$\times$ oil-immersion objective. Alternatively, attached cells (on six-well plates) were scraped and lysed in a buffer of 0.1 M Tris containing 10% (vol/vol) SDS, and the TMRE fluorescence was measured with a microplate reader as described above. The excitation and emission wavelengths were set at 508 $\pm$ 20 nm and 580 $\pm$ 40 nm, respectively. The

fluorescence was normalized for the protein concentration of individual extracts. To ensure that mitochondrial uptake of TMRE was related to membrane potential, a control study was carried out simultaneously with an uncoupling agent (FCCP, 5 mg in 2 ml) that is known to abolish the $\Delta\psi m$. For experiments with TMRE and MTGFM, concentrations of proteins were determined with the bicinchoninic acid protein reagent assay with BSA as the standard. All of the experiments described herein were repeated at least three times.

Results

Because most apoptotic pathways are mediated by mitochondria, we investigated whether mitochondrial aberrations are involved in Mn(II)-induced apoptosis to study the mechanism by which Mn(II) induces apoptosis of HeLa cells.

Mn(II)-Mediated Caspase-3 Activation. Because caspases are involved in the execution of apoptosis, protease activity was measured fluorometrically in HeLa cells by following the cleavage of the caspase-3 substrate Ac-DEVD-AFC in the cytosolic fraction of cells treated with various concentrations of Mn(II) ranging from 0.1 to 2 mM. The fluorescence intensity was quantitated and correlated with the enzymic activity. Fig. 1*A* shows that low (0–0.2 mM) Mn(II) concentrations exerted no effect on caspase-3 activity; however, at higher concentrations (0.5–2.0 mM) there was a dose-dependent increase in caspase-3 activity up to ≈5-fold above the control values. Similar results were obtained when activated caspase-3 levels were measured by Western blotting with antibodies that react with both active and inactive (precursor) forms of caspase-3 (Fig. 1*B*). Furthermore, Fig. 1*C* shows that there is no significant difference in LDH release (an indicator for cell necrosis) between control and Mn(II)-treated cells. This observation indicates that the integrity of the plasma membrane seems to be preserved. However, uptake of propidium iodide, a cell-impermeable dye, was observed in cells undergoing apoptosis induced by Mn(II) (data not shown). The thiol-containing compounds in cell lysates, determined spectrophotometrically after derivatization with 5,5′-dithiobis(2-nitrobenzoic acid), showed a net decrease as a function of increasing concentrations of Mn(II) (data not shown).

PARP Cleavage in Mn(II)-Treated HeLa Cells. To confirm the involvement of caspase-3 in Mn(II)-mediated cell death, we analyzed the cleavage of PARP in lysates of HeLa cells treated with different concentrations of Mn(II) (0.1–2 mM) for 24 hr and as a function of time from 2 to 24 hr at 2 mM Mn(II). Results obtained with untreated cells and with cells incubated with 0.1–0.5 mM Mn(II) for 24 hr showed the presence of intact ≈116-kDa PARP. However, when the concentration of Mn(II) was raised to 1 or 2 mM, the cleavage of PARP was clearly detected. Fig. 2*B* shows the time course of PARP cleavage caused by 2 mM Mn(II).

Detection of Apoptotic Cells by Hoechst Staining. To verify the presence of apoptotic cells further, we examined changes in nuclei by Hoechst staining in Mn(II)-treated and control cells. Fig. 3 shows that, when HeLa cells were treated with concentrations of Mn(II) ranging from 0 to 2 mM for 24 hr at 37°C, a significant number of apoptotic cells were observed only in the presence of 1 and 2 mM Mn(II). The nuclei of apoptotic cells were condensed and fragmented as revealed by the Hoechst 33342 nuclear-staining dye and are identified with arrows in Fig. 3 *c* and *d*. In the nonapoptotic cells, nuclei are revealed as rounded contours. Apoptotic cells also were observed when cells were treated with 2 mM Mn(II) for 6 hr (data not shown). These results are in agreement with the caspase-3-activation and PARP-cleavage data shown above.

Estimation of $\Delta\psi m$ Alterations by Mn(II) Treatment. Binding of the fluorescent dye, TMRE, in a variety of mammalian cells has been shown to depend on the high transmembrane potential maintained in functional mitochondria (16). Fig. 4 shows that the TMRE fluorescence displayed a punctuated pattern typical of mitochondrial distribution in treated and untreated cells. However, Mn(II)-treated cells (0.5 and 1 mM for 24 hr) bind and retain more TMRE compared with control cells. In an effort to determine whether this difference in TMRE uptake reflected a difference in mitochondrial transmembrane potential, FCCP, a drug known to disrupt the transmembrane potential of mitochondria (17), was used to monitor the TMRE–mitochondria interaction in treated and untreated cells. Indeed, the addition of FCCP was accompanied by a huge decrease in TMRE fluorescence. These results indicate that

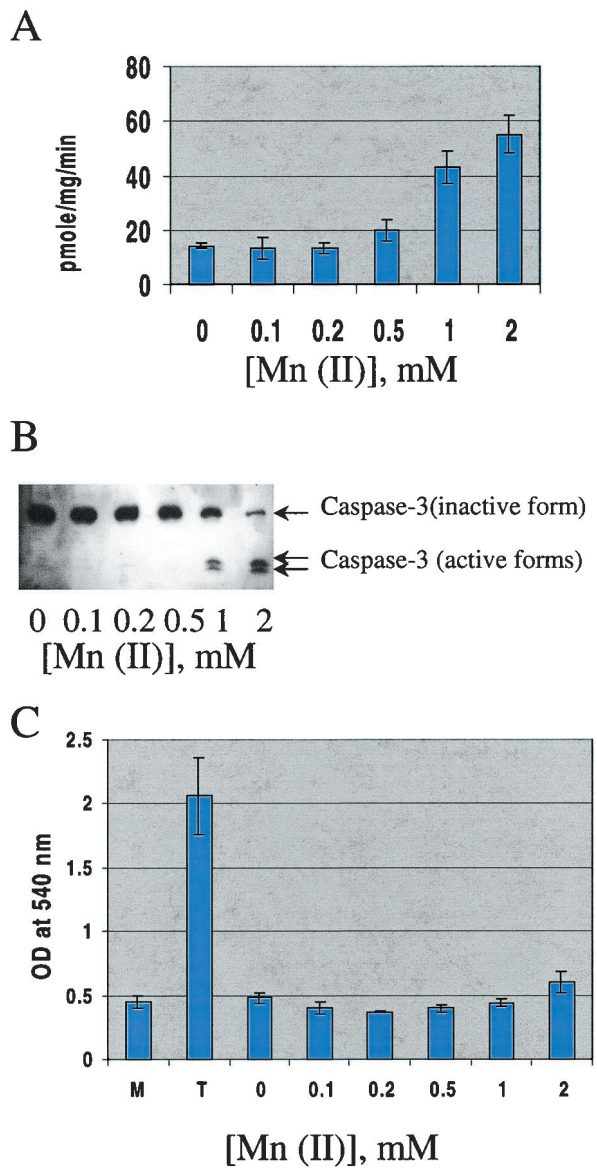

Fig. 1. Caspase-3 activation in HeLa cells and LDH released from cells after treatment with Mn(II). HeLa cells were treated with the indicated Mn(II) concentration for 24 hr. (*A*) Caspase-3 activity in the cell extracts was measured fluorometrically with Ac-DEVD-AFC as substrate. (*B*) Equal amounts of protein extracts were separated electrophoretically and caspase-3 was identified by Western blotting with polyclonal antibody that crossreacted with both the 32-kDa procaspase-3 and the 17-kDa activated enzyme. (*C*) The release of LDH from the intact Mn(II)-treated cells was determined by LDH activity in the culture media by using an LDH assay kit. M and T, culture medium alone and cells lysed with Triton X-100, respectively.

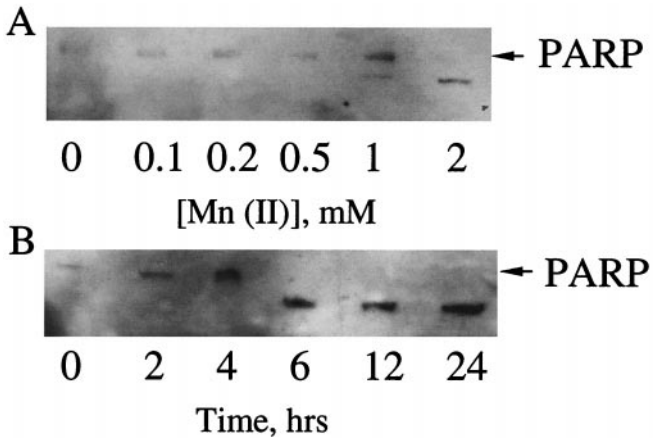

Fig. 2. Mn(II) induces the cleavage of PARP in HeLa cells. PARP immunoreactive protein was detected by Western blotting with a polyclonal antibody (as described in *Materials and Methods*) in HeLa cells treated with Mn(II) (0–2 mM) for 24 hr (*A*) or with 2 mM Mn(II) for 2, 4, 6, 12, and 24 hr (*B*). Fifty micrograms of cell protein extract was loaded in each lane.

Mn(II)-treated cells still have energized mitochondria. To rule out the possibility that the accumulation of TMRE was caused by an increase in mitochondrial volume, an electron microscopy experiment was carried out. When 0.5 or 1.0 mM of Mn(II) was used to treat the HeLa cells for 24 hr, the sizes of their mitochondria were comparable to those of the control cells (Fig. 5*A*). These results suggest that the increase in TMRE uptake in treated cells is caused by the increase in mitochondrial inner membrane potential rather than by an increase in mitochondrial volume.

To study the role of the permeability transition pore (PTP) in Mn(II)-mediated apoptosis in HeLa cells, a technique based on calcein loading and Co^{2+} quenching was used as described (18). The data revealed that Mn(II) (0.5 and 1 mM) had no effect on PTP (Fig. 5*B*). These results are in agreement with the electron microscopy and TMRE results, because PTP opening leads to an increase in mitochondrial volume and to the dissipation of $\Delta\psi m$ (19).

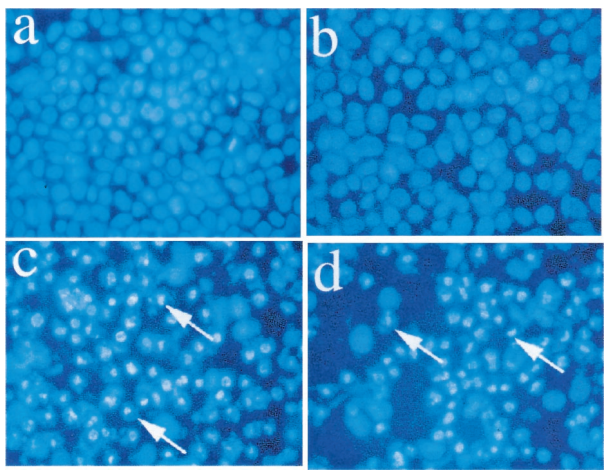

Fig. 3. Apoptotic morphology of HeLa cells after 24-hr treatment with Mn(II). Control and treated cells were fixed with 3.7% (vol/vol) paraformaldehyde in PBS for 10 min and permeated with 0.1% Triton X-100 for 10 min at room temperature. The fixed cells were stained with Hoechst 33342 (1 mg/ml PBS) for 5 min and washed. The morphology of the stained cells was examined with a fluorescent microscope (340-nm excitation and 510-nm barrier filter). Apoptotic cells were identified by the presence of highly condensed or fragmented nuclei (arrows highlight two examples of many). (*a*) Control. (*b–d*) Cells treated with 0.2, 1, and 2 mM Mn(II), respectively.

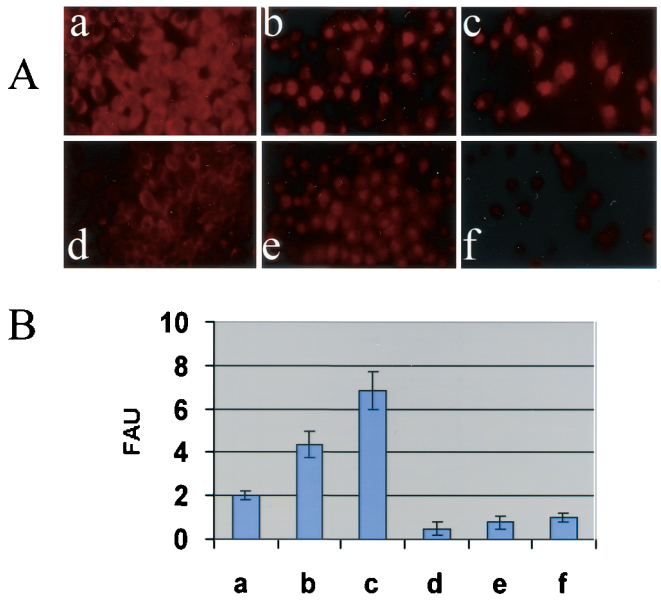

Fig. 4. Analysis of $\Delta\psi m$ by TMRE uptake in HeLa cells treated with different concentrations of Mn(II) (0–1 mM) for 24 hr at 37°C in the presence or the absence of FCCP. After Mn(II) treatment, cells were loaded with TMRE and then analyzed with a fluorescence microscope. (*Aa*) Control. (*Ab*) Cells treated with 0.5 mM Mn(II). (*Ac*) Cells treated with 1 mM Mn(II). (*Ad*) *a* plus FCCP. (*Ae*) *b* plus FCCP. (*Af*) *c* plus FCCP. (*B*) TMRE fluorescence intensity measured by a fluorometer for Mn(II)-treated and control cells. (Bar a) Control. (Bar b) Cells treated with 0.5 mM of Mn(II). (Bar c) Cells treated with 1 mM Mn(II). (Bar d) Bar a plus FCCP. (Bar e) Bar b plus FCCP. (Bar f) Bar c plus FCCP. Data are expressed as the means of three independent experiments. The data in *B* are expressed after normalizing for protein content (see *Materials and Methods*). FAU, fluorescence arbitrary units.

Estimation of Mitochondrial Mass. Mn(II) treatment (0.5 and 1 mM for 24 hr at 37°C) of HeLa cells causes an increase in the fluorescence intensity of mitochondria after staining with NAO (Fig. 6), a dye that binds to cardiolipin in the mitochondrial inner membrane independent of the transmembrane potential (20, 21). A similar observation was made when MTGFM was used (data not

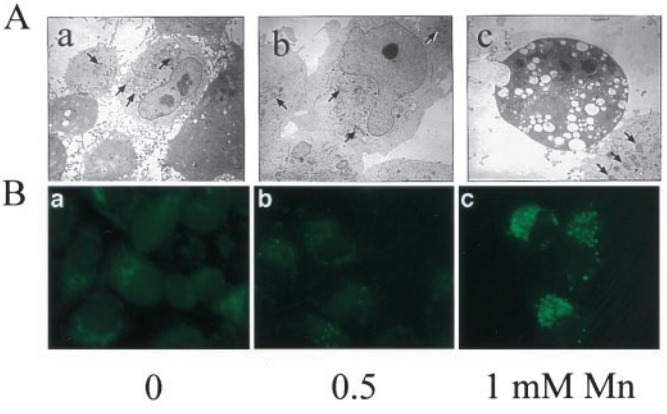

Fig. 5. Transmission electron microscopy and mitochondrial membrane permeability of normal untreated cells and Mn(II)-treated cells. (*A*) HeLa cells were treated with the indicated concentration of Mn(II) for 24 hr at 37°C. After treatment, the cells were fixed, and the mitochondrial morphology (highlighted by arrows) was analyzed by electron microscopy. (*B*) After treatment with the indicated concentration of Mn(II), cells were loaded with 1 μM of calcein for 15 min at room temperature and then incubated with 1 mM $CoCl_2$ in PBS for 1 hr. The cells were washed extensively with PBS and analyzed with fluorescence microscopy.

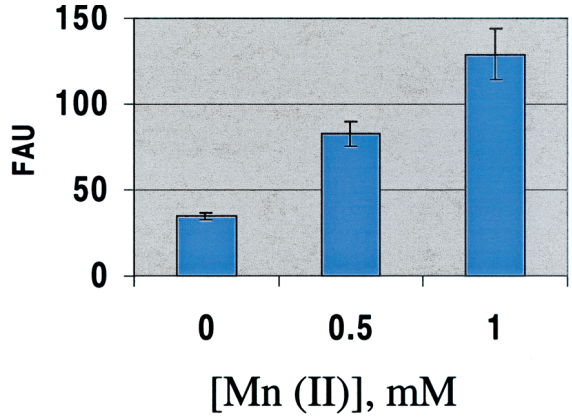

Fig. 6. Analysis of mitochondrial mass by NAO uptake in HeLa cells treated with different concentrations of Mn(II). HeLa cells were incubated with 0, 0.5, and 1.0 mM Mn(II) for 24 hr at 37°C. The attached cells were then loaded with NAO for 15 min at room temperature and analyzed with a fluorometer. FAU, fluorescence arbitrary units.

shown), suggesting that an increase in mitochondrial inner membrane mass may be responsible for the observed net increase in inner membrane potential per cell after Mn(II) treatment.

Production of ROS Induced by Mn(II). To determine whether the changes observed in Mn(II)-treated cells can be correlated with Mn(II)-induced intracellular ROS production, the ROS-sensitive dye DCFH was used to monitor the levels of intracellular ROS. Fig. 7 shows that there was a substantial increase in DCF fluorescence intensity in Mn(II)-treated cells (0–1 mM for 24 hr) relative to that of the control cells. The DCF fluorescence increase in Mn(II)-treated cells seemed to be concentration-dependent. When the fluorescence of rhodamine, caused by the oxidation of dihydrorhodamine 123 by ROS, in HeLa cells treated with 0.5 and 1 mM of Mn(II) and in untreated cells was monitored by fluorescent microscopy, similar results were obtained (data not shown). These observations clearly demonstrated that Mn(II) induces the production of ROS in HeLa cells. Because the oxidation of DCFH and dihydrorhodamine by ROS is not specific with respect to individual ROS species, we measured the concentration of H_2O_2 released into the culture medium by cells treated with different concentrations of Mn(II). When HeLa cells were treated with Mn(II) for 6 hr, the release of H_2O_2 increased as a function of Mn(II) concentration (data not shown). H_2O_2 release at 2 mM Mn(II) was enhanced by

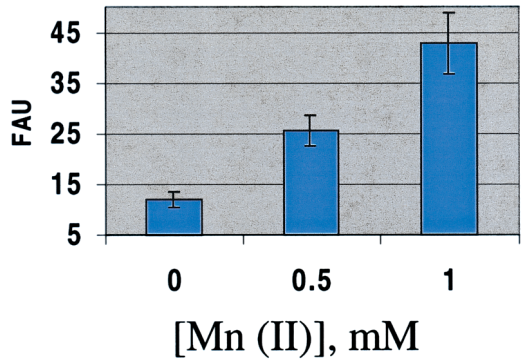

Fig. 7. Generation of ROS in Mn(II)-treated HeLa cells as measured by oxidation of DCFH. HeLa cells were treated with the indicated concentration of Mn(II) for 24 hr at 37°C. These cells then were stained with 50 μM DCFH-DA for 15 min at 37°C, and their DCF fluorescence was measured by fluorometry. FAU, fluorescence arbitrary units.

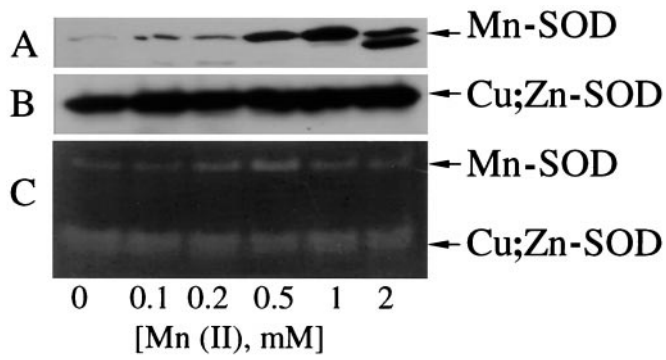

Fig. 8. Effect of Mn(II) treatment on the protein levels and activities of Mn-SOD and Cu,Zn-SOD. HeLa cells were treated with the indicated concentration of Mn(II) for 24 hr at 37°C. (*A*) Equal amounts of protein extracts from the treated cells were applied to SDS/2–20% polyacrylamide gels for electrophoresis under reducing conditions. The Mn-SOD was identified by Western blot analysis. (*B*) The procedure was similar to that described in *A*, except that anti-Cu,Zn-SOD antibody was used to identify Cu,Zn-SOD. (*C*) Equal amounts of protein extracts were applied to native polyacrylamide gels for electrophoresis, and the activities of Mn-SOD and Cu,Zn-SOD were quantitated on the gels.

about 6.5-fold relative to that of control cells. It is interesting to note that at this Mn(II) concentration and incubation time, PARP was almost completely cleaved (data not shown), indicating that caspase-3 was activated.

Induction of Mn-SOD and Catalase by Mn(II). Fig. 8 shows that treatment of HeLa cells with increasing concentrations of Mn(II) (0.1–2 mM) for 24 hr leads to a marked increase in Mn-SOD protein when the Mn(II) concentration was raised from 0.5 to 2 mM. However, the Mn-SOD activity appeared to peak at 0.5 mM Mn(II). At 1 and 2 mM Mn(II), the Mn-SOD was degraded, as indicated by the appearance of a second band, particularly that obtained with 2 mM Mn(II). This degradation seemed to be accompanied by a decrease in its enzymic activity. However, results from Western blotting and activity analysis revealed that there are no significant differences in Cu,Zn-SOD caused by Mn(II) treatment (0.1–2 mM). Interestingly, we found that the level of catalase activity was significantly elevated in cells treated with 2 mM of Mn(II) for 24 hr (data not shown).

Discussion

It has been shown that low concentrations of Mn(II) protect cells against oxidative stress (3). This protective effect likely derives from the fact that Mn(II) can catalyze the dismutation of superoxide radical anions and H_2O_2 under physiological conditions (1, 22). In this study, we discovered that sub- and low-millimolar amounts of Mn(II) caused HeLa cells to undergo apoptosis. This conclusion is based on the observed classical markers found in apoptotic cells that include caspase-3 activation, PARP cleavage, DNA condensation, and typical cell morphological changes (Figs. 1–3). At this lethal concentration range, Mn(II) also induced the production of ROS (Fig. 7) that are known to cause mitochondria-mediated apoptosis (21, 23). Mitochondria-mediated apoptosis has been shown to be associated with the reduction in $\Delta\psi$m (19, 24–26). This decrease in $\Delta\psi$m leads to the release of cytochrome *c* from the intermembrane space of mitochondria into the cytosol, which is required for the formation of Apaf-1·cytochrome *c* complex in the presence of dATP or ATP. This multimeric complex activates procaspase-9, which then activates downstream caspases such as caspase-3 (27). However, our data clearly show that with the Mn(II)-induced apoptosis, there was no decrease in $\Delta\psi$m and no increase in cytosolic cytochrome *c* levels. In addition, Mn(II)

treatment also failed to cause procaspase-9 activation (data not shown). Thus, the Mn(II)-induced apoptosis seems to be unique.

Consistent with the notion that Mn(II)-induced apoptosis does not proceed via a mitochondria-mediated pathway, we found that the value of $\Delta\psi m$ increased as a function of the Mn(II) concentration (Fig. 4). A similar increase also was observed when other indicators such as TMRE or chloromethyl-x-rosamine were used (data not shown). The observed increase in the uptake of indicator such as TMRE by Mn(II)-treated cells was not caused by enhanced mitochondria volume, as judged by the electron microscopic data (Fig. 5). Addition of the uncoupling agent FCCP drastically reduced TMRE uptake of the Mn(II)-treated cells, suggesting that these cells can be depolarized by this protonophore. Furthermore, using calcein-loading and Co(II)-quenching methods (18) to detect permeability changes in inner mitochondrial membranes, we also did not observe the opening of permeability transition pores. It is interesting to note that Mn(II)-treated cells exhibited enhanced NAO fluorescence levels. Because NAO is known to bind cardiolipin, a unique phospholipid exclusively located in the inner mitochondrial membrane (28), our results suggest that the observed increase of $\Delta\psi m$ in Mn(II)-treated cells may be derived from the increase in cardiolipin.

As mentioned earlier, Mn(II) also induced the generation of H_2O_2. A number of studies have revealed that Mn-SOD can be induced by oxidative stress such as treatment with TNF and lipopolysaccharide (29), 2-*O*-tetradecanoylphorbol-13-acetate (30, 31), and x-ray irradiation (32). Western blot analysis clearly showed that 0.5 or 2.0 mM of Mn(II) induced an increase in Mn-SOD protein that was accompanied by an elevation of its activity (Fig. 8). However, at 2.0 mM Mn(II), the Mn-SOD seemed to be significantly degraded, suggesting the presence of an active protease other than caspase-3 induced by Mn(II). The degradation of Mn-SOD, as reflected by the loss of its activity, can be observed even at 1.0 mM of Mn(II). This bell-shaped Mn-SOD induction by Mn(II) also was observed with human breast cancer cells, except that they did not show the degraded protein (33). It is reasonable to suggest that Mn(II) may substitute Ca(II) to activate calpain, which could catalyze the cleavage of Mn-SOD.

In summary, our data show that Mn(II) is able to induce apoptosis in HeLa cells through the activation of caspase-3; however, this apoptosis seems to deviate from most apoptotic pathways that are mediated by mitochondria. The Mn(II)-induced apoptosis does not correlate with mitochondrial depolarization, release of cytochrome *c* from mitochondria, and the activation of procaspase-9. The mitochondrial hyperpolarization observed in Mn(II)-treated cells could be derived from the elevation of cardiolipin induced by Mn(II), particularly in view of the fact that Mn(II) is known to activate cardiolipin synthase (34). In addition, Mn(II) also induces the elevation of ROS, Mn-SOD, and catalase activity as well as a protease other than caspase-3 that can degrade Mn-SOD at high Mn(II) concentrations. It will be interesting to investigate further this unique apoptotic pathway activated by Mn(II).

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