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# Original Research Communication

# Selenium Attenuates Expression of MnSOD and Uncoupling Protein 2 in J774.2 Macrophages: Molecular Mechanism for Its Cell-Death and Antiinflammatory Activity

SHANI SHILO, MICHAL AHARONI-SIMON, and OREN TIROSH

## ABSTRACT

**Selenium can activate cell death. However, the mechanism of action is not yet fully defined. We hypothesized that selenium may impede mitochondrial superoxide dismutation to $H_2O_2$ and $O_2$, leading to cell death in macrophages and that this effect may be relevant to antiinflammatory treatment by selenium. In this study, the mechanism of action of selenium was investigated in nonactivated and activated (immune-stimulated) J774.2 macrophages. Sodium selenite treatment decreased dichlorodihydrofluorescein-reacting intracellular reactive oxygen species (ROS) (mainly peroxides and hydroxyl radicals), with no correlation to glutathione peroxidase activity. However, selenite decreased the transcription and expression of manganese superoxide dismutase (MnSOD) and uncoupling protein 2 (UCP2). This cellular effect was due to inhibition of specificity protein-1 (Sp1) binding to its DNA binding site. Following immune stimulation of macrophages using lipopolysaccharides plus interferon-γ, MnSOD was up-regulated. Activated macrophages showed higher mitochondrial membrane potential, intracellular ROS levels, and cellular resistance to cell death. Selenite treatment attenuated all of these parameters. Selenite prevented nuclear factor-κB (NF-κB) activation as a mechanism of its inhibitory activity on MnSOD expression in the immune-stimulated cells. In addition, overexpression of human MnSOD protected against death induced by selenite treatment. It is therefore concluded that selenium at high nanomolar to low micromolar concentrations shifts the balance between inflammatory response and cell death toward the latter, through a direct effect on the transcription factors Sp1 and NF-κB, and down-regulation of MnSOD and UCP2.** *Antioxid. Redox Signal.* 7, 276–286.

## INTRODUCTION

SELENIUM is recognized as essential in animal and human nutrition (8, 9). In the form of the amino acid selenocysteine, it is a component of a number of antioxidant enzymes, *e.g.,* glutathione peroxidase (GPx) (1, 5). 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, 29, 32).

Excessive and continuous cytokine production in response to bacterial lipopolysaccharides (LPS) or superantigens is a hallmark of the systemic inflammatory response (SIRS), which can be life-threatening (19, 31). Selenium administration to SIRS patients dramatically improves prognosis, indicating an inhibitory effect on an overstimulated immune system (12, 14, 15). Nevertheless, the precise underlying mechanism is not known.

A mechanism by which various pharmacological agents might modulate the functioning of the immune system could

The School of Nutritional Sciences, Institute of Biochemistry, Food Science and Nutrition, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, Rehovet, Israel.

involve direct interaction with inflammatory cells sensitizing them to die and to decrease their reactive oxygen species (ROS) and cytokine production (13, 26). Provoking such effects in macrophages may be a key event in modulating the inflammatory response by selenium. So far, pro-cell death activity of selenium has been connected mainly to anticancer effects (22).

We recently reported that micromolar levels of sodium selenite increase superoxide levels and decrease hydrogen peroxide ($H_2O_2$) production in the mitochondria of immune-system cells, leading to cell death (34). We therefore hypothesized that selenium could impede mitochondrial superoxide dismutation to $H_2O_2$ and $O_2$ and that this could strongly sensitize cell death in macrophages and decrease peroxide production in immune-stimulated inflammatory cells. Such a mechanism of action for selenium could be relevant in attenuating an overstimulated immune system, explaining its beneficial effect in inflammation.

Possible targets that might explain the change in the redox balance are the mitochondrial proteins manganese superoxide dismutase (MnSOD) and uncoupling protein 2 (UCP2). MnSOD is responsible for the dismutation of superoxide radicals into $H_2O_2$ in the mitochondria. MnSOD homozygous knockout is lethal in mice, whereas partial knockout creates increased sensitivity to cell death (23, 40). UCP2 belongs to a family of proteins located at the inner mitochondrial membrane that act as proton channels or transporters (11, 16). UCP2 is widely expressed in mammalian tissues, and strongly in macrophages. UCP2 knockout mice have been found to be highly resistant to bacterial infection. The macrophages of the UCP2 knockout mice generate more superoxide as measured by nitro blue tetrazolium reduction (3). Nutrients that can mimic this knockdown effect could be clinically important.

The aim of this study was to elucidate the mechanisms of action of selenium in executing its cell-death and antiinflammatory effects, and to investigate the possible interaction of selenium with the mitochondrial proteins MnSOD and UCP2.

## MATERIALS AND METHODS

### *Cell culture*

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 macrophages reached 60% confluence, the cells were exposed to treatments as indicated in the figure legends. Sodium selenite was used as the source of selenium in all experiments.

### *Determination of cell viability*

Cell-membrane integrity was detected by flow cytometry (FACSort, BD) as a measurement of cell viability. For this assay, the nonpermeant DNA-interchelating dye propidium iodide, 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 (33, 38).

### *Intracellular ROS*

Intracellular ROS were detected using dichlorodihydrofluorescein diacetate ($H_2$DCF-DA) (37). This probe has high reactivity to $H_2O_2$, lipid hydroperoxide, and hydroxyl radicals and low reactivity to superoxide anions (39). After the different treatments, the cells were washed three times with phosphate-buffered saline (PBS). Cells were centrifuged (2,000 rpm, 5 min), resuspended in PBS, and incubated with 25 μ*M* $H_2$DCF-DA 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 dichlorofluoroscein (DCF) emission was recorded at 530 nm. Data were collected from at least 10,000 cells.

Alternatively, DCF fluorescence was recorded using a microfluorometer plate reader (GENios, Tecan, Austria). Cells were washed twice with PBS, and then 25 μ*M* $H_2$DCF-DA was added and incubated for 30 min at 37°C. Cells were washed again, and cell lysis was performed using PBS/0.2% Triton X-100. Total fluorescence was recorded and calibrated according to protein content.

### *Western blots [specificity protein-1 (Sp1), MnSOD, UCP2]*

Cells were grown in six-well tissue culture plates, treated with selenium for 24 or 72 h. The wells were washed with PBS to remove detached cells, scraped, and centrifuged (5 min, 2,000 rpm). Boiling lysis buffer was added to the pellets [1% sodium dodecyl sulfate (SDS), 1 m*M* sodium orthovanadate, 10 m*M* Tris, pH 7.4]. Protein content was measured (Bio-Rad protein assay kit) and equalized. Sample buffer (containing SDS) was added, and the samples were boiled. The samples were subjected to SDS–polyacrylamide gel electrophoresis followed by western blot analysis.

Separated proteins were transferred electrophoretically from the gel onto a nitrocellulose membrane (Amersham International plc, Buckinghamshire, U.K.). Equal protein gel loading and membrane transfer were validated by Ponceau S (Sigma–Aldrich, St. Louis, MO, U.S.A.) staining before blotting. The membrane was blocked in TBS (0.15 *M* NaCl/10 m*M* Tris-HCl, pH 7.4) containing 5% (vol/vol) skimmed milk (Blotto), and then incubated overnight with primary antibodies at room temperature (diluted 1:2,000 in Blotto). After being washed six times in TBS containing 0.05% (vol/vol) Tween 20, the membrane was incubated for 2 h at room temperature with secondary antibody (diluted 1:5,000 in Blotto). Immunoreactive bands were detected with ECL western blotting detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) and developed on film (Fuji Super RX, Dusseldorf, Germany). Primary antibodies for Sp1 and MnSOD were from BD Transduction Laboratories (San Jose, CA, U.S.A.) and for UCP2 from Alpha Diagnostics (San Antonio, TX, U.S.A.). Secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.).

### *Preparation of nuclear extracts*

Nuclear extracts were prepared using a slight modification of the method of Dignam *et al*. (10). Treated cells were washed with cold PBS, scraped, and centrifuged. Hypotonic

buffer (1 ml) was added to the cell pellets [20 m*M* HEPES, pH 7.0, 10 m*M* KCl, 1 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol (DTT), 0.1% Triton X-100, 20% glycerol, 2 m*M* phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml aprotonin and leupeptin]. Pellets were disrupted by pipetization and centrifuged at 3,000 rpm for 5 min. The pellets were resuspended in 100 µl of cold extraction buffer (20 m*M* HEPES, pH 7.0, 10 m*M* KCl, 1 m*M* $MgCl_2$, 0.5 m*M* DTT, 0.1% Triton X-100, 20% glycerol, 2 m*M* PMSF, 1 mg/ml aprotonin and leupeptin, 420 m*M* NaCl). The samples were rotated for 20 min at 4°C and then centrifuged at 15,000 rpm for 10 min. The supernatant was collected. Protein content was measured using Bradford reagent. Samples were kept frozen at −70°C.

### *Electromobility shift assay (EMSA) analysis*

Gel shift assays were performed according to the gel shift assay protocol from Promega Corp. (San Luis Obispo, CA, U.S.A.). A 22mer double-stranded nuclear factor-κB (NF-κB) consensus oligonucleotide, 5′-AGTTGAGGGGACTTT CCCAGGC-3′, and Sp1 consensus oligonucleotide, 5′-ATT CGATCGGGGCGGGGCGAGC-3′, were labeled with $^{32}P$. For each reaction, 2 µg of nuclear extract was incubated with 2 µl of 5× binding buffer for 5 min. Then, 5 µl of water and 1 µl of the $^{32}P$-labeled oligonucleotide was added at room temperature for 20 min. To the samples was added 1 µl of dye-free gel loading solution, and then the samples were separated using 6% nondenaturing acrylamide gel electrophoresis. The gel was dried, exposed to a phosphoimaging screen, and then visualized with a phosphoimager (FLA5OO, Fujifilm, Japan). For specificity, 1 µl of unlabeled consensus oligonucleotide was used to compete with the labeled probe. For the *in vitro* experiment, 5 µ*M* sodium selenite was added to the nuclear extracts prior to the binding buffer and incubated for 5 min.

### *Evaluation of mitochondrial membrane potential (MMP) in cells*

MMP was detected by flow cytometry (FACSort, BD) using the membrane potential-sensitive fluorescent probe Mito-Tracker Orange (CMTMRos) (Molecular Probes, Eugene, OR, U.S.A.). The following fluorescence setting was used: excitation at 488 nm and emission at 575 nm (FL2 channel). Macrophage cells (1 million cells/ml) were stained with 0.1 µ*M* CMTMRos for 30 min at 37°C. Accumulation of the dye in the mitochondria was evaluated by flow cytometer analysis as already described (2, 27). Data were collected from 10,000 cells.

### *GPx activity*

GPx activity was determined by following the rate of NADPH oxidation at 340 nm in the presence of the substrates: 3 m*M* reduced glutathione, 1.2 U of glutathione reductase, and 1.5% $H_2O_2$, using a microfluorometer plate reader (GENios). The reaction contained 500 µl of Tris reaction buffer (100 m*M* Tris-HCl, 300 m*M* KCl, 5 m*M* EDTA, 1 m*M* $NaN_3$, pH 7.0), substrates, and 100 µl of cell extract.

Macrophages were treated with selenium for 24 h. Wells were washed twice with PBS, then permeabilized and scraped using 200 µl of 0.2% Triton X-100. The reaction was recorded every minute for 25 min. The graph's slope was calculated, and the results were adjusted according to the protein amount calculated with the Bradford reagent (24, 25).

### *RT-PCR*

Total RNA was prepared and isolated by the Tri-Reagent method according to the manufacturer's protocol (Sigma–Aldrich). Total RNA (1 µg) was converted into cDNA using Reverse-iT first strand synthesis kit (ABgene, Epsom, U.K.). Amplification of MnSOD and UCP2 was performed by incubating 10 ng equivalents of DNA with the following oligonucleotide primers: for detection of mouse MnSOD (mMn-SOD), left: 5′-ATGTTGTGTCGGGCGGCG-3′, and right: 5′AGGTAGTAAGCGTGCTCCCACACG-3′ (6); for detection of UCP2, left: 5′-TTCAAGGCCACAGATGTGCC-3′ and right: 5′-TCGGGCAATGGTCTTGTAGGC-3′ (30). The sequence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as a control using the following primers: left: 5′-TCCGCCCCTTCCGCTGATG-3′ and right: 5′-CACGGAAGGCCATGCCAGTGA-3′. PCR was performed with 28 cycles (25 cycles for GAPDH) of 30 s at 96°C, 30 s at 58°C, and 1 min at 72°C. PCR products were analyzed on 1.3% (wt/vol) agarose gels.

### *Overexpression of human MnSOD (hMnSOD) in J774.2 macrophages*

Mouse J774.2 macrophages ($3 \times 10^5$ cells) were transiently transfected with 2 µg of a hMnSOD expression vector pEGFPN1/MnSOD (kindly provided by Sonia C. Flores, Webb–Waring Institute for Cancer, Aging and Antioxidant Research, University of Colorado Health Sciences Center, Denver, CO, U.S.A.) carrying the hMnSOD gene, by using Superfect Transfection Reagent (Qiagen Inc., Valencia, CA, U.S.A.). Protein transcription and translation were verified by RT-PCR for hMnSOD (primers: left: 5′-TTGGCCAAGGG-GATGTTAC-3′, and right: 5′-CGTGGTTTACTTTTTCAA-GC-3′) and by FACS analysis for EGFP-increased fluorescence in cells, respectively. Forty-eight hours following transfection, cells were treated with 30 µ*M* selenium for 24 h.

### *Statistical analysis*

Comparisons between two groups were performed by *t* test. For multiple groups, data were analyzed by 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

### *Effect of selenite on ROS production and GPx activity*

We have previously shown that an early event in the cell-death cascade facilitated by selenite treatment is the decrease in DCF-sensitive ROS production (34). Here we show that

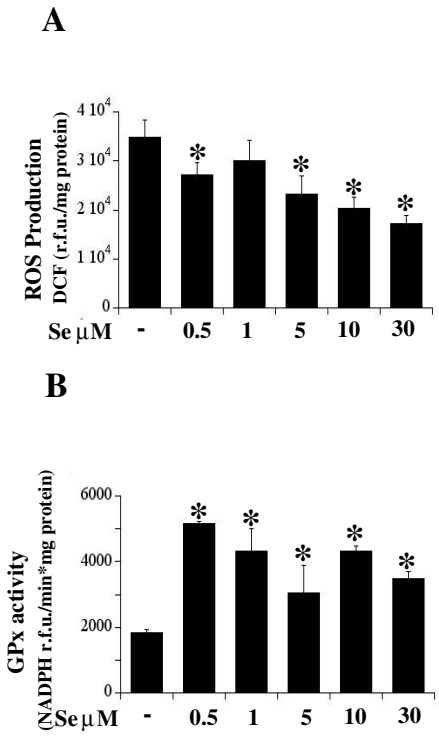



**FIG. 1. Sodium selenite decreases macrophage ROS production and regulates GPx activity.** J774.2 macrophages were treated with increased concentrations of selenite (Se) for 24 h. **(A)** Decrease in DCF-sensitive ROS ($H_2O_2$) production in cells. *$p < 0.05$, significantly different compared with control. **(B)** Results of GPx activity. Selenite at 0.5 μ*M* substantially increased the GPx activity; no further increase in activity was observed with higher concentrations of selenite, indicating maximal GPx activity. *$p < 0.05$, significantly different compared with control.

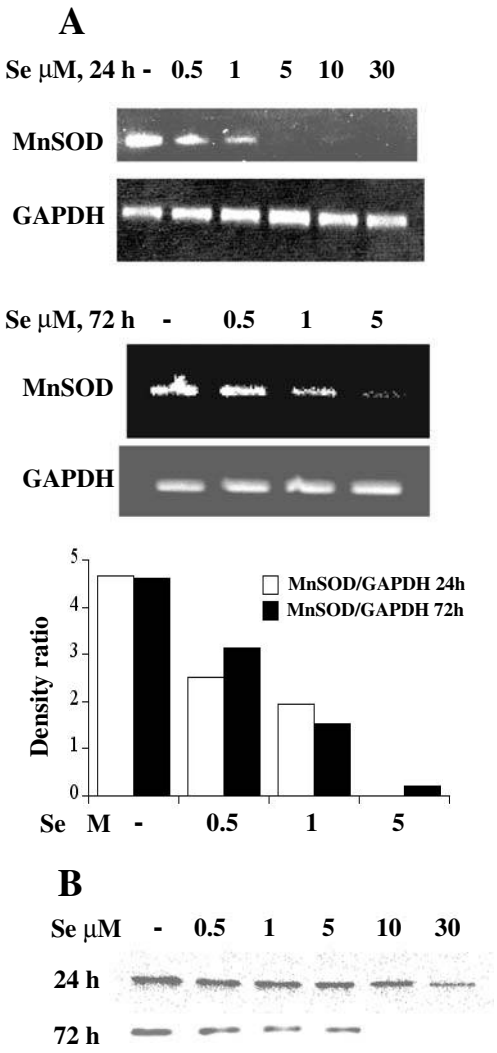



**FIG. 2. Selenite down-regulates MnSOD transcription and expression in a dose-dependent manner.** J774.2 macrophage cells were treated with 0.5–30 μ*M* sodium selenite (Se) for 24 and 72 h. MnSOD mRNA levels and protein levels were evaluated. **(A)** RT-PCR analysis of MnSOD and calculated density ratio of MnSOD/GAPDH of the presented gel run. Data are representative of three independent experiments. **(B)** Western blot analysis of MnSOD. Data are representative of at least three independent experiments.

treating cells with increasing concentrations of selenite (0.5–30 μ*M*) results in a dose-dependent decrease in DCF-sensitive ROS production (Fig. 1A). Activity of the enzyme GPx due to selenite treatment was maximal at 0.5 μ*M* selenite (Fig. 1B). Higher doses of selenite decreased cellular ROS production dose-dependently, but did not further increase GPx activity. Therefore, selenite may affect expression and activity of other ROS-modulating enzymes.

### *Effect of selenite on the mitochondrial proteins MnSOD and UCP2*

MnSOD is the mitochondrial enzyme responsible for the dismutation of superoxide to $H_2O_2$; therefore, changes in MnSOD levels may explain the effect of selenite on decreasing DCF-sensitive ROS production (23, 39). We exposed macrophages to 0.5–30 μ*M* sodium selenite for 24 h and 72 h. MnSOD mRNA levels and protein levels were checked. Treating cells with increased concentrations of selenite resulted in a significant decrease in MnSOD transcription (Fig. 2A). Figure 2B shows that selenite treatment also decreased the MnSOD protein expression.

There is evidence connecting UCP2 and changes in ROS (superoxide) production in macrophages (3). Figure 3 shows selenite's effect on UCP2 transcription and expression. Macrophage cells were treated with sodium selenite (0.5–30 μ*M*) for 24 and 72 h. UCP2 mRNA levels and protein levels were checked. Selenite treatment for 24 and 72 h caused a dose-dependent decrease in mRNA level (Fig. 3A). Western blot analysis showed lower protein levels of UCP2 after selenite treatment (Fig. 3B).

Selenite's effect on the *sod2 (MnSOD)* and *Ucp2* genes in J774.2 macrophages (decreasing transcription and expression of the two genes) was also observed in Jurkat T-cell lympho-

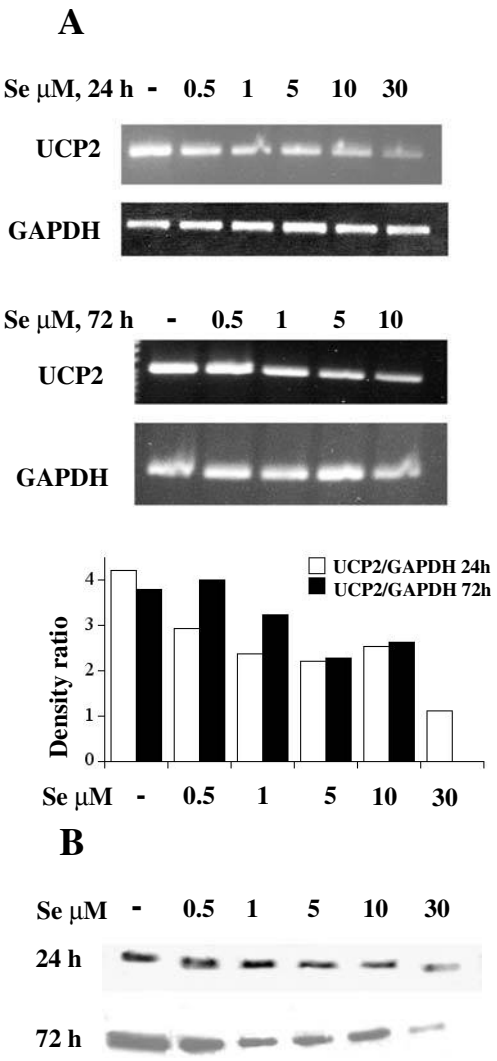



**FIG. 3. Selenite down-regulates UCP2 transcription and expression.** J774.2 macrophages were treated with 0.5–30 μ*M* sodium selenite (Se) for 24 and 72 h. UCP2 mRNA levels and protein levels were evaluated. **(A)** RT-PCR analysis of UCP2 and calculated density ratio of UCP2/GAPDH of the presented gel run. Data are representative of three independent experiments. **(B)** Western blot analysis of UCP2. Data are representative of at least three independent experiments.

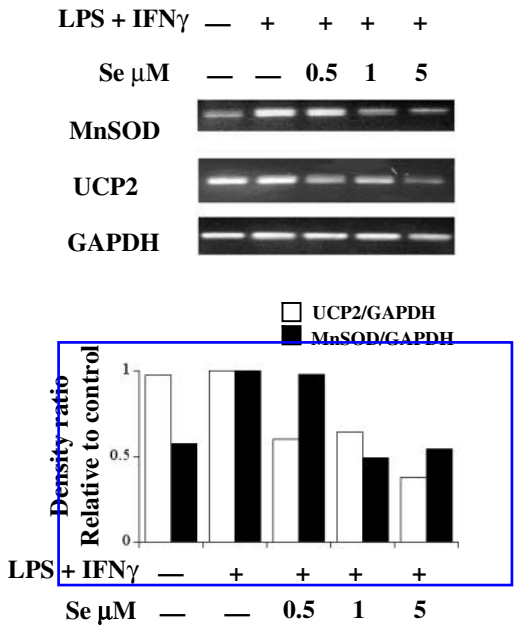



**FIG. 4. Selenite down-regulates mRNA levels of MnSOD and UCP2 in immune-stimulated (activated) macrophages.** Activation of macrophages by 10 ng/ml LPS and 50 units/ml IFNγ for 24 h up-regulates MnSOD mRNA levels. No effect was observed on UCP2 mRNA levels. Selenite (Se) treatment abrogated this overexpression of MnSOD. UCP2 mRNA levels also decreased following selenite treatment. **(Top)** RT-PCR of MnSOD, UCP2, and GAPDH. Data are representative of three independent experiments. **(Bottom)** Densitometry analysis of MnSOD/GAPDH and UCP2/GAPDH mRNA levels following RT-PCR of the presented gel run.

cytes, although Jurkat cells were much less sensitive to selenite treatment (data not shown).

## *Effect of selenite on MnSOD and UCP2 in activated macrophages*

Selenium has been shown to be beneficial in patients suffering from sepsis and to reduce mortality in intensive care units patients (12, 15). Macrophage maturation plays a central role in the immune-system response and causes activation of >100 genes that encode mediators of inflammatory and immune responses (19). We therefore investigated sodium selenite's effect in activated macrophages. J774.2 macrophages were treated with 0.5–5 μ*M* selenite for 72 h and then activated using 10 ng/ml LPS and 50 units/ml interferon-γ (IFNγ) for 24 h. Activation of control macrophages up-regulated MnSOD mRNA levels. Little effect was observed on UCP2. Selenite treatment attenuated the overexpression of MnSOD following activation. UCP2 mRNA levels were also decreased following selenite treatment (Fig. 4).

## *Effect of selenite on transcription factors that regulate sod2* (MnSOD) *and* ucp2 *gene expression*

*Effect of selenite on the transcription factor NF-κB.* To study *NF-κB* in activated macrophages, an EMSA of the transcription factor was carried out (Fig. 5). After 72 h, selenite-treated and control cells were activated for 6 h by LPS and IFNγ. Selenite treatment for 72 h at 0.5–5 μ*M* did not affect basal levels of NF-κB DNA binding. Activation significantly increased NF-κB DNA binding in nuclear extracts, and treatment with increased concentrations of selenite resulted in a dose-dependent decrease of NF-κB DNA binding (Fig. 5A). In a cell-free system, selenite decreased NF-κB binding activity after addition of 5 μ*M* to nuclear extracts of activated cells

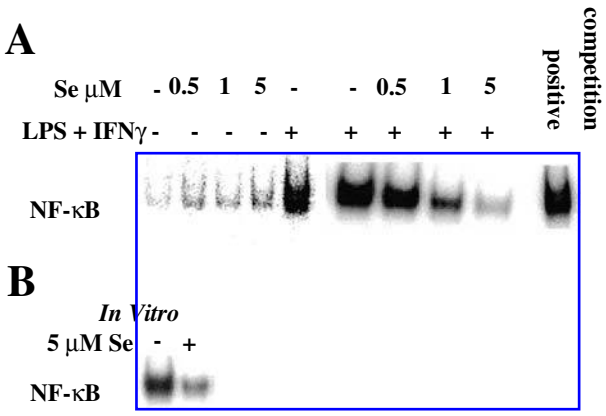



**FIG. 5. Selenite down-regulates NF-κB DNA binding activity in activated macrophages.** Cells were treated with 0.5–5 μ*M* selenite (Se) for 72 h and then activated with 10 μg/ml LPS and 50 units/ml IFNγ for 6 h. Nuclear extracts were prepared and EMSA was performed. **(A)** Selenite treatment in nonactivated macrophages did not affect the transcription factor NF-κB. Activated macrophages showed NF-κB binding activity in the nucleus, but selenite treatment abrogated this effect. Competition with 100× cold probe was performed on a positive control nuclear extract. **(B)** Cell-free system EMSA of nuclear extracts of activated macrophages treated or not with 5 μ*M* selenite for 5 min. Data are representative of at least three independent experiments.

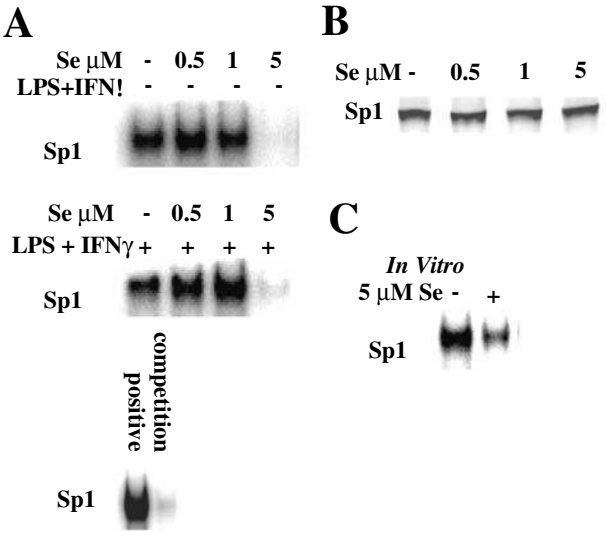



**FIG. 6. Selenite treatment in macrophages attenuates Sp1 transcription activity.** Cells were treated with 0.5–5 μ*M* selenite (Se) for 72 h and activated with 10 μg/ml LPS and 50 units/ml IFNγ for 6 h. Nuclear extracts were prepared and EMSA was performed. **(A)** EMSA of Sp1. **(B)** Western blot of Sp1. Cells were treated with increased concentrations of selenite for 72 h. **(C)** Cell-free system EMSA of control nuclear extracts treated or not with 5 μ*M* selenite for 5 min. Data are representative of at least three independent experiments.

(Fig. 5B). This suggests that selenite directly prevents NF-κB binding to its DNA site.

*Effect of selenite on the transcription factor Sp1.* Macrophages were treated with 0.5–5 μ*M* selenite for 72 h and were then activated or not by LPS and IFNγ for 6 h. Low levels of selenite (0.5–1 μ*M*) did not change Sp1 DNA binding activity, but the higher level treatment (5 μ*M*) significantly decreased Sp1 binding activity. Activation did not affect Sp1 levels in the nucleus compared with the control. Selenite treatment in activated macrophages showed a similar effect (Fig. 6A).

The major regulation on Sp1 transcription factor is its transcription rather than nuclear translocation (4). We therefore checked whether the reduced DNA binding activity following selenite treatment was due to a decrease in Sp1 protein expression. Western blot analysis for Sp1 (Fig. 6B) showed that the total protein extracted from macrophages treated with 0.5–5 μ*M* sodium selenite for 72 h was not affected, indicating no change in Sp1 protein expression.

Sp1 contains three Cys2His2 zinc fingers in the DNA-interaction domain of the protein. These zinc fingers contain

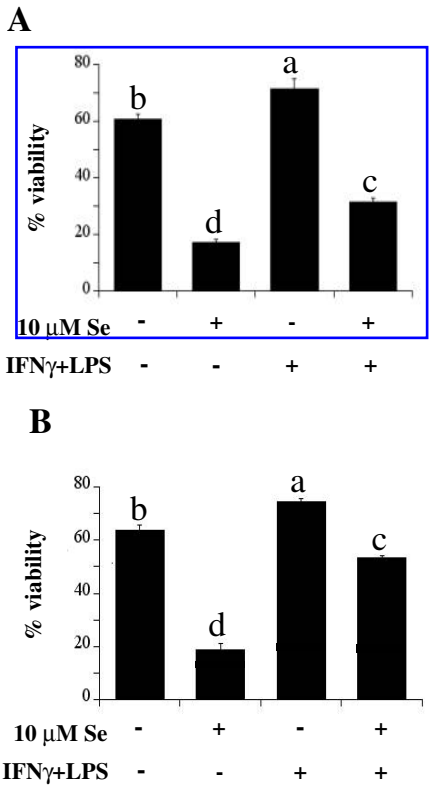



**FIG. 7. Activated macrophages are more resistant to selenite-induced cell death.** Activation of J774.2 macrophages increased their viability, whereas selenite (Se) potentiated cell death. **(A)** Simultaneous activation using LPS + IFNγ and treatment with 10 μ*M* selenite for 24 h. **(B)** Preactivation for 24 h by LPS + IFNγ followed by 24 h of treatment with 10 μ*M* sodium selenite. Means without the same letter differ ($p < 0.05$).

cysteine residues that are redox-sensitive and may be susceptible to modification, thus destroying the DNA-binding properties of the transcription factor (42). In a cell-free system, 5 μ*M* sodium selenite directly affected Sp1 binding activity (Fig. 6C).

### *Selenite-induced cell death is inhibited in activated macrophages*

Cells were activated using LPS and IFNγ. Activation for 24 h increased macrophage viability, whereas 10 μ*M* selenite treatment for 24 h decreased viability (Fig. 7). Simultaneous activation of macrophages combined with selenite treatment resulted in partial protection against the selenite treatment (Fig. 7A). When the activation treatment was given 24 h prior to the selenite treatment, selenite-induced death was suppressed (Fig. 7B).

### *Overexpression of MnSOD inhibits selenite-induced cell death*

hMnSOD was overexpressed in the murine macrophage cell line. Transcription and translation of the hMnSOD was verified by RT-PCR using specific PCR primers and by increased fluorescence of EGFP (Fig. 8A and B). Transfected cells were treated or not with 30 μ*M* selenite for 24 h and were compared with nontransfected controls (Fig. 8A). Control cells (nontransfected) treated with selenite were analyzed for mMnSOD mRNA. In these cells, selenite depleted the endogenous MnSOD. However, in cells that overexpressed hMnSOD (transfected), a significant amount of mMnSOD mRNA was detected with or without selenite treatment. These data indicate a protective effect of hMnSOD against depletion of the endogenous mMnSOD by high-dose selenium. Overexpression of hMnSOD completely protected the cells against selenite induced cell death (Fig. 8C).

### *Effect of selenite on DCF-sensitive ROS production and MMP in activated macrophages*

In correlation with the higher levels of MnSOD, activated macrophages showed high levels of intracellular DCF-sensitive ROS. Selenite reduced oxidation of $H_2$DCF to fluorescent DCF, indicating an antioxidant effect of selenite (Fig. 9A).

We correlated macrophage ROS production to the MMP. Macrophages were activated for 24 h, and then selenite treatment was used. Indeed, activated macrophages had a higher

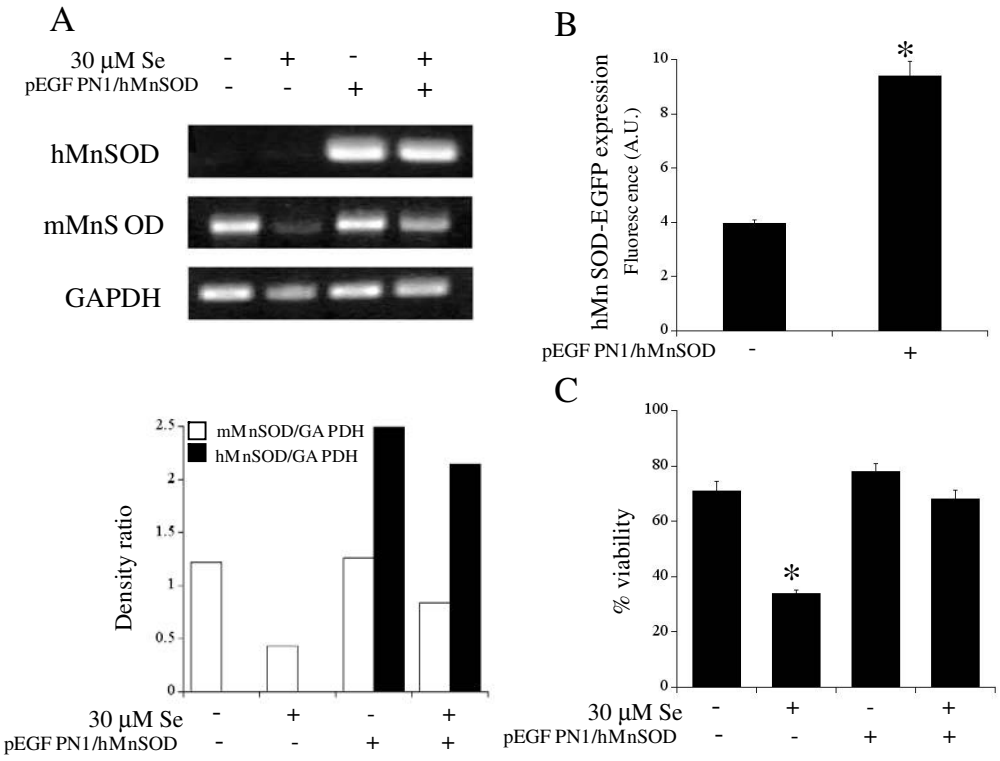



**FIG. 8. Overexpression of hMnSOD protects against high-dose selenite-induced cell death.** Mouse J774.2 macrophages ($3 \times 10^5$ cells) were transiently transfected with 2 μg of hMnSOD expression vector pEGFPN1/MnSOD. **(A) (Top)** RT-PCR analysis of hMnSOD and mMnSOD mRNA levels in control cells and cells transfected for 48 h, followed or not by 30 μ*M* sodium selenite (Se) treatment for additional 24 h. **(Bottom)** Calculated density ratio of hMnSOD/GAPDH and of mMnSOD/GAPDH of the presented gel run. **(B)** FACS analysis for the presence of hMnSOD-EGFP in the macrophages after transfection. The data indicate expression of hMnSOD in the transfected cells. *$p < 0.05$, significantly different compared with control. **(C)** FACS analysis of cell viability in macrophages transfected or not with the hMnSOD gene and treated with 30 μ*M* sodium selenite for 24 h. *$p < 0.05$, significantly different compared with control.

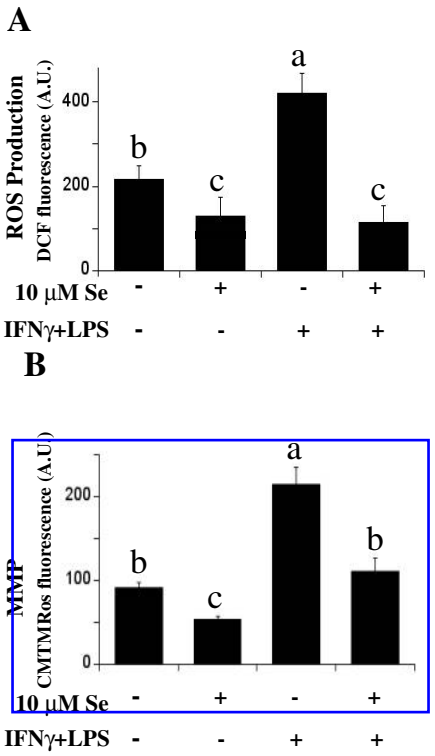



**FIG. 9. Activation of macrophages increases ROS production and MMP, whereas selenite treatment attenuates the effect.** Macrophages were activated for 24 h using LPS + IFNγ where indicated. This was followed by 10 μ*M* sodium selenite (Se) treatment for 24 h. **(A)** ROS production. **(B)** MMP. Means without the same letter differ ($p < 0.05$).

MMP and higher ROS production compared with nonactivated macrophages. Selenite-treated macrophages showed a marked decrease in MMP and ROS production (Fig. 9).

## DISCUSSION

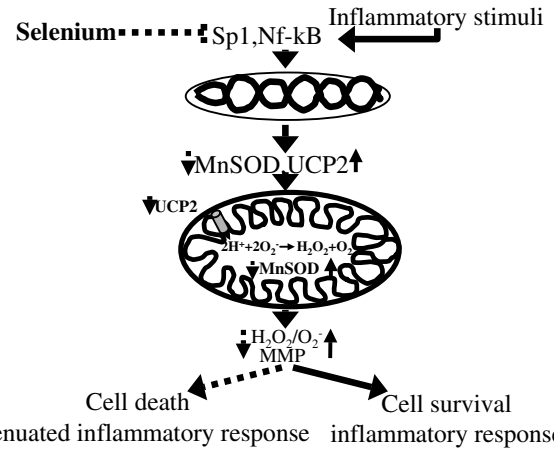
Selenium is essential in diet as it is incorporated into several antioxidant enzymes. The first selenoenzyme discovered was GPx, which oxidizes glutathione and thus eliminates peroxides. Maximal GPx activity is a criterion for maximal incorporation of selenium into selenoenzymes. In recent years, selenium has been shown to have other properties important in inducing apoptosis and cell death. This effect has been investigated in cancer cells as a mechanism for selenium's anticancer effects (8, 14, 22). It was reported that selenium can potentiate mitochondrial permeability transition *in vivo* as a putative mechanism for chemoprotection (35).

The dose-dependent decrease in DCF-sensitive ROS production induced by selenium could not be attributed merely to the increased GPx activity. Sodium selenite (0.5 μ*M*) increased GPx activity, indicating that cells in culture could be selenium-deficient. However, higher levels (>0.5 μ*M* selenite) did not further increase GPx activity, but rather reduced $H_2O_2$ production (Fig. 1), suggesting a different cause for reduced ROS production. Our observations suggest that selenium can impede mitochondrial superoxide dismutation due to its influence on the mitochondrial enzyme MnSOD. Sodium selenite treatment down-regulated, in a dose-dependent manner, MnSOD mRNA and protein levels in murine macrophages.

Another candidate protein that could be involved in selenium's action is UCP2, which transfers protons through the inner membrane of the mitochondria into the matrix. Matrix proton levels are necessary for the enzymatic reaction of MnSOD, which is a pH-sensitive enzyme (17). Studies in UCP2 knockout mice, which are resistant to the systemic inflammatory response induced by infection, indeed showed more superoxide production in isolated macrophages (3). The data may therefore shed new light on the connection between selenium and its antiinflammatory and antiinfectious roles (12). Sodium selenite treatment of 0.5–30 μ*M* dose-dependently down-regulated UCP2 transcription and expression, although to a lesser extent than MnSOD.

Focusing on the two proteins, a common denominator regulating their transcription was sought. Transcription factor Sp1 has been reported to be essential for basal transcription in MnSOD (43) and UCP2 (28). Sp1 belongs to the Sp/XKLF transcription factor family, which is united by a combination of three conserved Cys2His2 zinc fingers that form the DNA binding domain (4). Sp1 recognizes and specifically binds to GC-rich sites (20). The Cys2His2 zinc fingers may be susceptible to oxidation, thus destroying the DNA-binding properties of the transcription factor (42).

Selenite treatment resulted in reduced Sp1 binding. Western blot analysis showed that the decreased binding was not due to less Sp1 synthesis. In a cell-free system, analysis showed that selenium can directly reduce this binding activ-




**SCHEME 1. Proposed molecular mechanism for selenium's effect on cell death/survival and redox balance in inflammatory cells.** Treatment of macrophages with selenium reduces DNA binding activity of transcription factors Sp1 (in naive and stimulated condition) and NF-κB (following inflammatory stimulation). This leads to reduced transcription and expression of the mitochondrial proteins MnSOD and UCP2. As a consequence, there is impaired dismutation of superoxide ($O_2^-$) to $H_2O_2$ in the mitochondria, and reduced MMP. This leads to cell death and to an attenuated inflammatory response.

ity. Therefore, sodium selenite treatments may lead to a modification in the binding domains of Sp1 and thereby to reduced transcription of MnSOD and UCP2 (Scheme 1).

Macrophage activation plays a central role in the immune-system response. In activated macrophages, MnSOD is up-regulated, resulting in increased ROS production, increased MMP, and increased viability. Selenium treatment in such activated macrophages decreased the effect of the activation by inflammatory cytokines and decreased MnSOD levels.

Treatment with LPS and IFNγ induces NF-κB binding in the nucleus in mononuclear phagocytes and T cells. The nuclear import and binding of NF-κB allows transcriptional activation of >100 genes that encode mediators of inflammatory and immune responses (19). Activation of the macrophages increased nuclear NF-κB content, whereas selenium treatment dose-dependently decreased the binding activity (Scheme 1). NF-κB binding domain is rich with cysteine residues and can be modified by selenium; this was previously shown in cells and in a cell-free system. Addition of DTT restored the NF-κB binding activity, indicating that selenium induces a reversible oxidative modification to block NF-κB activation (21).

Selenium intraction with transcription factors Sp1 and NF-κB could be relevant *in vivo* as well, because subcellular distribution analysis of selenium in human liver samples showed that half of the total selenium content existed in the nuclei (7).

Changes in MMP are integral to the cell's life–death transition (36). Increased MnSOD levels following macrophage activation resulted in higher MMP, peroxide production, and cell viability. The reaction catalyzed by MnSOD leads to the removal of matrix protons and contributes to maintaining MMP. Decreased MnSOD levels by selenium would result in reduced MMP, which may contribute to selenium-induced cell death. Although under physiological conditions only a small percentage of the oxygen leaks into the matrix as superoxide radicals, it is still possible that the dismutation reaction has a role to eliminate protons from the mitochondria matrix to elevate membrane potential. This effect is further provoked under $pO_2$ of 21% where the fraction of superoxide/oxygen can be close to 10% (18). UCP2 may transfer protons to the mitochondria. A lower UCP2 level induces mitochondrial coupling and higher membrane potential. However, UCP2 levels are not affected following activation of macrophages. The main effect in activated macrophages is the increased expression of MnSOD leading to removal of superoxide and matrix protons, thereby elevating the MMP.

In immune-system cell lines, selenium sensitizes cell death. This effect was blocked by the superoxide scavenger Tiron and by the SOD-mimicking compound tempol (34). Therefore, a defense system against superoxide accumulation, *e.g.,* MnSOD, is pivotal in controlling macrophage survival. Indeed, activated macrophages had up-regulated MnSOD levels and were resistant to cell death, whereas selenium-treated macrophages showed lower MnSOD levels. Interfering with mitochondrial superoxide dismutation by selenium leads to higher superoxide in the mitochondria, less $H_2O_2$ production, and low MMP. As a result of such redox effect, cells may be resensitized to various death signals and the inflammatory cellular response could be inhibited.

Zhong *et al.* reported that MnSOD overexpression in prostate cancer cells showed an increase in sensitivity to the cytotoxicity of buthionine sulfoximine, a glutathione-depleting agent, and vitamin C, but a decrease in sensitivity to sodium selenite (44). Overexpression of the hMnSOD in J774.2 macrophages protected the cells from cell death induced by high-dose selenium. Interestingly, increased MnSOD levels due to hMnSOD transfection protected the cells against selenite-induced depletion of the endogenous mMnSOD. This effect may be due to signaling properties of increased steady state of $H_2O_2$ activating the mMnSOD transcription. Exogenous addition of $H_2O_2$ was shown to increase MnSOD mRNA, supporting the hypothesis that ROS directly affect expression of MnSOD (41). We have shown that addition of a moderate amount of *tert*-butyl hydroperoxide to cells also provided partial protection against cytotoxicity of high-dose selenium (34).

In conclusion, in J744.2 macrophages, sodium selenite inhibits the transcription factors Sp1 and NF-κB, which down-regulate the mitochondrial proteins MnSOD and UCP2, leading to reduced ROS, MMP, and viability of inflammatory cells. This may be one of the basic mechanisms underlying selenium's antiinflammatory and pro-cell death effects (Scheme 1).

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## ABBREVIATIONS

DCF, dichlorofluorescein; DTT, dithiothreitol; EMSA, electromobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPx, glutathione peroxidase; $H_2$DCF-DA, dichlorodihydrofluorescein diacetate; hMnSOD, human manganese superoxide dismutase; $H_2O_2$, hydrogen peroxide; IFNγ, interferon-γ; LPS, lipopolysaccharides; mMnSOD, mouse manganese superoxide dismutase; MMP, mitochondrial membrane potential; MnSOD, manganese superoxide dismutase; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl sulfate; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SIRS, systemic inflammatory response syndrome; Sp1, specificity protein-1; UCP2, uncoupling protein 2.

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Address reprint requests to:
*Oren Tirosh, Ph.D.*
*Institute of Biochemistry, Food Science and Nutrition*
*The Hebrew University of Jerusalem*
*Rehovot 76100, Israel*

*E-mail:* otirosh@agri.huji.ac.il

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