Selenite sensitizes mitochondrial permeability transition pore opening *in vitro* and *in vivo*: a possible mechanism for chemo-protection

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There is a known connection between selenium supplementation and chemo-protective anti-cancer activity. This biological phenomenon may be due to the ability of selenium to instigate cellular apoptosis. However, the mechanism by which selenium promotes cellular apoptosis is still obscure. The present study shows that sodium selenite, a common dietary form of selenium, promotes the mitochondrial permeability transition (MPT) in isolated rat liver mitochondria both *in vitro* and following *in vivo* supplementation. A low selenium concentration (0.1–10 μM) strongly induced cyclosporin A-sensitive mitochondrial swelling. Selenium also promoted both calcium release from the matrix of isolated mitochondria and uncoupled respiration. The MPT-inducing effect of selenium provoked the release of cytochrome *c*, a pro-apoptotic factor, into the incubation medium. Selenium did not increase intra-mitochondrial peroxide production, but did consume endogenous mitochondrial glutathione. Moreover, the effect of MPT induction was greatly potentiated in the presence of thiol-bearing antioxidants, e.g. *N*-acetylcysteine and lipoamide. During MPT progression, selenium induced NADH oxidation via electron acceptance from complex I. Supplementation for 20 days with 16 p.p.m. selenium in the drinking water of rats increased the propensity of mitochondria to undergo the MPT. More marked mitochondrial swelling in response to calcium and lower calcium-uptake capacity were observed, in the absence of liver damage or the intensive oxidation of reduced glutathione. Therefore selenite facilitates MPT pore opening via its thiol- and NADH/complex I-dependent reduction, and thereby may provide chemo-protection by potentiation of the capacity of the mitochondria to regulate programmed cell death. Data from the present study suggest that selenium can regulate important mitochondrial functions both *in vivo* and *in vitro*.

Key words: apoptosis, calcium, cytochrome *c*, mitochondrial permeability transition, selenium.

INTRODUCTION

Sodium selenite is a common dietary form of selenium, which is recognized as essential in animal and human nutrition [1,2]. In the form of the amino acid selenocysteine, selenium is a component of a number of antioxidant enzymes, e.g. glutathione peroxidase and thioredoxin reductase [3,4]. Selenium supplementation induces immune boosting, chemo-protective and anti-cancer activities. Such activities have been associated with selenium intake that corrects for nutritionally deficient status in animals. A higher intake of selenium in mice prevented mammary tumorigenesis more effectively, and independently of glutathione peroxidase expression levels [5]. Therefore selenium intake at concentrations higher than those associated with maximal expression of the selenocysteine-containing enzymes is beneficial [1,2].

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 [4,6,7]. Induction of apoptosis in cancer cells is the preferred way of eliminating them. In addition, an efficient apoptotic process in normal tissues prevents malignant transformation and helps to correct age-related tissue damage [8,9]. There is a known connection between selenium and apoptosis [10–14]. Selenium may facilitate the reactions of cysteine residues via the transient formation of more reactive S–Se intermediates, leading to cell death. [14]. In recent years, efforts have been made to explain the pro-apoptotic effect of selenium. It was shown in a human hepatic cell line and a human hepatoma cell line that SeO_2 prompts apoptosis in correlation with down-regulation of Bcl-2 and up-regulation of p53 levels [10,11]. However, selenium can trigger apoptosis independently of DNA damage in cells with a p53 null phenotype [14]. It has been suggested that the cell-cycle protein kinase cdk2 and protein kinase C are strongly inhibited by various forms of selenium, due to the formation of selenium adducts of the selenotrisulphide (S–Se–S) or selenenylsulphide (S–Se) type, or catalysis of disulphide formation [14]. Ebselen, a selenium-containing compound, has been found to induce apoptosis via induction of the mitochondrial permeability transition (MPT) [13].

Loss of mitochondrial integrity is a critical event in the apoptotic process [15]. Mitochondria are known to regulate cell death, in addition to their critical function in energy metabolism [16–20], via four inter-related pathways: (i) the MPT and the release of cell-death/apoptosis-promoting factors; (ii) release of cytochrome *c* by caspase-dependent activation of pro-apoptotic members of the Bcl-2 family of proteins [21]; (iii) disruption of ATP production; and (iv) alteration of the redox status of cells and production of reactive oxygen species (ROS) [22–27]. The permeability transition (PT) that occurs in the mitochondria

Abbreviations used: CsA, cyclosporin A; DCF, dichlorofluorescein; H_2DCF-DA, dichlorodihydrofluorescein diacetate; ICP, inductively coupled plasma; MPT, mitochondrial permeability transition; NAC, *N*-acetylcysteine; PT, permeability transition; RLM, rat liver mitochondria; ROS, reactive oxygen species.

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involves a sudden increase in inner membrane permeability to solutes with molecular masses below approx. 1500 Da. This phenomenon is most easily observed after matrix accumulation of Ca^{2+}, and it is widely believed to be caused by the opening of a cyclosporin A (CsA)-regulated channel [28–31]. In the present study, the effects of selenium in the form of sodium selenite on rat liver mitochondria (RLM) *in vitro* and following *in vivo* supplementation were investigated.

EXPERIMENTAL

Materials

Materials were obtained at the highest available purity from the following sources: calcium chloride, rotenone, sodium selenite, *N*-acetyl-L-cysteine (NAC), Bradford reagent, glutamic acid, *t*-butylhydroperoxide, mannitol, sucrose, Hepes, EDTA, succinate, GSH and lipoamide (Sigma, St. Louis, MO, U.S.A.); dichlorodihydrofluorescein diacetate (H_2DCF-DA) and maleic acid (Merck, Whitehouse Station, NJ, U.S.A.); purified anti-cytochrome *c* monoclonal antibody and anti-mouse IgG immunoglobulin-specific polyclonal antibody conjugated to horseradish peroxidase (PharMingen, San Diego, CA, U.S.A.); full-range Rainbow recombinant protein molecular mass markers and ECL® Western blotting detection reagents (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.); super RX X-ray film (Fuji, Dusseldorf, Germany).

Animals

Male Sprague–Dawley rats (4 months old; approx. 250 g) received food and water *ad libitum*. They were kept in plastic cages with wire tops in a light-controlled room. All animals were cared for under the guidelines set forth by the Animal Care Committee of the Hebrew University, Jerusalem, Israel.

Isolation of RLM

After overnight fasting, the animals were anaesthetized and killed by decapitation. Livers were removed with scissors and immersed immediately in ice-cold 210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.35 (MSH buffer), containing 1 mM 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 in MSH buffer [32]. The respiratory control ratio of the mitochondria was above 3 in succinate-energized mitochondria, indicating intact coupled mitochondria.

The protein content of the final mitochondrial suspension was determined by the Bradford method using an ultra microplate reader (ELX 808; Bio TEK Instruments).

Standard incubation procedure

Isolated mitochondria (0.5–1 mg of protein/ml) were incubated at ambient temperature with 60 nmol of calcium/mg of mitochondrial protein with stirring, unless stated otherwise, in a buffer consisting of 210 mM mannitol, 70 mM sucrose and 5 mM Hepes, pH 7.4. Mitochondria were energized with 5 mM succinate (in the presence of 2 μM rotenone) or 5 mM glutamate plus 5 mM malate as respiration substrates. Following the incubation, various treatments were imposed as indicated in the Figure legends. For respiration control, a different incubation medium was prepared containing 200 mM sucrose, 20 mM KCl, 3 mM K_2PO_4, 5 mM Tris base and 6 mM $MgCl_2$.

ROS (peroxide) 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 MnSOD [33] to produce a constant flux of hydrogen peroxide. Intra-mitochondrial peroxides were detected using H_2DCF-DA [34]. The mitochondria were resuspended and incubated with 25 μM H_2DCF-DA for 10 min at room temperature for the detection of intra-mitochondrial fluorescence. The fluorochrome-loaded mitochondria were excited using a 488 nm argon-ion laser in a flow cytometer (FACSort, BD). Dichlorofluorescein (DCF) emission was recorded at 530 nm. Data were collected from at least 50000 mitochondria. Hydrogen peroxide was also measured using a microfluorimeter plate reader (GENios, Tecan, Austria), to evaluate kinetically (for 10 min) total mitochondrial ROS production, using 25 μM H_2DCF-DA and recording DCF emission.

Swelling measurements

Mitochondrial swelling was measured by the change in absorbance at 540 nm with a Spectronic Unicam spectrophotometer. Mitochondria were incubated in the standard incubation medium at 0.5 mg of mitochondrial protein/ml.

Calcium measurements

Calcium efflux and influx were monitored with an Orion Ca^{2+} ion selective electrode (Orion Research Inc., Beverly, MA, U.S.A.). Mitochondria were incubated in the standard incubation medium with stirring at 1 mg of protein/ml.

NADH oxidation rate

NADH oxidation was measured by the decrease in autofluorescence of NADH (excitation wavelength 390 nm, emission 465 nm). The oxidation was evaluated using a microfluorimeter plate reader (GENios).

Western blot analysis of cytochrome *c*

Mitochondria (0.5 mg/ml) were incubated at 25 °C for 20 min in 210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.4, 2 μM rotenone plus 5 mM succinate or 5 mM glutamate plus 5 mM malate, and treated with selenium. The incubated samples were centrifuged at 14000 ***g*** at 4 °C for 5 min. Aliquots of the supernatant were frozen, then lyophilized and resuspended in SDS buffer. In addition, the mitochondrial pellet was dissolved in 10 % (w/v) SDS. All samples were diluted 1:1 (v/v) with sample buffer (Sigma) and boiled. The samples were subjected to SDS/PAGE followed by Western blot analysis. Briefly, separated proteins were transferred electrophoretically from the gel to a nitrocellulose membrane (Amersham). The membrane was blocked in TBS (0.15 M NaCl/10 mM Tris/HCl, pH 7.4) containing 5 % (v/v) skimmed milk (Blotto), then incubated overnight with the primary antibody at 4 °C (diluted 1:1000 in Blotto). After washing six times in TBST [TBS containing 0.05 % (v/v) Tween 20], the membrane was incubated for 2 h at room temperature with the secondary antibody (diluted 1:1000 in Blotto). Immunoreactive bands were detected with ECL® Western blotting detection reagents and developed on film.

In vivo supplementation

Control rats (six animals) received food and water *ad libitum*. The selenium-treated group (six animals) received food and

selenium-supplemented water (16 p.p.m. sodium selenite) *ad libitum* for 20 days. The diet provided approx. 2 μg/g per day of selenium, which is considered an acceptable excess supplementation diet for this element [35,36]. Experiments were performed in pairs. On each day, one animal from each group was killed and RLM were isolated and analysed.

Measurement of incorporation of selenium into liver by the inductively coupled plasma (ICP) method

Samples were prepared for analysis by microwave-assisted digestion using an MLS 1200 mega microwave digestion unit (Milestone). Batches of liver tissue (550–564 mg) were weighed into 120 ml Teflon PFA digestion vessels. A 5 ml volume of concentrated nitric acid was added to each vessel. Blanks were processed in advance for each vessel. The rotor was transferred to the microwave oven and the samples were exposed for 10 min to 500 W and for another 10 min to 580 W of microwave power. At the conclusion of the digestion period, the vessels were allowed to cool down to room temperature and were uncapped. Liquid residues were taken up in water, transferred into 25 ml calibrated flasks and brought up to volume with water.

Analyses were conducted on portions of these solutions, and compared with multi-element standards (Merck) in the same solvent. Selenium (Se) was determined by ICP/atomic emission spectrometry at 196.090 nm. An ICP/atomic emission spectrometry system, model Spectroflame Modula E (Spectro, Kleve, Germany), was used with a cross-flow nebulizer. The power level was 1.2 kW, coolant flow was 15 litres/min, auxiliary flow was 0.5 litre/min and nebulizer flow was 0.5 litre/min. The observation height was 10 mm above the coil.

Histology of liver sections

Liver sections from control rats and selenium-treated rats were fixed in 10 % (v/v) buffered formalin, embedded in paraffin, cut into slices of approx. 5 μm width and stained in routine haematoxylin and eosin.

Evaluation of GSH content: HPLC with electrochemical detection

Isolated mitochondria treated with m-phosphoric acid were frozen immediately in liquid nitrogen and stored at -80 °C, until HPLC analysis. Immediately before the assay, samples were thawed, vortexed and then centrifuged at 15000 ***g*** for 2 min. The clear supernatant was removed and injected into the HPLC system.

A C-18 reverse-phase column (15 cm $\times$ 4.6 mm; GL Science, Inc., Tokyo, Japan) and a coulometric detector (ESA; Coularray, Chelmsford, MA, U.S.A.) were used. The mobile phase, consisting of 98 % (v/v) 50 mM NaH_2PO_4 (pH 2.7) and 2 % (v/v) acetonitrile, was delivered using an isocratic solvent delivery module (ESA) set at a flow rate of 1 ml/min. The retention time for GSH was 2.7 min.

Statistics

Data were analysed by Student's t test for comparison of two groups, and by one-way ANOVA or two-way ANOVA for factorial experiments. Where necessary, data were log-transformed before analysis in order to stabilize variances. Differences were considered significant at probability levels of $P < 0.05$ using the Fisher's protected least significant difference method or Dunnett t test. SPSS 8 was used for all analyses.

RESULTS

Selenium induces high-amplitude mitochondrial swelling

Addition of selenium (sodium selenite) directly to RLM loaded with calcium induced the MPT, as reflected by exacerbated mitochondrial swelling. Selenium at 0.1, 1 or 10 μM dose-dependently induced significant mitochondrial swelling (Figure 1A). RLM energized with glutamate plus malate were more susceptible to calcium-induced swelling compared with mitochondria energized with succinate (in the presence of rotenone) (Figures 1B and 1C). Addition of selenium to mitochondrial preparations exacerbated swelling in both glutamate + malate- and succinate + rotenone-energized mitochondria. Since sodium selenite contains sodium, we checked whether sodium itself was causing the swelling, using 20 μM NaCl. NaCl did not facilitate mitochondrial swelling (results not shown). The selenium-induced swelling was CsA-sensitive: a concentration of 1 μM CsA prevented the swelling induced by 10 μM sodium selenite (Figure 1). ADP at 80 μM also prevented swelling (Figure 1C).

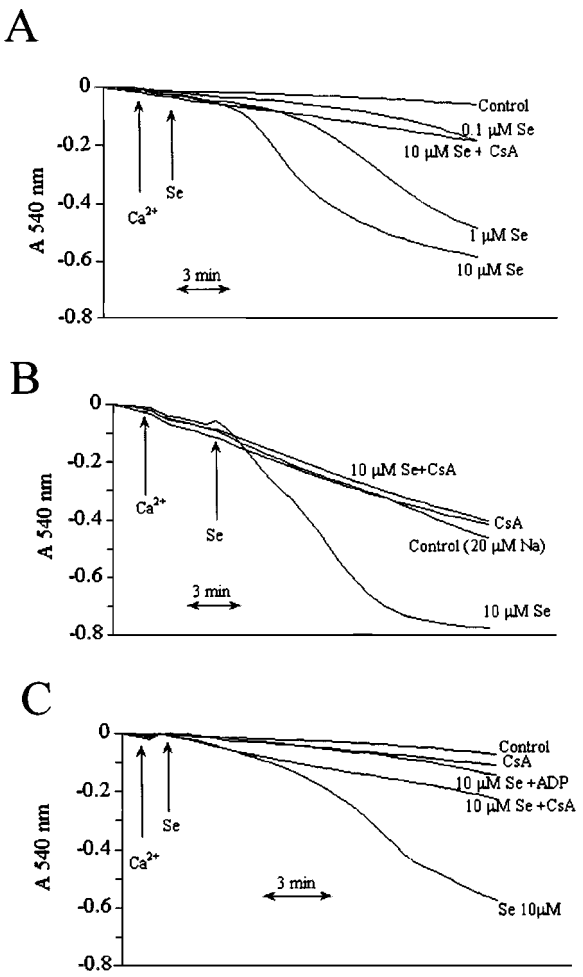

Figure 1 Effects of selenium on MPT pore opening

(**A**, **B**) Swelling effect monitored at A_{540} in mitochondria energized by glutamate plus malate and loaded with (**A**) 10 or (**B**) 60 nmol of calcium/mg of protein. In both (**A**) and (**B**), 0.1–10 μM sodium selenite was added where indicated, and 1 μM CsA was used to inhibit swelling induced by selenium. (**C**) Swelling effect monitored at A_{540} as in (**B**), except that mitochondria were energized with succinate as the respiration substrate in the presence of 2 μM rotenone. Mitochondria were loaded with 60 nmol of calcium/mg of protein. Selenium induced high-amplitude mitochondrial swelling. Pore opening and swelling were inhibited by adding 1 μM CsA or 80 μM ADP.

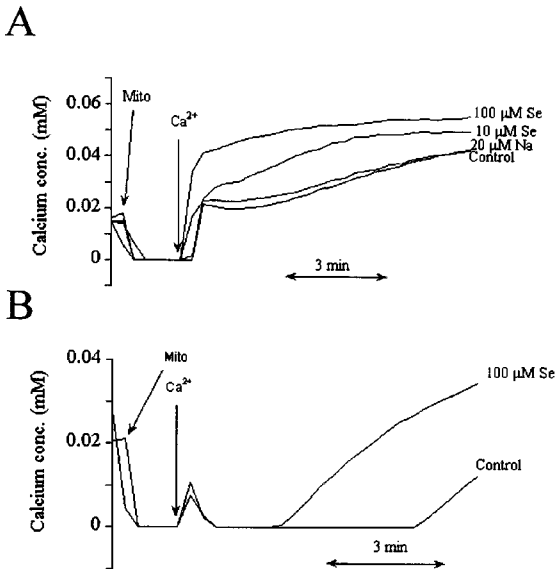

Figure 2 Effect of selenium on calcium efflux

(**A**) Glutamate + malate-energized mitochondria. The experiments were performed using an MPT-inducing treatment of 10 or 100 μM selenium; 60 nmol of calcium/mg of mitochondrial protein was added to the incubation medium before the mitochondria. When calcium was taken up by the mitochondria, a second, identical portion of calcium was added where indicated. A value of 0 indicates complete uptake of extra-mitochondrial calcium by the mitochondria. (**B**) Succinate-energized mitochondria in the presence of 2 μM rotenone.

Selenium induces mitochondrial calcium release

RLM loaded with calcium were exposed to selenium. Measurements of calcium-retention capacity showed that selenium induced the release of calcium from the mitochondria in a dose-dependent manner. Concentrations as low as 10 μM selenium were sufficient to accelerate the release of mitochondrial calcium, indicating MPT pore opening (Figure 2). Mitochondria energized with glutamate plus malate were more prone to respond to selenium treatment than were succinate-energized mitochondria (Figure 2). NaCl (20 μM, used as a control) did not facilitate mitochondrial calcium release (Figure 2A).

Selenium induces changes in mitochondrial respiration

An evaluation of oxygen consumption during succinate-dependent mitochondrial respiration showed that selenium allows rapid consumption of all dissolved oxygen (Figure 3A). Treating the mitochondria with calcium and 10 μM selenium facilitated rapid oxygen consumption in the absence of ADP. However, selenium inhibited oxygen consumption in glutamate + malate-energized mitochondria (Figure 3B). In the presence of ADP and ATP, the effect of selenium on mitochondrial respiration was minor (Figures 3B and 3C).

Effects of selenium on mitochondrial ROS production and GSH level

Oxidants can damage mitochondria and facilitate swelling [37]. To determine whether selenium elevates intra-mitochondrial ROS production, the oxidant-sensitive fluorescent probe H_2DCF-DA was loaded into the RLM. The results showed no change in peroxide production by energized mitochondria treated with selenium, measured as intra-mitochondrial DCF fluor-

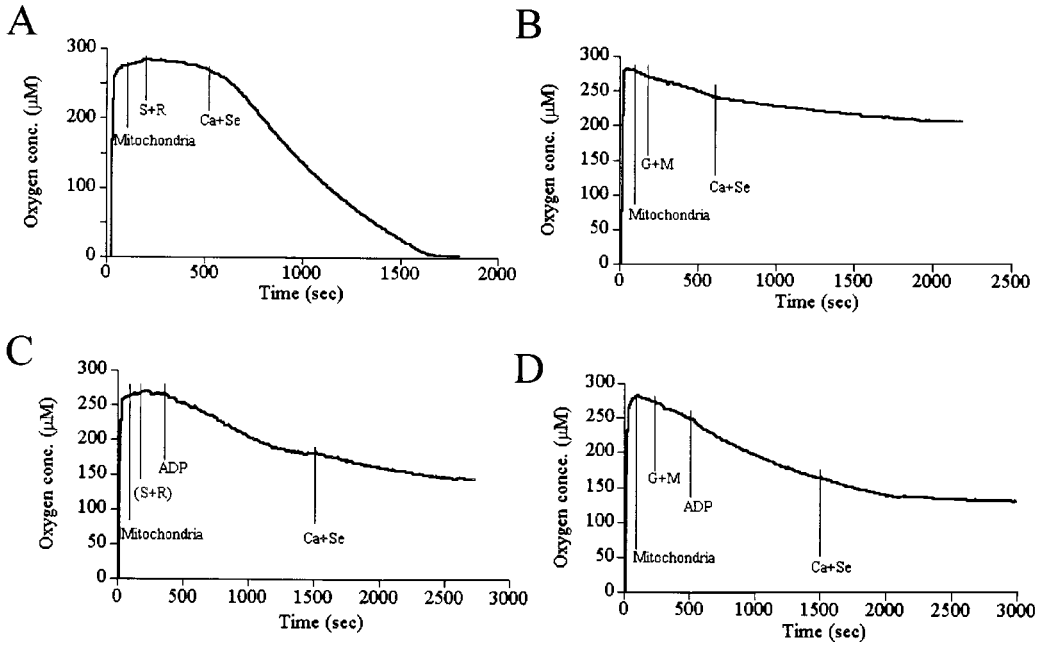

Figure 3 Mitochondrial respiration

(**A**) Mitochondrial respiration induced by 5 mM succinate (substrate) in the presence of 2 μM rotenone (S + R). Calcium (60 nmol/mg of protein) + 10 μM selenium induced accelerated mitochondrial respiration. (**B**) Mitochondrial respiration induced by 5 mM glutamate + 5 mM malate (G + M). Calcium (60 nmol/mg of protein) + 10 μM selenium inhibited mitochondrial respiration. (**C**) Mitochondrial respiration induced by 2 μM rotenone, 5 mM succinate (substrate) and 160 μM ADP. Calcium (60 nmol/mg of protein) + 10 μM selenium was used where indicated. (**D**) As in (**C**), but with glutamate + malate as respiration substrates.

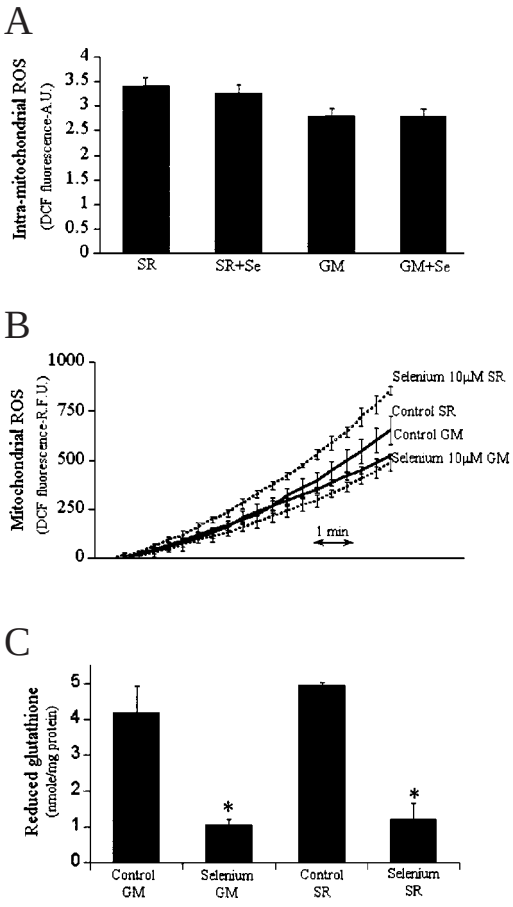

Figure 4 Effects of selenium on mitochondrial ROS (peroxide) production and GSH consumption

(**A**) RLM (0.5 mg of protein/ml) were incubated with 60 nmol of Ca^{2+}/mg of protein; 10 μM selenium was added as the MPT-inducing treatment, and the production of ROS was compared with that in control RLM following loading with 25 μM H_2DCF-DA for 10 min. The intra-mitochondrial ROS level was analysed by flow cytometry. Fluorescence emission was monitored on the FL1 channel and data were collected from at least 50000 mitochondria. Measurements were made in mitochondria energized with glutamate + malate (GM) or succinate in the presence of rotenone (SR). (**B**) Effect of selenium on mitochondrial ROS (peroxide) production as in (**A**), measured kinetically for 10 min using a fluorescence microplate reader. (**C**) Effect of selenium on mitochondrial GSH consumption. Mitochondria (0.5 mg of protein/ml) were incubated with 60 nmol of Ca^{2+}/mg of protein, 10 μM selenium and substrate for respiration. GSH was measured after 10 min using the HPLC method. *Significantly different from relevant control at $P < 0.05$.

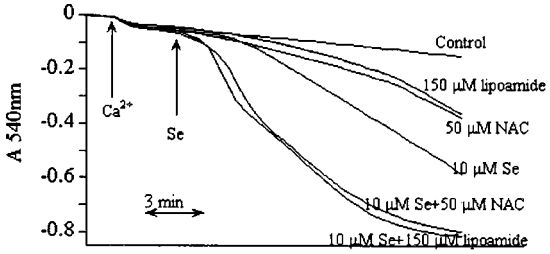

Figure 5 Effects of selenium and thiols on MPT pore opening

The swelling effect was monitored at A_{540} in succinate-energized mitochondria in the presence of rotenone: 10 μM sodium selenite, 150 μM lipoamide and/or 50 μM NAC were used to induce swelling. Adding thiols with selenium caused intensified high-amplitude mitochondrial swelling.

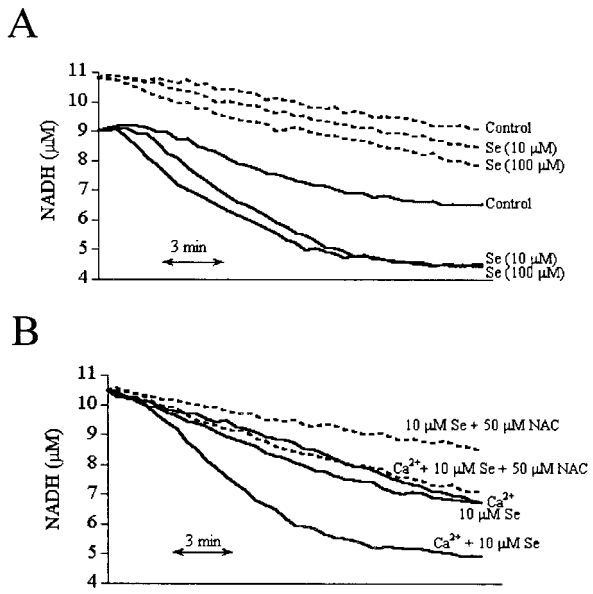

Figure 6 Effects of selenium, calcium and thiols on mitochondrial pyridine nucleotide oxidation

(**A**) Dose-dependent effect of selenium. The incubation medium contained MSH buffer, pH 7.4, with 5 mM glutamate plus 5 mM malate (solid lines) or with 5 mM succinate in the presence of 2 μM rotenone (broken lines); 0.5 mg of protein/ml of mitochondria and 60 nmol of Ca^{2+}/mg of protein were used. NAD(P)H oxidation was measured by the decrease in auto-fluorescence. (**B**) Effects of calcium and thiols. Mitochondria were energized with glutamate plus malate. The decrease in the autofluorescence of NAD(P)H was measured as an indication of NAD(P)H oxidation. Solid lines, effects of selenium with and without calcium; broken lines, effects of combined treatment of selenium + NAC with and without calcium.

escence after a 10 min incubation with H_2DCF-DA (Figure 4A). Measurements of oxidant production also showed only a minor effect of selenium treatment on ROS production in succinate + rotenone-energized mitochondria, and no effect with glutamate + malate as substrates (Figure. 4B).

Selenium treatment induced rapid glutathione consumption in isolated RLM. Treatment for 10 min resulted in $\sim 80\,\%$ loss of total glutathione (Figure 4C). Such utilization and consumption of the most abundant endogenous thiol, glutathione, was not accompanied by a burst of generated ROS (Figures 4A and 4B).

Synergism between selenium and thiols

To investigate further the mechanism of action by which selenium exerts its influence, thiols were added to calcium-loaded mitochondria. We assumed that thiol antioxidants would potentiate the effect. Indeed, incubating RLM with selenium and a thiol-bearing compound, i.e. NAC or lipoamide, resulted in significantly augmented high-amplitude mitochondrial swelling compared with RLM treated with selenium alone (Figure 5). NAC was more potent than lipoamide (cyclic disulphide), which needed to be reduced initially by the pyruvate dehydrogenase complex.

Selenium induces oxidation of mitochondrial pyridine nucleotides

Selenium increased mitochondrial NAD(P)H oxidation dose-dependently. The effect of selenium was significant only during

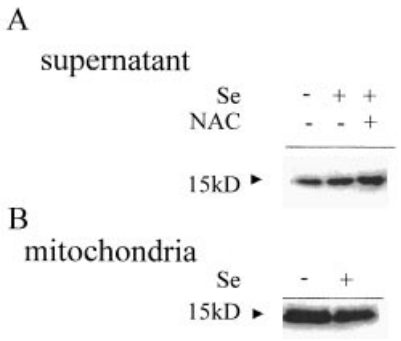

Figure 7 Cytochrome *c* release induced by selenium and thiols

(**A**) Presence of cytochrome *c* in the incubation medium of mitochondria energized with 5 mM glutamate plus 5 mM malate and treated or not with 10 μM selenium and/or 50 μM NAC. (**B**) Presence of cytochrome *c* in mitochondria energized with 5 mM glutamate plus 5 mM malate following treatment with 10 μM selenium.

the calcium-induced PT occurring in mitochondria respiring on complex I substrates (glutamate and malate) (Figures 6A and 6B). The accelerated consumption of reduced pyridine nucleotides by selenium was facilitated only in the presence of calcium (Figure 6B).

The consumption of reduced pyridine nucleotides was attenuated in the presence of thiol-bearing compounds compared with mitochondria treated with selenium alone (Figure 6B). In addition, the calcium effect observed for selenium was also observed for combined selenium and NAC treatment. Indeed, exacerbated NADH oxidation was seen only in the presence of calcium (Figure 6B).

NADH consumption was recorded in RLM diluted in MSH buffer to a concentration of 0.5 mg of protein/ml. Changes in the micromolar range (Figure 6) were recorded. Because of the small volume occupied by the suspended mitochondria, actual matrix changes in NADH levels are much higher.

Selenium induces cytochrome *c* release

There is a known connection between selenium and apoptosis, and the release of cytochrome *c* from the mitochondria may start or potentiate a cascade of events leading to apoptosis. Western blot analysis demonstrated that 10 μM selenium induced the release of cytochrome *c* from RLM energized with glutamate + malate (Figure 7A). Mitochondria treated with NAC plus selenium released more cytochrome *c* than those treated with selenium alone (Figure 7A). The amount of cytochrome *c* remaining in mitochondria treated with selenium was lower than that in controls (Figure 7B).

Selenium-supplemented animals

Selenium levels were measured in livers from control and selenium-supplemented rats. The concentration in livers from control animals was 2.82 ± 0.16 mg of Se/kg of tissue, and in those from selenium-supplemented animals it was 4.90 ± 0.09 mg of Se/kg of tissue (1.75 times higher). Since a further increase in tissue selenium following supplementation could have resulted in a toxic effect, which we sought to avoid, the high-selenium diet was terminated after 3 weeks, when only a minimal elevation in tissue selenium incorporation was observed. Histological analysis of liver sections showed no abnormal findings. Sections of livers from control and Se-supplemented rats presented virtually identical histological findings.

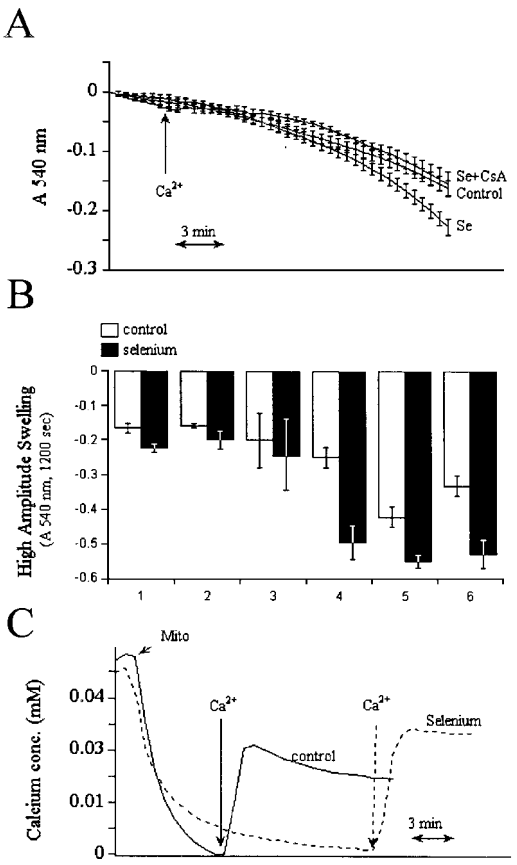

Figure 8 Effects of *in vivo* supplementation with selenium

RLM from animals supplemented with excess dietary selenium were compared with RLM from control, non-supplemented animals. The figure shows a typical experiment using succinate + rotenone-energized mitochondria (for more details, see the Experimental section). (**A**) Swelling monitored at A_{540}: exacerbated high-amplitude swelling occurred in the mitochondria isolated from the selenium-supplemented group which was significantly lower than control ($P < 0.05$). (**B**) Swelling effect monitored at A_{540}, comparing pairs of control (open bars) and selenium-supplemented (solid bars) animals. Student's paired *t* test was used to show significant differences ($P < 0.05$) between the mean high-amplitude swelling for RLM from the selenium-supplemented in comparison with the control animals. (**C**) Calcium retention capacity. RLM were obtained from control (solid line) and selenium-supplemented (broken line) animals. Calcium (60 nmol/mg of mitochondrial protein) was added to the incubation medium before the mitochondria were added. When calcium was taken up by the mitochondria, a second identical portion of calcium was added where indicated.

RLM prepared from selenium-supplemented animals were more susceptible to calcium-induced mitochondrial swelling than mitochondria isolated from non-supplemented animals. Treating the mitochondria (0.5 mg of protein) with 30 μM calcium caused exacerbated high-amplitude swelling in the selenium-supplemented group (Figure 8A). CsA prevented calcium-dependent mitochondrial swelling in RLM isolated from selenium-supplemented animals (Figure 8A). Comparing pairs of control and treated animals showed increased high-amplitude mitochondrial swelling following selenium supplementation which was statistically significant ($P < 0.05$; Figure 8B).

Measurements of mitochondrial calcium uptake and retention capacity showed a higher loading time for RLM isolated from selenium-supplemented compared with non-supplemented animals. A second dose of calcium in selenium-supplemented animals resulted in vigorous MPT pore opening and accelerated release of almost all of the retained calcium. This

effect was less apparent in calcium-loaded RLM isolated from non-supplemented animals (Figure 8C).

Selenium treatment did not induce glutathione consumption in the livers of selenium-supplemented animals. GSH levels in livers from control and selenium-supplemented animals were 20.5 ± 2.4 and 20.4 ± 5 nmol/mg of protein respectively. Therefore reduction of a minute amount of selenium by the large liver GSH and thiol pools facilitated the mitochondrial effect with no significant oxidation of thiols in the treated animals that could indicate oxidative stress.

DISCUSSION

Selenium-dependent glutathione peroxidases and other selenoproteins provide the molecular basis for selenium's antioxidant activity [4,38,39]. However, higher levels of selenium supplementation can be expected to affect other functions related to tumorigenesis, carcinogen metabolism, immune function, cell-cycle regulation and apoptosis.

Selenium and mitochondrial apoptosis

We demonstrate a mitochondrial pathway by which selenium may promote apoptosis. The MPT is one of the ways in which mitochondria can facilitate cell-death processes [16,18,20]. Selenium induced swelling and the release of calcium from intact mitochondria at low (nmol/l) physiological levels. CsA strongly inhibited selenium-dependent mitochondrial swelling, suggesting opening of the PT mega-pore as the reason for the effect of selenium. ADP, which is known to retard the progression of the PT, also blocked the effect of selenium. Induction of PT pore opening by a low concentration of selenium makes this element one of the most potent inducers of the MPT. Selenium treatment induced the release of cytochrome *c*, a pro-apoptotic factor, into the supernatant. This effect was exacerbated by thiols.

Selenium and the MPT

Selenium's biochemistry involves strong interactions with thiols. The reaction of selenite with GSH leads to the formation of ROS [12]. Such oxidizing activity may regulate the opening of the PT pore. However, monitoring of ROS production in the mitochondrial matrix showed no elevation of mitochondrially derived DCF-sensitive ROS. Hydrogen peroxide produced by selenite–thiol interactions could have been scavenged by glutathione peroxidase activity, since residual GSH can still serve as a substrate for the enzyme. Moreover, treatment with two potent thiol-bearing antioxidants not only did not prevent the effect of selenium, it accelerated it. Therefore the reducing power of mitochondrial thiols may have facilitated rapid reduction of sodium selenite, leading to selenium-dependent PT pore opening, swelling and cytochrome *c* release. Indeed, treatment of isolated mitochondria resulted in the rapid consumption of endogenous glutathione, indicating that selenium can utilize the reducing power of mitochondrial thiols when supplemented in the form of selenite. Moreover, selenium did not induce oxidative stress in the livers of supplemented animals, as reflected by the lack of change in liver GSH content (because the elevation of selenium was mild and not toxic) and the absence of histological pathology.

Exposure of intact, state 4 succinate-respiring mitochondria to selenium accelerated oxygen consumption, indicating an uncoupling-like effect. This effect of selenium could be due to a selenium-induced PT that resulted in the removal of coupling respiration inhibition and thereby increased electron-transfer chain activity. In state 4 mitochondria energized with glutamate + malate, selenium prevented the reduction of oxygen by the mitochondria, possibly due to a shift in the electron flow from oxygen to selenium. In support of this, selenium indeed promoted pyridine nucleotide oxidation in mitochondria. However, in the presence of ADP/ATP, selenium had only a minor effect, probably due to MPT inhibition and calcium chelation. The novel aspect emerging from this series of experiments is that the selenium-induced PT led to a loss of pyridine nucleotides, and not vice versa, since selenium did not facilitate pyridine nucleotide oxidation in the absence of calcium. Consumption of mitochondrial NADH was not substantial when complex I of the electron-transfer chain was blocked with rotenone and the mitochondria were respiring on succinate. Therefore selenium did not oxidize NADH directly, but rather accelerated NADH dehydrogenase-dependent NADH utilization.

In vivo sensitization of the MPT

Selenium supplementation of rats for 20 days resulted in RLM that were more prone to undergoing the PT. Parameters such as membrane potential, mitochondrial content of cytochrome *c* and respiration control were not affected (results not shown). Therefore the results suggest that mitochondria isolated from selenium-supplemented animals will still be as efficient at ATP production as those from controls, but will have an improved capacity to execute programmed cell death via the MPT when needed. In this way, selenium may exert its biological preventative effect by not allowing malignant transformation to occur. Exposure of cells to stress (oxidative or other) will result in an elevation of intracellular and mitochondrial calcium levels, allowing selenium to activate apoptosis in such damaged cells and to eliminate them.

Conclusion

Selenium facilitates the MPT, and this effect of selenium is regulated by its reduction by thiols. Following this reduction, selenium may bridge critical thiols in the PT pore and activate pore opening, as has been reported for various thiol-cross-linking agents [37]. Further reduction of selenium is then possible via complex I of the mitochondria, leading to NADH utilization. This activity may promote the mitochondrial pathway of cellular apoptosis. An improved capacity to execute cellular apoptosis when required may be a central mechanism by which supplementation with selenium prevents various diseases.

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