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Selenite Activates Caspase-Independent Necrotic Cell Death in Jurkat T Cells and J774.2 Macrophages by Affecting Mitochondrial Oxidant Generation

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ABSTRACT

Sodium selenite, a common dietary form of selenium, is recognized as essential in animal and human nutrition. Mechanisms regulating the inflammatory response of the immune system involve regulation of apoptosis and control of reactive oxygen species (ROS) production. In this study, the effect of sodium selenite on ROS production and cell-death rates in macrophages and T cells was investigated. Exposing Jurkat T cells or J774.2 macrophages to >5 μ*M* sodium selenite induced cell death. In both Jurkat T cells and J774.2 macrophages, rapid loss of the cell's capacity to generate dichlorofluorescein-sensitive ROS preceded cell death. The main cellular source of ROS was found to be the mitochondria electron-transfer chain. DEVDase activity in the cells remained unchanged and even decreased with time, as well as DNA fragmentation level, which was almost unaffected, indicating cell death with necrotic characteristics. *tert*-Butyl hydroperoxide at a concentration of 5 μ*M* was beneficial in attenuating the rate of cell death. The superoxide scavenger Tiron was tested for its ability to protect the cells against selenium. Tiron completely protected the J774.2 macrophage cell line against selenium and attenuated the cell death effect in Jurkat T cells. In the presence of the superoxide dismutase-mimicking compound tempol, selenium's macrophage-killing effect was inhibited. Therefore, our results show that, at least *in vitro,* selenite induces changes in the balance between mitochondrial superoxide and hydrogen peroxide production, which can facilitate cell death in immune system cells. This may be one mechanism by which selenium down-regulates the immune system's inflammatory response and protects against overproduction of peroxides. *Antioxid. Redox Signal.* 5, 273–279.

INTRODUCTION

SODIUM SELENITE is a common dietary form of selenium, recognized as essential in animal and human nutrition (9, 10). In the form of the amino acid selenocysteine, it is a component of a number of antioxidant enzymes, *e.g.,* glutathione peroxidase and thioredoxin reductase (1, 5).

Selenium supplementation is beneficial in inflammatory diseases such as multiple sclerosis and inflammatory bowel disease, and has been linked to the expression of blood glutathione peroxidase (21, 27). Selenium supplementation at levels between 125 and 500 μg/day (above the recommended daily allowance) to patients with sepsis-induced systemic inflammatory response syndrome (SIRS) dramatically improved prognosis, indicating an inhibitory effect on an overstimulated immune system (4, 12, 13, 15). Little information is available on the biological activity of selenium or on its function in its enzyme-free form; most experiments on the topic have involved its activity while incorporated into selenoproteins (1, 24, 29).

Among the functions of the immune system response are induction of apoptosis and production of reactive oxygen

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species (ROS) (14, 22). Modulating these functions may attenuate the inflammatory response. In this study, the effect of selenium in the form of sodium selenite on ROS production and cell-death rates in macrophages and T cells was investigated in order to further elucidate the beneficial mechanisms of selenium supplementation in regulating immune system function.

MATERIALS AND METHODS

Cell culture

Human Jurkat T cells (ATCC) and murine J774.2 macrophages (kindly provided by Prof. Dov Zipori from the Weizmann Institute of Science) were grown in RPMI medium supplemented with 10% (wt/vol) fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and 1 × glutamine at 37°C in a humidified atmosphere consisting of 95% air and 5% CO_2. When Jurkat cell density reached 0.5×10^6 cells/ml, and when macrophages reached 60% confluence, the cells were exposed to selenium (dissolved in water) to stimulate cell death.

Determination of cell viability

Cell membrane integrity was detected by flow cytometer (FACSort, BD) as a measurement of cell viability. For this assay, the nonpermeant DNA-interchelating dye propidium iodide (PI), which is excluded by viable cells, was used. Fluorescence settings were: excitation at 488 nm and emission at 575 nm. Data were collected from at least 10,000 cells (31, 34).

DNA integrity

Cells exposed to selenite stimulation were centrifuged (600 *g,* 5 min) and collected. The pellet was resuspended in a solution containing 50 μg/ml PI, 0.1% (wt/vol) sodium citrate, and 0.1% (vol/vol) Triton X-100. The permeabilized cells were kept in the dark for 2 h at 4°C, and their DNA integrity was analyzed using a flow cytometer. An argon-ion laser was used for excitation at 488 nm, and the emission was recorded at 575 nm. Data were collected from at least 10,000 cells (31).

Caspase 3-like (DEVDase) activity

Cell pellets were lysed in phosphate-buffered saline (PBS) containing 0.2% Triton X-100 on ice for 10 min. The cell lysates were centrifuged at 10,000 *g* for 5 min. The clear supernatant was collected and placed on ice. Cell extracts were incubated with 60 μ*M* fluorogenic caspase-3 substrate (Ac-DEVD-AMC, Calbiochem, La Jolla, CA, U.S.A.) in incubation buffer (PBS) with 5 m*M* dithiothreitol. Fluorescence was recorded following 30 min of incubation at ambient temperature in the dark using a microfluorometer plate reader (GENios, Tecan Austria), at 360 nm excitation and 460 nm emission (31). Protein content was calculated by the Bradford assay. Caspase activity was adjusted, or not, to the protein level as mentioned in the figure legends.

Intracellular ROS

Intracellular ROS were detected by using dichlorodihydrofluorescein diacetate (H_2DCF-DA) (33). After different treatments, the cells were washed three times with PBS. Cells were centrifuged (600 *g,* 5 min), resuspended in PBS, and incubated with H_2DCF-DA (25 μ*M*) for 30 min at 37°C. To detect intracellular fluorescence, the fluorochrome-loaded cells were excited using a 488-nm argon-ion laser in a flow cytometer. The dichlorofluorescein (DCF) emission was recorded at 530 nm. Data were collected from at least 10,000 cells.

Isolation of rat liver mitochondria

After overnight fasting, the animals (Sprague–Dawley rats, 250 g) were anesthetized by ether and killed by decapitation. Livers were removed with scissors and immediately immersed in ice-cold 210 m*M* mannitol, 70 m*M* sucrose, 5 m*M* HEPES, pH 7.35 (MSH buffer) containing 1 m*M* EDTA. Livers were freed of fat and connective tissue, cut to pieces with scissors, and homogenized in 60 ml of MSH/EDTA buffer per liver using a glass homogenizer with a Teflon pestle. Mitochondria were isolated by conventional differential centrifugation (2, 20).

Mitochondrial ROS (hydrogen peroxide and superoxide) measurements

The electron-transfer chain may produce a flux of superoxide radicals via the one-electron reduction of molecular oxygen, which is then dismutated by manganese superoxide dismutase (MnSOD) to produce a constant flux of hydrogen peroxide (7). Mitochondrial peroxides were detected using H_2DCF-DA (2). The isolated rat liver mitochondria (IRLM) were resuspended and incubated with 25 μ*M* H_2DCF-DA for 10 min at room temperature for the detection of mitochondrial fluorescence. For superoxide production, lucigenin-dependent chemiluminescence measurements were used (25).

Statistical analysis

Data were analyzed by one-way ANOVA. Differences were considered significant at probability levels of $p < 0.05$ using the Fisher's protected least significant difference method. Statistical analysis was performed using the statistical computer program SPSS version 8 (SPSS Inc., Chicago, IL, U.S.A.).

RESULTS

Exposing Jurkat T cells or J774.2 macrophages to elevated levels of sodium selenite (5–30 μ*M*) induced cell death. Selenite concentrations above 5 μ*M* effectively triggered cell death of cells in culture within 72 h (Fig. 1).

To elucidate the intracellular events preceding cell death, intracellular DCF-sensitive ROS production and cell death (by incorporation of PI) were monitored kinetically for 24 h. In both Jurkat T cells and J774.2 macrophages, rapid loss in the cellular capacity to generate DCF-sensitive ROS was observed (Fig. 2), and this event preceded cell death.

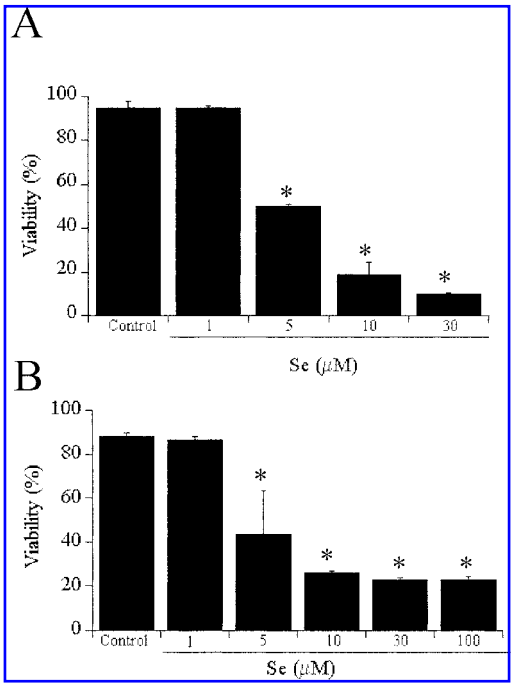


FIG. 1. Selenite-induced cell death. (**A**) J774.2 macrophages treated with elevated concentrations of selenite for 72 h. (**B**) Jurkat T cells treated with elevated concentrations of selenite for 72 h. *$p < 0.05$, significantly different compared with control.

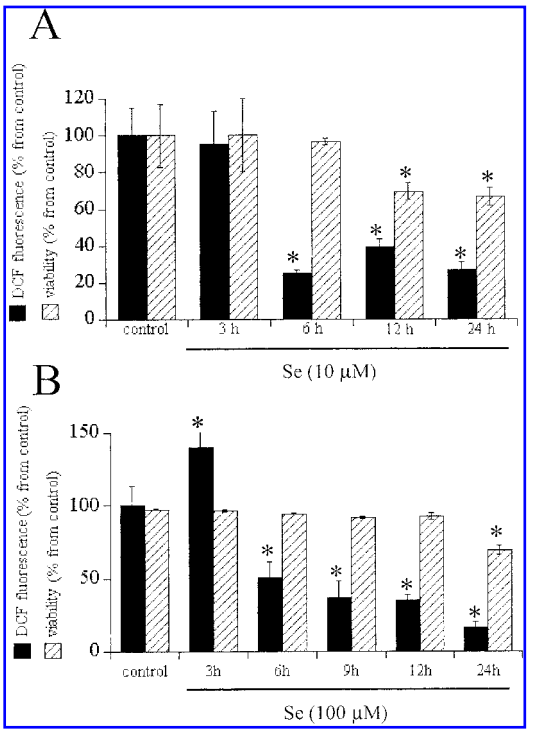


FIG. 2. Decrease in DCF-sensitive ROS (peroxides) production in cells treated with selenite. Loss of ROS production capacity preceded cell death. (**A**) J774.2 macrophages treated with 10 μ*M* selenite. (**B**) Jurkat T cells treated with 100 μ*M* selenite. *$p < 0.05$, significantly different compared with control.

Another intracellular event characteristic of apoptosis is activation of the caspase cascade. Several articles have reported elevated caspase activity in selenite compound-treated cells (17, 18, 32). Although the activation of caspase was not observed as an early event in the cell-death cascade initiated by selenite, enhanced DEVDase activity per protein was shown. However, this was due to lower protein content in the 24-h selenite-treated Jurkat T cells and macrophages and not due to pro-caspase activation (Fig. 3). In contrast to selenite, treatment with CH11 (a Fas-activating antibody) for 2 h elevated the net DEVDase activity in Jurkat T cells (Fig. 3B). If caspase were indeed a major player in the cell-death cascade, significant activation of endonucleases that fragment the nuclear DNA would be expected. However, only a minor rate of DNA fragmentation was recorded in selenite-treated cells prior to loss of membrane integrity (Fig. 4). Furthermore, cell shrinkage was not observed by the FACS forward scatter data (not shown).

We then sought to elucidate the main source of ROS in these cells. Treatment for 15 min with the mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) dramatically decreased the intracellular ROS produc-

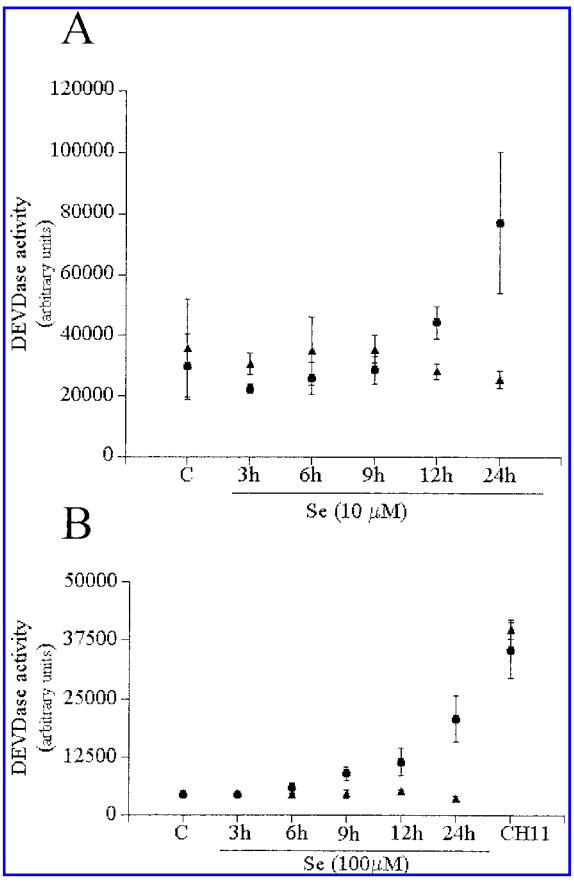


FIG. 3. DEVDase activity in macrophages and T cells. (●) DEVDase activity adjusted to protein. (▲) DEVDase activity. (**A**) J774.2 macrophages treated with 10 μ*M* selenite. (**B**) Jurkat T cells treated with 100 μ*M* selenite. Cells were treated with 0.1 μg/ml CH11 for 2 h.

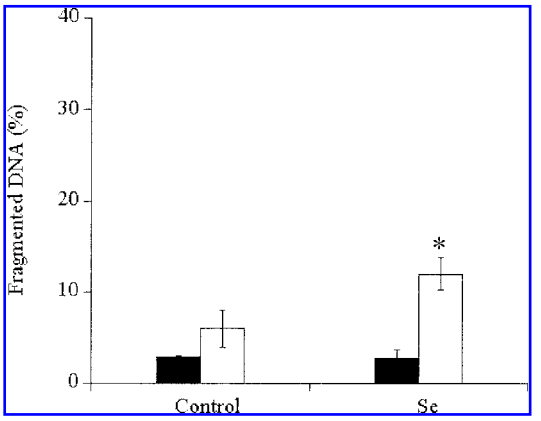


FIG. 4. Accumulation of fragmented DNA in selenite-treated cells. J774.2 macrophages were treated with 10 μ*M* selenite (solid bars) and Jurkat T cells were treated with 100 μ*M* selenite (open bars) for 24 h. *$p < 0.05$, significantly different compared with control.

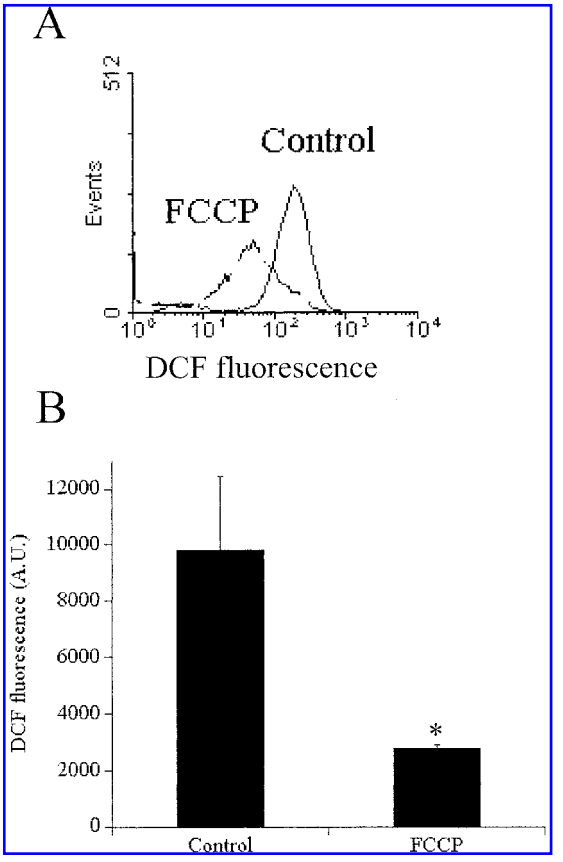


FIG. 5. FCCP-sensitive cellular ROS production. J774.2 macrophages and Jurkat T cells were treated with 10 μ*M* FCCP for 15 min, followed by incubation with H_2DCF-DA. DCF formation was evaluated. (**A**) Jurkat T cell DCF levels were analyzed by flow cytometer (histogram). (**B**) Macrophage DCF levels were evaluated after washing and extraction with PBS–Triton X-100 (0.5%) using a microfluorometer reader. *$p < 0.05$, significantly different compared with control.

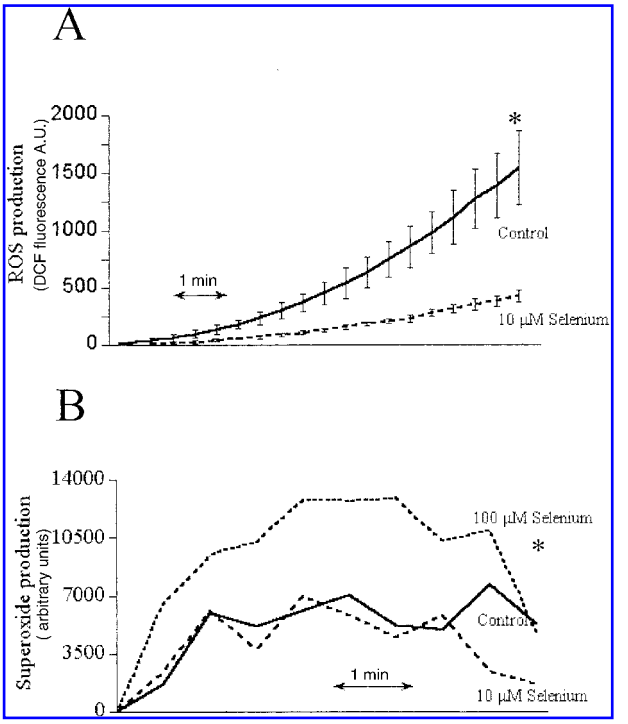


FIG. 6. Isolated rat liver mitochondria. ROS production was determined in the presence and absence of 10 μ*M* selenite. (**A**) DCF fluorescence (peroxides). (**B**) Lucigenin-dependent chemiluminescence (superoxide). *$p < 0.05$, significantly different compared with control.

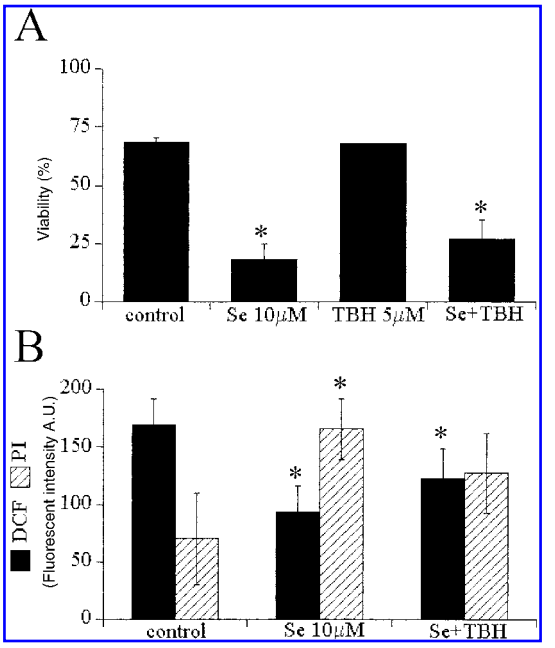


FIG. 7. Treatment with *tert*-butyl hydroperoxide. (**A**) Viability of J774.2 macrophages following treatment with selenite (10 μ*M*) for 24 h. *tert*-Butyl hydroperoxide (TBH; 5 μ*M*) was added 5 min before the selenite. (**B**) Changes in intracellular ROS and PI accumulation (total fluorescence) in J774.2 macrophages following 24-h treatment with selenite and *tert*-butyl hydroperoxide as indicated in A.

tion capacity measured by DCF fluorescence (Fig. 5). Therefore, mitochondria were identified as the main source of ROS.

To evaluate whether selenite can interact directly with mitochondria to attenuate ROS production, we incubated IRLM with selenite. At a concentration of 10 μ*M,* selenite strongly inhibited DCF-sensitive ROS generation from mitochondria in respiration state 3 (Fig. 6A). In contrast, mitochondria exposed to selenite at relatively high concentrations showed increased production of superoxide radicals (Fig. 6B).

To evaluate whether changes in the ratio and profile of ROS production in immune cells facilitate the effects of selenite-induced cell death, we added a low amount of oxidant to J774.2 macrophages. *tert*-Butyl hydroperoxide at a concentration of 5 μ*M* was beneficial in attenuating cell death induced by 10 μ*M* selenite (Fig. 7). No protective effect was observed in the Jurkat T cells when 100 μ*M* selenite was used (data not shown). The superoxide scavenger Tiron was tested for its ability to protect the cells against selenite. Tiron completely protected the J774.2 macrophage cell line against selenite and attenuated the cell-death effect in Jurkat T cells (Fig. 8A and B). In the presence of the superoxide dismutase (SOD)-mimicking compound tempol, the effect of selenite in macrophages was completely inhibited, indicating a significant role of superoxide-dismutation inhibition in selenite-induced cell death (Fig. 8C).

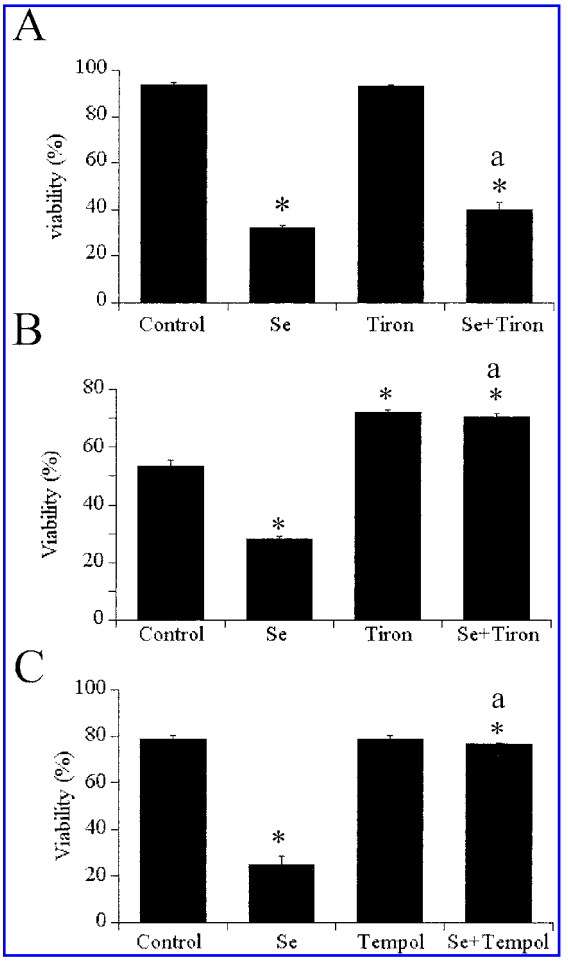


FIG. 8. Protection against selenite-induced cell death by the superoxide scavenger Tiron or SOD-mimicking compound tempol, 24-h treatment. (**A**) Jurkat T cells: Tiron (10 m*M*) and selenite (10 μ*M*) were used. **p* < 0.05, significantly different compared with control; [a]*p* < 0.05, significantly different compared with selenite-treated cells. (**B**) J774.2 macrophages: Tiron (10 m*M*) and selenite (10 μ*M*) were used. **p* < 0.05, significantly different compared with control; [a]*p* < 0.05, significantly different compared with selenite-treated cells. (**C**) J774.2 macrophages: Tempol (5 m*M*) and selenite (10 μ*M*) were used. **p* < 0.05, significantly different compared with control; [a]*p* < 0.05, significantly different compared with selenite-treated cells.

DISCUSSION

Type of cell death

Two types of cell death are generally recognized: necrotic and apoptotic. Activation of necrotic events was considered until recently to be promoted by extensive cellular trauma, whereas apoptosis is considered to be a mechanism of cell death driven by a genetically controlled cascade of events initiated by minor insults or an extracellular signal. Using models of receptor-mediated cell death, activation of necrotic pathways has been reported in cells that do not activate caspase (16, 23). Furthermore, it is now clear that a signal-transduction cascade is involved in cell-death processes with necrotic characteristics (16). Exposure of Jurkat T cells or J774.2 macrophages to selenium facilitated cell death (Fig. 1). If sodium selenite were activating the apoptosis-type cell-death cascade, increased DEVDase activity and chromatin fragmentation would have been observed. However, selenite facilitated cell-death activity with necrotic characteristics, without inducing unique, intracellular, apoptotic events (Figs. 3 and 4).

Selenium induces necrotic cell death in immune-system cells, which are known to generate significant amounts of ROS. As in these cells decreased amounts of ROS are detected using peroxide-sensitive probes following selenium treatment and protection is achieved by superoxide scavengers, inhibition of superoxide dismutation could be the necrotic cell-death activator.

Redox regulation of necrotic cell death

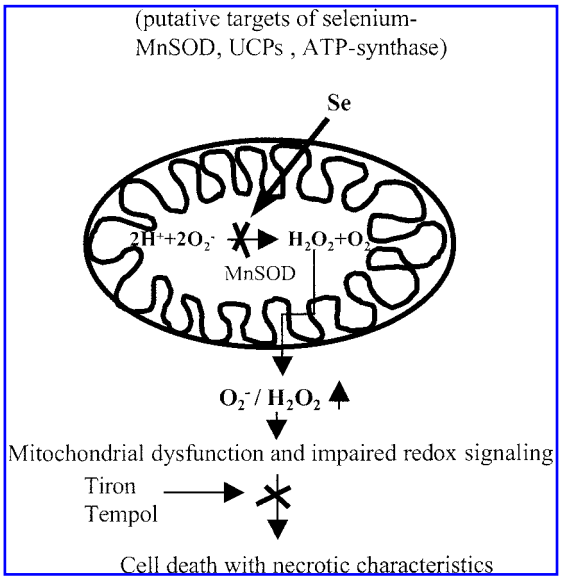
Mammalian cells exhibit a broad spectrum of responses to oxidative stress, depending on the stress level encountered. Low levels of hydrogen peroxide in the range of 3–15 μ*M* cause a significant mitogenic response (25–45% growth stimulation), whereas treatment with higher superphysiological concentrations of ROS can cause temporary growth arrest (11). It is widely held that cells possess a strong redox buffering capacity to maintain viability. Increased oxidative stress eventually leads to apoptosis and to necrotic cell death (11, 28). However, measurable levels of intracellular ROS are always produced in cells and serve as signaling molecules for numerous signal-transduction pathways (30). A case in point is TRAF (tumor necrosis factor receptor-associated factor)-mediated signal transduction-facilitated ROS production from the mitochondrial electron-transfer chain resulting in enhanced nuclear factor-κB activation (8). In human hepatoma

cells, low levels of the exogenous ROS hydrogen peroxide (1–10 μ*M*) significantly stimulated Akt/PKB activity and c-fos/c-jun expression, leading to cell growth, which was abolished by antioxidants (19). This suggests that the redox state may stimulate cell proliferation through changes in signal transduction. Therefore, mitochondria-derived ROS may orchestrate the redox tone of the cell and thereby serve a function in cell survival. Indeed, mitochondria-targeted catalase has previously been shown to potentiate apoptotic cell death in response to tumor necrosis factor and cycloheximide in HepG2 cells (3).

Direct measurement of ROS production indicated that selenite significantly attenuates intracellular DCF-sensitive ROS production (Fig. 2). As already mentioned, cells possess strong redox buffering capacity to maintain viability and cell-signaling capacity (6, 28). During selenium treatment, the capacity to produce ROS is altered. The conversion of superoxide to hydrogen peroxide appears to be blocked by selenium. Treatment of isolated mitochondria with selenium decreased mitochondrial superoxide dismutation (Figs. 5 and 6). Thus, selenium may lead to a shift in cellular redox balance (elevation in mitochondrial superoxide and decrease in hydrogen peroxide). Inhibition of mitochondrial superoxide dismutation will damage the mitochondria and facilitate cellular necrosis (26).

Tiron completely protected the J774.2 macrophage cell line against selenium and attenuated the cell-death effect in Jurkat T cells. (Fig. 8A and B). A SOD-mimicking compound that converts superoxide to hydrogen peroxide also protected the cells (Fig. 8C). The addition of the oxidant *tert*-butyl hydroperoxide was partially beneficial in preventing selenium-induced necrotic cell death (Fig. 7). All of these observations support the hypothesis that an increase in the cellular ratio of superoxide to hydrogen peroxide activates a caspase-independent type of cell death with necrotic characteristics. This selenium-induced ROS may also attenuate the immune-system cell's capacity to release ROS to the environment to trigger inflammation because of the lower ability of superoxide to cross biological membranes compared with hydrogen peroxide.

In conclusion, *in vitro,* selenium induces changes in the balance between mitochondrial superoxide and hydrogen peroxide production and can lead to accelerated necrotic cell death in immune-system cells (Scheme 1), *e.g.,* macrophages and T cells. This may be one mechanism by which selenium down-regulates the immune-system response and protects against an aggressive inflammatory response.



SCHEME 1. Effect of selenite on mitochondria and cell death. Treatment with sodium selenite on immune-system cells, *e.g.,* macrophages and lymphocytes, caused an increase in superoxide (O_2^-) and decrease in hydrogen peroxide (H_2O_2), probably influencing the enzymatic dismutation reaction by mitochondrial SOD (MnSOD) [possible targets for selenium action: MnSOD, uncoupling proteins (UCPs), and ATP-synthase]. This change in the ratio and profile of cellular ROS may lead to mitochondrial dysfunction and impaired redox signaling, culminating in cell death with necrotic characteristics. Treatment with Tiron (superoxide scavanger) and tempol (SOD-mimicking compound) protects cells from death.

ACKNOWLEDGMENTS

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ABBREVIATIONS

DCF, dichlorofluorescein; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; H_2DCF-DA, dichlorodihydrofluorescein diacetate; IRLM, isolated rat liver mitochondria; MnSOD, manganese superoxide dismutase; PBS, phosphate-buffered saline; PI, propidium iodide; ROS, reactive oxygen species; SOD, superoxide dismutase.

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