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Induction of the mitochondrial permeability transition by selenium compounds mediated by oxidation of the protein thiol groups and generation of the superoxide

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Abstract

The cancer chemopreventive effect of selenium compounds cannot be fully explained by the role of selenium as a component of antioxidant enzymes, suggesting that other mechanisms, such as thiol oxidation or free radical generation, also underlie this effect. The toxicities of six different selenium compounds (selenite, selenate, selenocystine, selenocystamine, selenodioxide, and selenomethionine) have now been compared in HepG2 human hepatoma cells and isolated rat liver mitochondria. Selenite, selenocystine, and selenodioxide induced apoptosis in HepG2 cells and mediated oxidation of protein thiol groups in both HepG2 cells and isolated mitochondria. Selenocystamine oxidized protein thiol groups in isolated mitochondria and crude extracts of HepG2 cells but not in intact HepG2 cells, suggesting that this compound is not able to cross the cell membrane. The selenium compounds capable of oxidizing thiol groups also induced the mitochondrial permeability transition (MPT) in isolated mitochondria. Furthermore, they generated the superoxide ($O_2^{\bullet -}$) on reaction with glutathione in the presence of mitochondria, and an $O_2^{\bullet -}$ scavenger inhibited their induction of the MPT. These results suggest that the pro-apoptotic action of selenium compounds is mediated by both thiol oxidation and the generation of $O_2^{\bullet -}$, both of which contribute to opening of the MPT pore.

Keywords: Selenium compounds; Apoptosis; Superoxide anion; Mitochondria; Mitochondria permeability transition

1. Introduction

Mitochondria play an important role in the regulation of apoptosis [1–5]. The intermembrane space of these organelles contains several pro-apoptotic proteins, including cytochrome *c*, procaspases 2, 3, and 9, and apoptosis-inducing factor, all of which are released into the cytosol as a result either of disruption of the outer mitochondrial membrane or of the opening of specific pores [6–8]. The opening of the MPT pore induced by apoptotic stimuli is thus thought to result in swelling of the mitochondrial matrix and consequent rupture of the outer membrane and release of pro-apoptotic proteins. The opening of the MPT pore is regulated by Ca^{2+}, thiol oxidants, ROS, and members of the Bcl-2 family of proteins [9–15].

The production of ROS by mitochondria contributes to a variety of conditions associated with cell death, including ischemia, aging, neurodegeneration, and cancer [3,16–18]. During mitochondrial respiration, electrons are released from their normal transport pathway to molecular oxygen at complexes I and III [19], resulting in formation of the superoxide ($O_2^{\bullet -}$). Mitochondria thus represent the main source of ROS in mammalian cells and accumulate oxidative damage more rapidly than do other cellular components [20]. Although the superoxide is itself damaging to cellular components, it also generates additional reactive oxidants both by dismutation to hydrogen peroxide, which is reduced, usually by a redox active metal ion, such as ferrous ion, to hydroxy radical and hydroxide. Oxidative

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Abbreviations: MPT, mitochondrial permeability transition; ROS, reactive oxygen species; $\Delta\Psi_m$, mitochondrial membrane potential; DTT, dithiothreitol; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); PAGE, polyacrylamide gel electrophoresis; NBT, nitroblue tetrazolium; Mn-TBAP, manganese(III) tetrakis(4-benzoic acid)porphyrin; TTFA, 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione.

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damage of mitochondria increases with aging, contributing to an age-related decrease in the efficiency of oxidative phosphorylation.

Selenium is an essential dietary nutrient for all mammalian species. Selenium compounds are also toxic both in intact animals and in cultured cells [21–24]. The toxicity of selenium is thought to be due to its ability to catalyze the oxidation of thiols and simultaneously to generate $O_2^{\bullet -}$ [25]. Selenium also exhibits a chemopreventive activity with regard to cancer [26,27], although the mechanism of this activity remains unclear.

We previously showed that selenite induces the MPT by promoting the oxidation of protein thiol groups [28]. To examine the possible role of $O_2^{\bullet -}$ generated by selenite in induction of the MPT, we have now compared the effects of various inorganic and organic selenium compounds on the opening of the MPT pore, the associated decrease in the $\Delta\Psi_m$, the release of cytochrome *c* into the cytosol, and the induction of apoptotic cell death with their abilities to oxidize the protein thiol groups and generate $O_2^{\bullet -}$ by reaction with glutathione (GSH).

2. Materials and methods

2.1. Reagents

Selenite, selenate, L-selenocystine [(*R*,*R*)-3,3′-diselenobis-(2-aminopropionic acid)], L-selenomethionine [(*S*)-(+)-2-amino-4-(methylseleno)butanoic acid], selenocystamine, and selenium dioxide were obtained from the Sigma Chemical Co. Antibodies to cytochrome *c* and IgG-HRP were obtained from PharMingen.

2.2. Cell culture

Human hepatoma HepG2 cells were maintained under a humidified atmosphere of 5% CO_2 at 37° in Dulbecco's modified Eagle's medium buffered with $NaHCO_3$ and supplemented with 10% fetal bovine serum (Gibco-BRL), penicillin (1000 units/mL), and streptomycin (1000 μg/mL).

2.3. Flow cytometric analysis of cellular DNA content

HepG2 cells were incubated with selenium compounds (10 μM) for various times and then stained with propidium iodide for flow cytometric analysis of DNA content. At least 20,000 events were analyzed with excitation set at 488 nm and emission monitored at 610 nm [29].

2.4. Assay of DNA fragmentation

Cells were suspended in a lysis buffer consisting of 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 12 mM β-glycerophosphate, 10 mM NaF, 5 mM EGTA, 1 mM Na_3VO_3, 3 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, aprotinin (5 μg/mL), leupeptin (5 μg/mL), and 0.2% SDS. After incubation for 1 hr at room temperature, the cell lysate was subjected to extraction with phenol–chloroform (1:1 v/v). The aqueous phase was mixed with ice-cold 70% ethanol containing 0.3 M sodium acetate (pH 5.2), and the resulting DNA precipitate was stored overnight at −20°. The DNA samples (25 μg per lane) were fractionated by electrophoresis through a 1.2% agarose gel, which was then stained with ethidium bromide and examined under ultraviolet illumination [30].

2.5. Isolation of mitochondria and monitoring of the MPT and $\Delta\Psi_m$

Mitochondria were isolated from rat liver and the MPT was monitored by measuring the decrease in optical density at 540 nm as described previously [31]. The $\Delta\Psi_m$ of HepG2 cells or isolated rat liver mitochondria was measured as described after incubation with 5 μM rhodamine 123 for 30 min; at least 20,000 events were analyzed by flow cytometry with excitation set at 488 nm and emission monitored at 530 nm [32].

2.6. Western blot for cytochrome c

For determination of cytochrome *c*, cytosolic extracts were obtained as described [33]. Cytosolic fractions (20 μg) prepared from cells treated with various selenium compounds for 24 hr were separated by electrophoresis on a 12% SDS–PAGE. The separated proteins were blotted onto a nitrocellulose membrane, and detection of cytochrome *c* was carried out using horseradish peroxidase-conjugated anti-mouse IgG (1/200 dilution, Pharmingen) and the ECL reagent (Amersham).

2.7. Detection of free thiol groups in proteins

The proportion of free thiol groups in the membrane or matrix fractions of mitochondria and in HepG2 cells or cell lysates was assessed by exposure to DTNB as described previously [34]. Absorption was measured at 415 nm, with GSH used as a standard for calibration.

2.8. Analysis of the formation of mitochondrial protein aggregates

Mitochondria (1 mg/mL) were incubated for 1 hr at room temperature with selenium compounds (1 mM) or with 1 mM diazenedicarbolic acid, bis-(*N*,*N*-dimethylamide), or diamide, in a solution (final volume: 1 mL) containing 10 mM Tris–HCl (pH 7.4), 0.25 M sucrose, and 0.5 μM rotenone. The mitochondria were then harvested by centrifugation at 10,000 *g* for 10 min at 4°. The organelles were solubilized by the addition of 100 μL of

SDS sample buffer (2% SDS, 50 mM Tris–HCl (pH 6.8), 0.1% bromophenol blue, 10% glycerol) in the presence or absence of DTT followed by incubation for 5 min at 80°. The samples (10 μL) were fractionated by SDS–PAGE on a 10% gel, after which the gel was stained with Coomassie brilliant blue [35].

2.9. Measurement of superoxide generation

Mitochondria (1 mg/mL) were incubated for 1 hr at room temperature with various concentrations of selenium compounds in a solution (final volume: 200 μL) containing 200 μM NBT, 0.25 M sucrose, 10 mM Tris–HCl (pH 7.4), and 5 mM succinate, with the reaction being initiated by the addition of the latter. The rate of NBT reduction was measured spectrophotometrically at 595 nm [36,37].

3. Results

3.1. Apoptosis of HepG2 cells induced by selenium compounds

We first examined the toxicity of various inorganic (selenite, selenate, selenodioxide) and organic (selenocystine, selenocystamine, selenomethionine) selenium compounds by determining whether they induce apoptosis in HepG2 cells (Fig. 1). Flow cytometry of HepG2 cells stained with propidium iodide revealed that exposure of

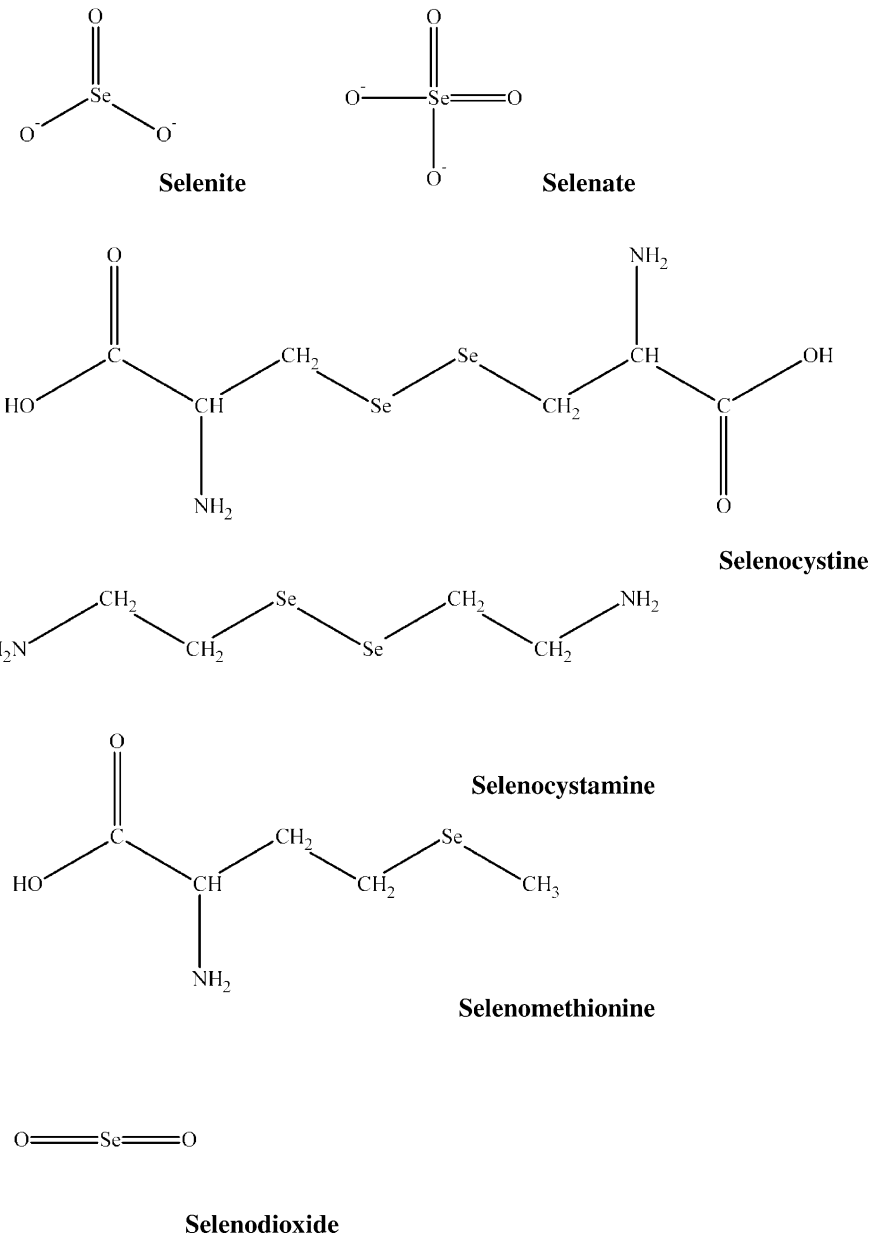

Fig. 1. Structures of selenium compounds.

the cells to 10 μM selenite, selenocystine, or selenodioxide induced a marked time-dependent increase in the proportion of apoptotic cells (as reflected by the subdiploid peak), whereas selenate, selenocystamine, and selenomethionine did not induce apoptosis (Fig. 2A and B). Analysis of the integrity of cellular DNA by agarose gel electrophoresis also demonstrated that incubation of HepG2 cells with the apoptosis-inducing selenium compounds (10 μM) for 24 hr induced internucleosomal DNA fragmentation (Fig. 2C).

3.2. Cytochrome c release and loss of $\Delta\Psi_m$ induced by pro-apoptotic selenium compounds

We next examined the effects of the six selenium compounds on mitochondrial events associated with apoptosis. These events are initiated by the MPT, which is accompanied by collapse of $\Delta\Psi_m$, impairment of respiration, and inhibition of ATP synthesis and results in the release of cytochrome *c* into the cytosol and cell death [1,5,7,9]. We incubated HepG2 cells for various times with selenium compounds (10 μM) and monitored $\Delta\Psi_m$ by flow cytometric measurement of the fluorescence emitted by rhodamine 123, a cationic lipophilic dye. Selenite, selenocystine, and selenodioxide (but not selenate, selenocystamine, or selenomethionine) each induced a time-dependent reduction in $\Delta\Psi_m$, with this effect being first apparent after treatment for 3–6 hr (Fig. 3A). Immunoblot analysis of the cytosolic fraction of HepG2 cells that had been incubated in the presence of selenium compounds (10 μM) for 24 hr also revealed that selenite, selenocystine, and selenodioxide each induced the release of cytochrome *c* into the cytosol (Fig. 3B). These results thus indicated that apoptosis induced by selenium compounds are mediated by mitochondria.

We also performed the same experiments with mitochondria isolated from rat liver. The mitochondria were preincubated with 5 mM succinate for 5 min, and were then incubated with the various selenium compounds (1 mM) for 30 min or 1 hr. The selenium compounds that induced apoptosis in HepG2 cells also induced a loss of $\Delta\Psi_m$ (Fig. 3C) and cytochrome *c* release (Fig. 3D) in isolated mitochondria. In addition, however, despite its inactivity with HepG2 cells, selenocystamine also induced these effects in isolated mitochondria.

3.3. Thiol oxidation induced by pro-apoptotic selenium compounds

The opening of the MPT pore is induced by an increase in the cytosolic Ca^{2+} concentration, thiol oxidants, and ROS [7,11,31,38]. To investigate the possibility that selenium compounds might induce the MPT by acting as thiol oxidants, we determined the effects of these agents on the proportion of free thiol groups in HepG2 cells and in isolated liver mitochondria. In previous study, we showed

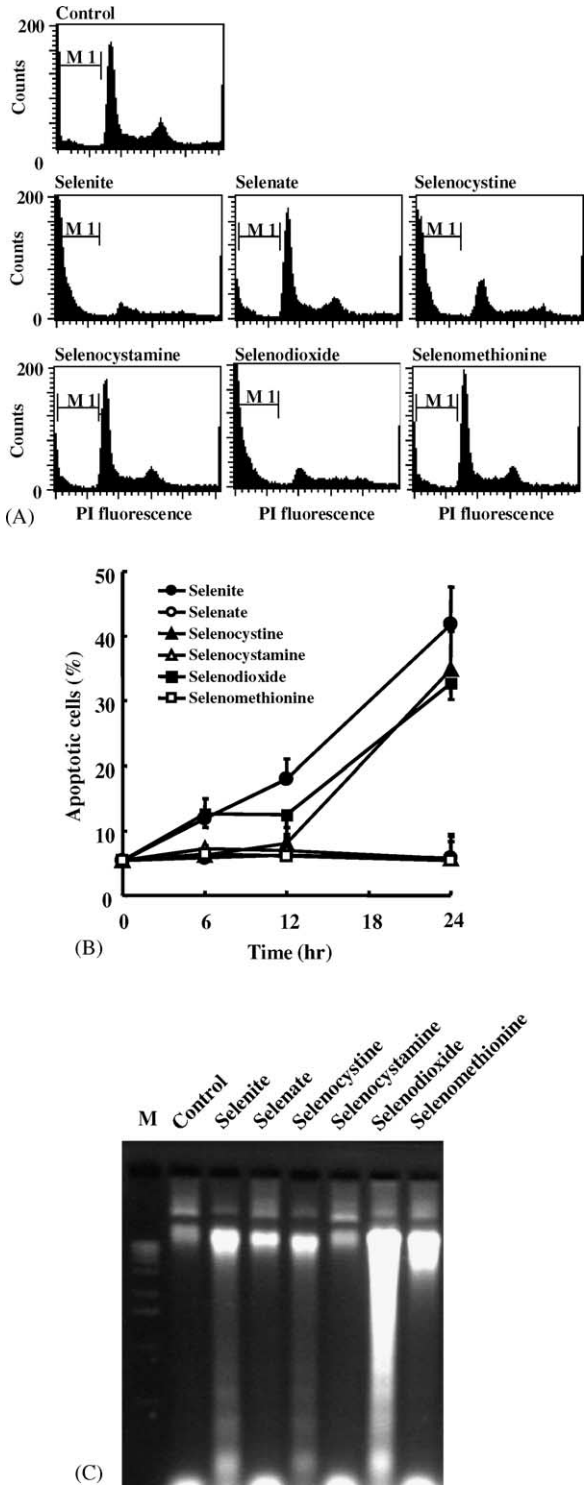

Fig. 2. Induction of apoptosis by selenium compounds in HepG2 cells. (A, B) Cells were incubated for 24 hr (A) or for the indicated times (B) at 37° in the absence (control) or presence of the indicated selenium compounds (10 μM). They were then stained with propidium iodide (PI) and analyzed by flow cytometry. Data in (B) represent the number of apoptotic cells (subdiploid cells, as indicated by the peak labeled M1 in (A)) expressed as a percentage of total cells and are means ± SE of values from three independent experiments. (C) Cells were incubated for 24 hr in the absence or presence of selenium compounds (10 μM), after which cellular DNA was analyzed by agarose gel electrophoresis and ethidium bromide staining. Lane M: molecular size standards.

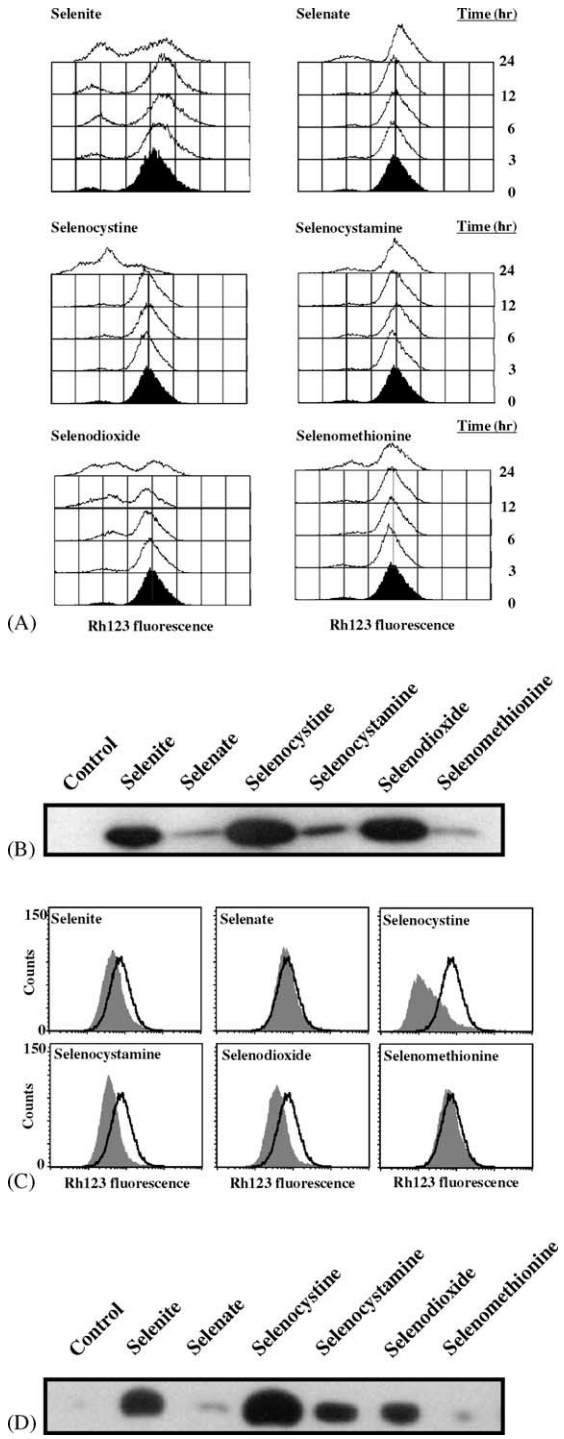

Fig. 3. Cytochrome *c* release and loss of $\Delta\Psi_m$ induced by selenium compounds in HepG2 cells and in isolated mitochondria. (A) HepG2 cells were incubated for various times at 37° in the presence of the indicated selenium compounds (10 μM). They were then stained with rhodamine 123 (Rh123) for 30 min and analyzed by flow cytometry for measurement of $\Delta\Psi_m$. (B) Cells were incubated for 24 hr in the absence (control) or presence of selenium compounds (10 μM), after which the cytosolic fraction was prepared and subjected to immunoblot analysis with antibodies to cytochrome *c*. (C) Isolated rat liver mitochondria (1 mg/mL) were suspended in a solution containing 0.25 M sucrose and 10 mM Tris–HCl (pH 7.4), preincubated for 5 min with 5 mM succinate, and incubated with selenium compounds (1 mM) for 30 min at room temperature in the presence of Rh123. They were then analyzed by flow cytometry for measurement of $\Delta\Psi_m$. (D) Mitochondria were treated as in (C) with the exception that the incubation with selenium compounds was performed for 1 hr in the absence of Rh123. They were then separated by centrifugation, and the resulting supernatant was subjected to immunoblot analysis with antibodies to cytochrome *c*.

that MPT is induced by various concentration of selenite (10–1000 μM), which lead to oxidation of protein thiol group, decrease in $\Delta\Psi_m$, and release of cytochrome *c* [28]. In this study, to observe evident effects of selenium compounds on mitochondria, we used high concentrations (10–1000 μM) of selenium compound.

Isolated mitochondria were incubated with selenium compounds (10 μM) for 1 hr, after which membrane and matrix fractions were prepared and assayed for free thiol groups with DTNB. Selenite, selenocystine, selenocystamine, and selenodioxide (but not selenate or selenomethionine) each induced a depletion of protein free thiol groups that was apparent in both mitochondrial fractions (Fig. 4A and B). Incubation of HepG2 cells with selenium compounds (10 μM) for 24 hr revealed that selenite, selenocystine, and selenodioxide each induced oxidation of protein free thiol groups (Fig. 4C); selenate, selenomethionine, and, in contrast to its action in isolated mitochondria, selenocystamine did not affect the abundance of free thiols in the cultured cells. These observations thus suggested that selenocystamine has the potential to induce mitochondria-mediated apoptosis but that it is prevented from doing so by its inability to penetrate the cell membrane. This conclusion was supported by the observation that selenocystamine, like selenite, selenocystine, and selenodioxide, induced thiol oxidation on incubation with crude extracts of HepG2 cells (Fig. 4D). We further examined whether selenium compounds acted as thiol cross-linkers of mitochondrial proteins by SDS–PAGE analysis. Like the thiol cross-linker diamide used as a positive control, the selenium compounds that induced thiol oxidation (selenite, selenocystine, selenocystamine, selenodioxide) also induced the formation of protein aggregates of high molecular mass (Fig. 4E). These results thus suggested that the ability of selenium compounds to mediate thiol oxidation and cross-linking might contribute to their induction of the MPT and cell death.

3.4. Induction of mitochondrial swelling by selenium compounds

During the MPT, the entry of water and solutes into mitochondria results in swelling of the matrix, rupture of the outer membrane, and the release of pro-apoptotic proteins from the intermembrane space [1,11]. We next examined whether the various selenium compounds (100 μM) induce the large-amplitude mitochondrial swelling attributable to the MPT by measuring the associated decrease in optical density at 540 nm. Isolated rat liver mitochondria were preincubated with 5 mM succinate for 5 min and then exposed to selenium compounds (Fig. 5A).

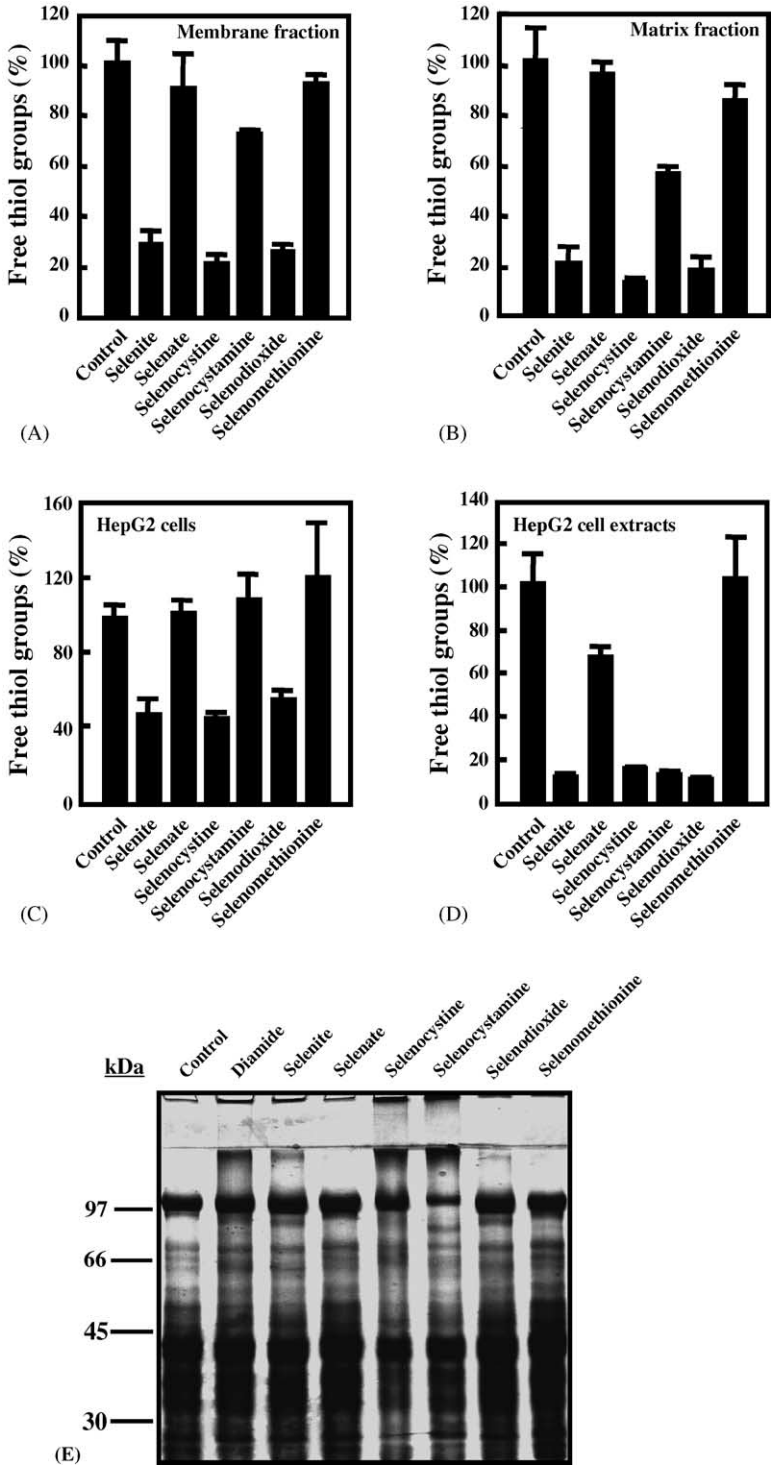

Fig. 4. Protein thiol oxidation induced by selenium compounds in isolated mitochondria and HepG2 cells. (A, B) Rat liver mitochondria (1 mg/mL) were suspended in a solution containing 0.25 M sucrose, 10 mM Tris–HCl (pH 7.4), and 0.5 μM rotenone and were incubated for 1 hr at room temperature in the absence (control) or presence of selenium compounds (10 μM). Membrane (A) and matrix (B) fractions were then prepared and assayed for their content of free thiol groups with the use of DTNB. (C) HepG2 cells were incubated with selenium compounds (10 μM) for 24 hr and then assayed for their content of free thiol groups. (D) Crude extracts of HepG2 cells (1 mg/mL) were incubated with selenium compounds (1 mM) for 1 hr at room temperature, after which their content of free thiol groups was determined. Data in (A)–(D) are expressed as a percentage of the total number of thiol groups and are means ± SE of values from three to five independent experiments. (E) Rat liver mitochondria were treated as in (A) and (B), with the exception that one incubation was performed with 1 mM diamide in the place of a selenium compound. They were then harvested by centrifugation, solubilized in SDS sample buffer in the absence of DTT, and subjected to SDS–PAGE. The positions of molecular size standards are shown (in kDa) on the left.

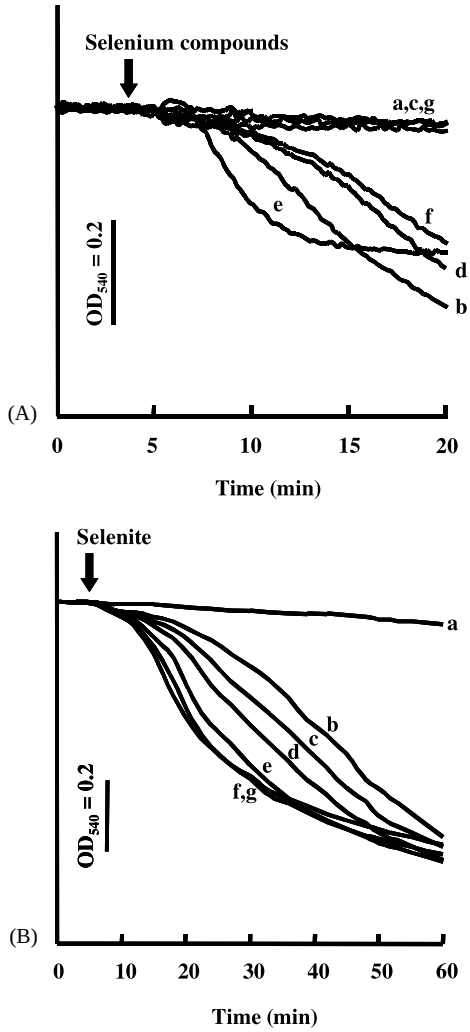

Fig. 5. Induction of MPT pore opening in isolated mitochondria by selenium compounds. (A) Rat liver mitochondria (1 mg/mL) were suspended in a solution containing 0.25 M sucrose and 10 mM Tris–HCl (pH 7.4), and were preincubated for 5 min at room temperature with 5 mM succinate. They were then incubated in the absence (trace a) or presence of selenite (trace b), selenate (trace c), selenocystine (trace d), selenocystamine (trace e), selenodioxide (trace f), or selenomethionine (trace g), each at a concentration of 100 μM, the addition of which is indicated by the arrow. (B) Mitochondria were suspended and preincubated as in (A), after which selenite at concentrations of 0, 10, 50, 100, 200, 500, or 1000 μM (traces a–g, respectively) was added as indicated by the arrow. In both (A) and (B), large-amplitude mitochondrial swelling was monitored by measurement of the decrease in optical density at 540 nm (OD_{540}).

Mitochondrial swelling was induced by selenite, selenocystine, selenocystamine, and selenodioxide, the same four compounds that induced thiol cross-linking of mitochondrial proteins. The mitochondrial swelling induced by these selenium compounds was completely inhibited by 5 μM cyclosporin A (an MPT pore blocker), 10 μM EGTA (a Ca^{2+} chelator), or 100 μM *N*-ethylmaleimide (a monofunctional thiol oxidant) (data not shown). The rate of mitochondrial swelling induced by selenite increased in a concentration-dependent manner from 10 μM to 1 mM (Fig. 5B).

3.5. Superoxide generation by reaction of selenium compounds with GSH

The MPT has been shown to be induced by thiol oxidants (diamide, alloxan, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid, phenylarsine oxide) and by ROS (peroxynitrite, *tert*-butylhydroperoxide, hydrogen peroxide) [15,35,39–41]. We also previously showed that the MPT induced by selenite is mediated by thiol oxidation [28]. Given that the reaction of selenite with GSH generates $O_2^{\bullet -}$, we investigated the possibility that $O_2^{\bullet -}$ generated by the reaction of selenium compounds and GSH contributes to the induction of the MPT by these compounds. We first measured the ability of the various selenium compounds to generate $O_2^{\bullet -}$ on incubation with GSH in the presence of isolated mitochondria as described above. In addition to selenite, selenocystine, selenocystamine, and selenodioxide (but not selenate or selenomethionine) also each generated $O_2^{\bullet -}$ by reacting with GSH (Fig. 6).

3.6. Mitochondrial swelling in response to superoxide generation by selenite

We next examined whether $O_2^{\bullet -}$ produced from the reaction of selenium compounds with GSH induces the MPT in isolated mitochondria. We compared the effect of selenite with that of diamide, which induces mitochondrial swelling through thiol oxidation but does not react with GSH to generate $O_2^{\bullet -}$. In this study, we used 100 μM selenite and 2 mM GSH because these concentrations generated the most amount of superoxide. The swelling

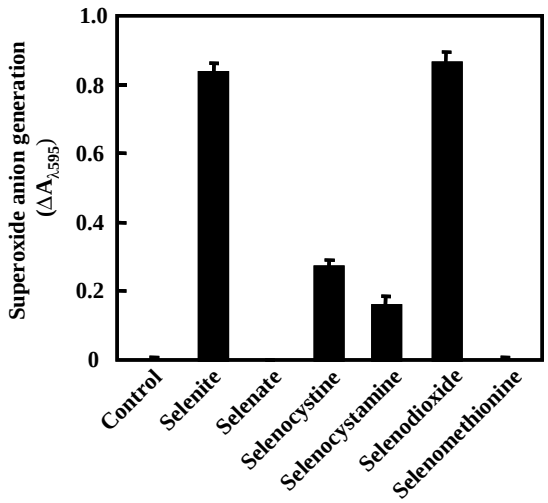

Fig. 6. Generation of the superoxide by the reaction of selenium compounds with GSH. Mitochondria (1 mg/mL) were suspended in a solution containing 200 μM NBT, 5 mM succinate, 0.25 M sucrose, and 10 mM Tris–HCl (pH 7.4). They were preincubated for 5 min with 2 mM GSH before the addition of the indicated selenium compounds (1 mM) and incubation for 1 hr at room temperature. Control was not added by selenium compounds. The rate of NBT reduction was measured spectrophotometrically at 595 nm. Data are expressed as the increase in A_{595} per minute and are means ± SE of values from three independent experiments.

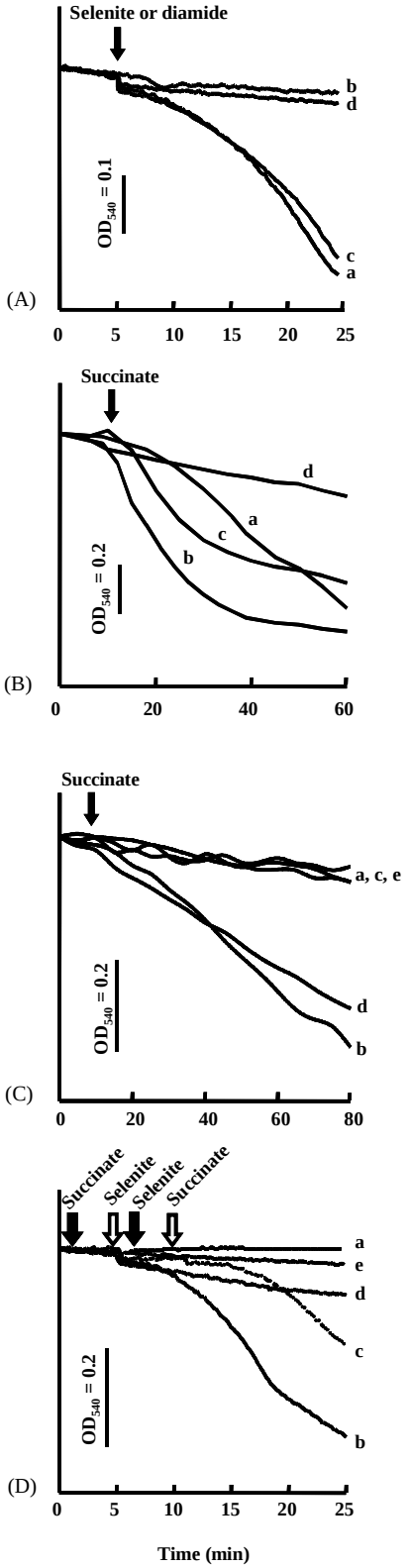

Fig. 7. Induction of the MPT by $O_2^{\bullet -}$ generated as a result of the reaction of selenite with GSH. (A) Rat liver mitochondria (1 mg/mL) were suspended in a solution containing 0.25 M sucrose and 10 mM Tris–HCl (pH 7.4), preincubated for 5 min at room temperature with 5 mM succinate, and then exposed (arrow) to 100 μM selenite (traces a and b) or 100 μM diamide (traces c and d) in the absence (traces a and c) or presence (traces b and d) of 2 mM GSH. (B) Mitochondria were suspended as in (A), preincubated for 1 hr with 100 μM selenite (traces a and b) or 100 μM diamide (traces c and d) in the absence (traces a and c) or presence (traces b and d) of 2 mM GSH, and then exposed (arrow) to 5 mM succinate. (C) Mitochondria were suspended as in (A) and preincubated for 1 hr at room temperature either with 100 μM selenite and 2 mM GSH, in the absence (trace b) or presence (trace c) of 0.5 mM Mn-TBAP, or with 0.2 mM xanthine and xanthine oxidase (20 mU/mL), in the absence (trace d) or presence (trace e) of 0.5 mM Mn-TBAP. The mitochondria were then exposed (arrow) to 5 mM succinate. Trace a: no addition. (D) Mitochondria were suspended as in (A), preincubated (closed arrow) for 5 min at room temperature in the absence (trace e) or presence of 5 mM succinate (trace b) or 5 mM succinate plus 5 μM TTFA (trace d), and then exposed (closed arrow) to 100 μM selenite. Alternatively, mitochondria were preincubated (open arrow) with 100 μM selenite for 5 min at room temperature and then exposed (open arrow) to 5 mM succinate (trace c). Trace a: no addition. Large-amplitude mitochondrial swelling was monitored by measurement of the decrease in optical density at 540 nm (OD_{540}).

induced by the addition of selenite or diamide, each at a concentration of 100 μM, to energized mitochondria was completely inhibited by 2 mM GSH (Fig. 7A). This inhibitory effect of GSH is likely attributable to prevention of the diamide- or selenite-induced oxidation of thiol groups present on a component of the MPT pore. However, the addition of 5 mM succinate to mitochondria that had been preincubated with GSH and selenite for 1 hr resulted in large-amplitude mitochondrial swelling (Fig. 7B); such swelling was not observed when diamide replaced selenite in this protocol. These data indicated that $O_2^{\bullet -}$ generated by the reaction of selenite with GSH induced the MPT. To confirm that $O_2^{\bullet -}$ is able to induce the MPT, we showed that another $O_2^{\bullet -}$ generation system, xanthine and xanthine oxidase, triggered opening of the MPT pore in isolated mitochondria and that this effect was inhibited by Mn-TBAP [42], an $O_2^{\bullet -}$ scavenger (Fig. 7C). Mn-TBAP also completely inhibited the swelling induced by the reaction of selenite with GSH before succinate treatment.

The energy state of the respiratory chain is an important determinant of MPT pore opening [43,44]. We therefore investigated the effect of mitochondrial energy state on induction of the MPT by selenite and GSH with the use of TTFA, an inhibitor of complex II. TTFA inhibited the MPT induced by preincubation of mitochondria with selenite and GSH before exposure to succinate (Fig. 7D).

4. Discussion

We have examined the toxicities of the inorganic selenium compounds selenite, selenate, and selenodioxide and the organic selenium compounds selenocystine, selenocystamine, and selenomethionine. By reaction with GSH, selenite and selenodioxide form selenodiglutathione, which is reduced to the selenopersulfide anion; the latter in turn produces $O_2^{\bullet -}$ through redox cycling [24,42]. Selenocystine is metabolized by reduced GSH and/or GSH reductase to hydrogen selenide via selenocysteine-glutathione selenenyl sulfide, which also produced $O_2^{\bullet -}$ by redox cycling [45]. Selenocystamine, which is a symmetrical diselenide

(RSeSeR), is reduced by thiol agents such as GSH to yield two selenoate anions (RSe^-) [46]; the latter also produce $O_2^{\bullet -}$ by redox cycling. Selenate and selenomethionine ($RSeCH_3$) do not produce $O_2^{\bullet -}$ by redox cycling or thiol oxidation [47]. We have now shown that selenite, selenocystine, and selenodioxide induced apoptosis in HepG2 cells, as revealed by flow cytometric analysis of DNA content and electrophoretic detection of internucleosomal DNA fragmentation.

In addition to being the principal site of ROS generation in cells, mitochondria are also the primary target of these molecules. Mitochondria thus play a key role in apoptosis induced by the accumulation of ROS and consequent changes in the cellular redox state. The MPT is a critical event in mitochondria-dependent apoptosis. The opening of the MPT pore, a large, high-conductance, and nonspecific channel that spans both mitochondrial inner and outer membranes, results in a loss of $\Delta\Psi_m$, mitochondrial swelling, and the release of cytochrome *c*, the latter of which activates the caspase mediators of apoptotic cell death [3,5,34,39]. We have now shown that the pro-apoptotic selenium compounds induced a decrease in $\Delta\Psi_m$ and the release of cytochrome *c* both in HepG2 cells and in isolated rat liver mitochondria. Our observation that selenocystamine induced these effects in isolated mitochondria but not in HepG2 cells suggested that this compound did not trigger apoptosis because it was not able to penetrate the cell membrane.

The opening of the MPT pore is induced by thiol oxidation [11,15,16,38,40]. We showed that the pro-apoptotic selenium compounds selenite, selenocystine, and selenodioxide were able to oxidize thiol groups in isolated mitochondria, in HepG2 cells, and in HepG2 cell lysates. Again, selenocystamine oxidized protein thiols in isolated mitochondria and HepG2 cell lysates but not in intact HepG2 cells, consistent with the conclusion that this compound is not able to cross the cell membrane.

The release of cytochrome *c* and other pro-apoptotic proteins such as Smac (DIABLO) from mitochondria induced by $O_2^{\bullet -}$ is thought to play an important role in apoptosis under a variety of pathological conditions, including ischemia-reperfusion injury, drug toxicity, and inflammation [1,48–51]. Large amounts of $O_2^{\bullet -}$ are produced as a result of xanthine oxidase-mediated catabolism of purine nucleotides, an increased activity of the electron transport chain, and, especially in neutrophils, by activation of NADPH oxidase. Furthermore, an increased production of $O_2^{\bullet -}$ has been described as an early event in several apoptotic paradigms [3,7,12]. In cells, $O_2^{\bullet -}$ is converted to ROS (H_2O_2, $OONO^-$), which in turn have been shown to trigger the MPT and the associated loss of $\Delta\Psi_m$, mitochondrial swelling, and cytochrome *c* release in isolated mitochondria [12,47,52].

We have now shown that mitochondrial swelling induced by selenite or diamide after preincubation of isolated mitochondria with succinate was prevented by the presence of GSH, consistent with the notion that these effects of selenite and diamide were mediated by thiol oxidation. In contrast, GSH potentiated rather than inhibited swelling when mitochondria were preincubated with this agent and selenite before exposure to succinate. Mitochondria preincubated with GSH and diamide before exposure to succinate did not exhibit swelling. These results thus suggest that the preincubation of mitochondria with selenite and GSH before exposure to succinate results in the generation of $O_2^{\bullet -}$ and that this anion might then affect the redox state of the pyridine nucleotide pool. Several studies have suggested that oxidation of the pyridine nucleotide pool, which is regulated by respiratory substrates such as succinate and glutamate–malate, results in induction of the MPT [35,53,54]. The MPT induced by selenite and GSH was prevented by the $O_2^{\bullet -}$ scavenger, Mn-TBAP. Our data thus suggest that $O_2^{\bullet -}$ plays an important role in the opening of the MPT pore induced by selenium compounds. From these data, we suggest the possibility that $O_2^{\bullet -}$, generated by reaction with selenite and GSH, plays an important role in the opening of the MPT, which may be mediated by thiol oxidation in isolated mitochondria.

In conclusion, we have shown that both organic and inorganic selenium compounds are able to induce apoptosis by triggering the MPT, which results in a loss of $\Delta\Psi_m$ and the release of cytochrome *c* into the cytosol. Furthermore, the induction of the MPT by selenium compounds appears to be mediated both by cross-linking of protein thiol groups and by the generation of $O_2^{\bullet -}$ through reaction with GSH.

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