The subcellular distribution of MnSOD alters during sodium selenite-induced apoptosis

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It was reported that high doses of sodium selenite can induce apoptosis of cancer cells, but the molecular mechanisms are poorly understood. Manganese superoxide dismutase (MnSOD) converts superoxide radical to hydrogen peroxide within the mitochondrial matrix and is one of the most important antioxidant enzymes. In this study, we showed that 20 μM sodium selenite could alter subcellular distribution of MnSOD, namely a decrease in mitochondria and an increase in cytosol. The alteration of subcellular distribution of MnSOD is dependent on the production of superoxide induced by sodium selenite.

INTRODUCTION

Antioxidant enzymes play a vital role in controlling the redox status of the cell. Manganese superoxide dismutase (MnSOD), a tetrameric enzyme located in the mitochondrial matrix, is an essential antioxidant enzyme responsible for converting the leaked superoxide radical from the respiratory chain to hydrogen peroxide (1, 2). In the absence of MnSOD, the superoxide radical is increased and cells undergo apoptosis (3). In consistent with its cellular function, the MnSOD knockout mice die embryonically and are hypersensitive to apoptosis (4, 5). Furthermore, the over-expression of MnSOD blocks apoptosis induced by praline oxidase, TNF-α, IL-3 withdrawal or ionizing irradiation (6-9). During Fas-mediated apoptosis, MnSOD is inactivated by caspase-specific degradation (10).

Selenium, an essential trace element, can induce apoptosis in cancer cells (11, 12). In previous studies, we demonstrated that 20 μM sodium selenite markedly induced apoptosis of NB4 cells, which was associated with the production of ROS and mitochondrial damage (13-15). In this study, we found that 20 μM sodium selenite was able to trigger MnSOD decrease in mitochondria and increase in cytosol. This alteration of subcellular distribution of MnSOD is dependent on the production of superoxide. The reduction of MnSOD within mitochondria probably damaged the mitochondrial oxidative defense system and thus jeopardized the cellular redox balance.

RESULTS

Sodium selenite alters the subcelluar distribution of MnSOD

We previously reported that sodium selenite could induce the production of ROS and mitochondrial damage (13-15). To investigate the molecular mechanism of this induction, we examined the subcellular localization of MnSOD protein. We found that although the expression level of MnSOD was not changed in NB4 cells upon the treatment of 20 μM sodium selenite (Fig. 1A), MnSOD appeared in cytosol by 3 hours and undetectable in mitochondria but present in cytosol at 24 hours upon 20 μM sodium selenite treatment (Fig. 1B). Cytochrome C, another key component of the respiratory chain, released from mitochondria to cytosol after 20 μM sodium selenite treatment (Fig. 1B). To further confirm the relocation of MnSOD protein, we performed immunofluorescent staining using MnSOD antibody and MitoTracker was used to mark the mitochondria. In control cells, MnSOD was present exclusively in mitochondria (red fluorescence). In contrast, MnSOD decreased in mitochondria and increased in cytosol when exposure to a cytotoxic dose of sodium selenite (Fig. 1C).

To address whether the alteration of subcellular distribution of MnSOD from mitochondria to cytosol after 20 μM sodium selenite treatment is specific for NB4 cells, we examined other human leukemia cell lines. We found that 20 μM sodium selenite could also induce the alteration of subcellular distribution of MnSOD from mitochondria to cytosol in Jurkat and HL60 cells (Fig. 1D).

The reduction of MnSOD in mitochondria induces apoptosis

Next, we asked whether the reduction of MnSOD in mitochondria could lead to cell apoptosis. To address this question, we first blocked the gene expression of MnSOD in NB4 cells with siRNA and found that MnSOD siRNA treatment was able to induce NB4 cell apoptosis (Fig. 2), suggesting that sup-
The subcellular distribution of MnSOD alters during sodium selenite-induced apoptosis
Liyang Cuan, et al.

Fig. 1. Sodium selenite induces the relocation of MnSOD from mitochondria to cytosol. NB4 (A to C), Jurkat and HL60 (D) cells were treated with 20 μM sodium selenite for different times. (A) Immunoblotting for MnSOD in total cell lysates. (B) The fraction of MnSOD in mitochondria and cytosol was examined by western blotting. (C) Immunofluorescent staining of the subcellular localization of MnSOD in NB4 cells. (D) Immunofluorescent staining of the subcellular localization of MnSOD in Jurkat and HL60 cells after 24 hours-treatment of sodium selenite.

Fig. 2. The reduction of MnSOD induces cell apoptosis. NB4 cells were transfected with MnSOD siRNA or scrambled siRNA. Twenty four hours later, cells were treated with or without 20 μM sodium selenite for 24 hours. The effect of MnSOD-siRNA on expression of MnSOD was analyzed by Western-blot and cell apoptosis were analyzed by flow cytometry. MnSOD siRNA were compared with scrambled siRNA with or without sodium selenite treatment (P < 0.05, n = 3).

Expression of MnSOD expression alone was sufficient to cause NB4 cell apoptosis. But removal of MnSOD by siRNA did not influence the sensitivity of cells to selenite (Fig. 2). The siRNA approach presumably reduced MnSOD expression in mitochondria by lowered expression levels, whereas sodium selenite decreased MnSOD in mitochondria by altering the subcellular distribution (see above). Both treatments could thereby likely induce cell apoptosis through different mechanisms. However, it is likely that a reduction of MnSOD in mitochondria is sufficient to give apoptosis.

Sodium selenite mediates the accumulation of superoxide in cells
What are the physiological consequences of reduction of MnSOD in mitochondria? A plausible assumption is that the decrease of MnSOD in mitochondria may inhibit its superoxide scavenging activity and thus lead to the accumulation of superoxide in cells. To test this possibility, DHE oxidation was used to detect the superoxide anions by flow cytometry. After sodium selenite treatment for 12 hours, DHE fluorescence was elevated from 96.85 ± 2.19 to 129.9 ± 0.71 (P < 0.05) (Fig. 3, left histogram). In contrast, after sodium selenite treatment, the hydrogen peroxide level, evaluated with DCFH fluorescence, was decreased from 228.65 ± 5.30 to 168.2 ± 9.33 (P < 0.05) (Fig. 3, right histogram). These results demonstrated that 20 μM sodium selenite treatment induced the accumulation of superoxide with concomitant lowered levels of hydro-
The subcellular distribution of MnSOD alters during sodium selenite-induced apoptosis

Liying Guan, et al.

Fig. 3. Sodium selenite induces an increase of superoxide but a decrease of hydrogen peroxide. NB4 cells treated with or without 20 μM sodium selenite for 12 hours. Fluorescence intensity of DHE and DCF in cells were analyzed by flow cytometry. *P < 0.05 compared with control (n = 3).

The alteration of subcellular distribution of MnSOD is superoxide dependent

High doses of sodium selenite could trigger DNA damage or oxidative damage, thus result in cell death (14, 16). When we treated NB4 cells with camptothecin (CPT) (0.5 μM), a topoisomerase inhibitor or oxidative reagent H2O2 (100 μM), we found that both reagents could indeed induce apoptosis (data not shown), but the subcellular localization of MnSOD was not changed. These data indicated that selenite-induced MnSOD relocation is an independent event of DNA damage or H2O2-mediated oxidative damage (Fig. 4A).

How does MnSOD protein relocation occur after 20 μM sodium selenite treatment then? Preincubation NB4 cells with MnTMPyP, a cell-permeable superoxide scavenger, not only significantly suppressed the selenite-induced apoptosis (Fig. 4B), but also inhibited the relocation of MnSOD from mitochondria to cytosol (Fig. 4C). The results indicate that the alteration of subcellular distribution of MnSOD induced by sodium selenite is superoxide dependent. MnTMPyP also inhibited mitochondrial membrane damage, the release of cytochrome c and the activation of caspase-9, -3 induced by sodium selenite (Fig. 4C, D, E).

DISCUSSION

Superoxide, hydrogen peroxide and hydroxyl radicals are believed to play important roles during apoptosis (17). Previous studies have demonstrated that the superoxide production in mitochondria is a key event during selenite-induced apoptosis process (18-21). However, the molecular mechanism of how sodium selenite triggers the accumulation of superoxide remains to be determined.

MnSOD, an antioxidant enzyme, converts the leaked superoxide radical from the respiratory chain to hydrogen peroxide and is essential for maintaining the proper cellular redox level. Inhibiting MnSOD gene expression by siRNA resulted in apoptosis, suggesting that MnSOD acts as a protective factor during cell death. Biochemistry analysis and immunofluorescent staining confirmed that 20 μM sodium selenite provoked MnSOD decrease in mitochondria and increase in cytosol in NB4, Jurkat and HL60 cells. The reduction of MnSOD in mitochondria may damage the mitochondrial oxidative defense system and mediate the accumulation of superoxide in cells. As we expected, 20 μM sodium selenite treatment induced the accumulation of superoxide in cells.

Currently, our results show that a cytotoxic dose of sodium selenite (20 μM) induces the alteration of the subcellular distribution of MnSOD during the thereby triggered cell apoptosis. But we could not tell the alteration of subcellular distribution of MnSOD results from the release of MnSOD from mitochondria to cytosol or the prevention nuclear-encoded MnSOD to be imported into mitochondria, with the mitochondrial MnSOD being degraded. Greco et al have reported the release of mitochondrial matrix proteins from mitochondria to cytosol (22). The generation of oxygen radicals increases the mitochondrial membrane permeability, and in turn results in release of matrix proteins (23, 24). In our study, Neither H2O2...
The subcellular distribution of MnSOD alters during sodium selenite-induced apoptosis
Liying Guan, et al.

Fig. 4. Superoxide causes the relocation of MnSOD from mitochondria to cytosol and mitochondrial dysfunction. (A) Immunofluorescent staining of MnSOD in NB4 cells treated with 100 μM H2O2 or 0.5 μM CPT for 24 hours. NB4 cells were pre-treated with or without 10 μM MnTMPyP for 1.5 hours and then treated with sodium selenite for 24 hours. (B) Flow cytometry analysis of the effect of MnTMPyP on sodium selenite-induced NB4 cell apoptosis. (C) Western-blot analysis of the effects of MnTMPyP on sodium selenite-induced the relocation of MnSOD and cytochrome c from mitochondria. (D) Flow cytometry analysis of the effect of MnTMPyP on activation of caspase-9 and -3. β-actin was used as a loading control.

nor CPT treatment would alter the subcellular localization of MnSOD. Scavenging superoxide by MnTMPyP could prevent the membrane permeability and the relocation of MnSOD from mitochondria to cytosol, suggesting that the alteration of subcellular localization of MnSOD is associated with superoxide accumulation. At the same time, MnTMPyP could inhibit cytochrome c release from mitochondria and the activation of caspase-9, -3 induced by 20 μM sodium selenite. Xiang et al. also reported that the overexpression of MnSOD could suppress selenite-induced mitochondrial membrane potential decrease and meanwhile inhibit the release of cytochrome c from mitochondria to cytosol (19). Combined together, these results indicate that the alteration of subcellular localization of MnSOD is likely a primary effect of sodium selenite treatment and not a consequent event of apoptosis.

Under normal physiological condition, the mitochondrial inner membrane is resistant to superoxide export (25). After sodium selenite treatment, the reduction of MnSOD in mitochondria probably impairs its scavenging superoxide capacity and thus leads to the accumulation of superoxide in cells. Therefore, the mitochondrial localization of MnSOD is crucial for its role in maintaining cell viability. Our observation that a cytotoxic dose of sodium selenite could trigger the subcellular compartment relocation of MnSOD revealed a previously unknown event that may prove to be of importance during selenite-induced apoptosis.

MATERIALS AND METHODS

Reagents and antibodies
Sodium selenite, Rh123 and anti-β-actin antibody were purchased from Sigma-Aldrich. MnTMPyP was purchased from Calbiochem. Anti-MnSOD antibody was purchased from BD Transduction Laboratories. Anti-cleaved caspases-9, -3 and anti-cytochrome C antibodies were purchased from Cell Signaling Technology. Dihydroethidium (DHE) and DCFH-DA were purchased from Beyotime Company (Jiangsu, China).

Cell culture
NB4, Jurkat, and HL60 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2.

Immunoblotting analysis
Cells were lysed in RIPA buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na3VO4, 1 μg/ml Leupeptin, 1 mM PMSF) for 5 minutes on ice and then subjected to sonication for 20 seconds. The lysates were centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was collected, and protein concentration was determined by the Bradford assay. Equal amounts of protein were separated by SDS-PAGE and transferred onto nitrocellulose
membranes. Then the samples were first reacted with primary antibodies, following by secondary antibodies conjugated with HRP. The blots were developed with ECL Kit (Armacia Biotech).

Mitochondria fractionation
Cells were resuspended in isotonic mitochondrial extraction buffer (Applegent Technologies), homogenized with a homogenizer for thirty bursts in ice, and then centrifuged at 800 g for 5 minutes to pellet the nuclei and unbroken cells. The supernatant was centrifuged at 13,000 g for 10 minutes to spin down the mitochondria, and the resulting supernatant was the cytosolic fraction. The isolated mitochondria were resuspended in RIPA buffer to extract mitochondrial protein.

Immunofluorescent staining
Cells were incubated with 100 nM MitoTracker (Invitrogen) in culture medium for 3 hours. After washing 3 times with ice-cold PBS, the cells were transfected onto the slides, fixed with 4% formaldehyde for 30 minutes, permeabilized with 1% Triton X-100 for 10 minutes at room temperature. After blocking with 1% BSA, anti-MnSOD antibody was added (1:100 dilution) and incubated at 4°C overnight followed by incubation with anti-mouse IgG-FITC (1:100 dilution) for 2 hours at 37°C. Images were immediately observed and captured using a Nikon Eclipse microscope (TE2000-U, magnification 100×).

Reactive oxygen species (ROS) measurement
Cells were washed with serum-free culture medium and incubated with 10 μM DCFH-DA and DHA respectively at 37°C for 30 minutes. After washing with PBS, ethidium oxidized by DHE or DCF Fluorescence was examined by flow cytometry analysis.

Small interference RNA
The MnSOD-specific siRNA: 5'-GGAACCUCACAUCACGC GCA-3' (26) and non-silencing scrambled siRNA: 5'-UUCUCC GACACUGUCAACGU-3' were synthesized by GenePharma (Shanghai Co. Ltd China). Cells were transfected with siRNAs by using Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer’s protocol.

Flow cytometric analysis of apoptosis
Cells were washed twice with ice-cold PBS, and fixed with 70% ethanol at 4°C overnight. After washing with PBS, cells were incubated in 0.5 ml PBS containing 50 μg/ml RNase A for 30 minutes at 37°C, and then added propidium iodide to achieve a final concentration of 50 μg/ml for 30 minutes on ice in the dark. The resultant cell suspension was then subjected to flow cytometry analysis.

Mitochondrial membrane potential (Δψm)
Cells were washed twice with ice-cold PBS, and incubated in 1 ml staining solution (PBS containing 10 μg/ml Rh123) for 30 minutes in the dark at 37°C. Then, after rinsing with PBS, cells were resuspended in 0.5 ml PBS. The fluorescent intensities of Rh123 were determined by flow cytometry.

Statistical analysis
Data were analyzed by the Student t test. A value of $P < 0.05$ was considered statistical significance.

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The subcellular distribution of MnSOD alters during sodium selenite-induced apoptosis

Liying Guan, et al.

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