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Low doses of selenium specifically stimulate the repair of oxidative DNA damage in LNCaP prostate cancer cells

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Abstract

Epidemiological studies have demonstrated an inverse relationship between selenium (Se) intake and cancer incidence and/or mortality. However, the molecular mechanisms underlying the cancer chemopreventive activity of Se compounds remain largely unknown. The objective of this study was to investigate the effect of low doses of Se on the stimulation of DNA repair systems in response to four different qualities of DNA damage. P53-proficient LNCaP human prostate adenocarcinoma cells were grown either untreated or in the presence of low concentrations of two Se compounds (30° nM sodium selenite, or 10 µM selenomethionine) and exposed to UVA, H₂O₂, methylmethane sulfonate (MMS) or UVC. Cell viability as well as DNA damage induction and repair were evaluated by the alkaline Comet assay. Overall, Se was shown to be a very potent protector against cell toxicity and genotoxicity induced by oxidative stress (UVA or H₂O₂) but not from the agents that induce other types of deleterious lesions (MMS or UVC). Furthermore, Se-treated cells exhibited increased oxidative DNA repair activity, indicating a novel mechanism of Se action. Therefore, the benefits of Se could be explained by a combination of antioxidant activity, the reduction in DNA damage and the enhancement of oxidative DNA repair capacity.

Keywords

DNA repair; hOGG1; oxidative DNA damage; selenium; Comet assay

Introduction

Selenium (Se) is an essential trace element of fundamental importance to human health. It is present in the diet mostly in organic forms [1]. However, both organic and inorganic forms of Se appear to be utilized with similar efficacy in the body to produce selenoproteins [2], although they enter Se metabolic pathways at different points, depending on their chemical

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Declaration of interest

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forms [3]. Se is bimodal in nature and its beneficial properties are apparent in a fairly narrow range of daily intake [4]. Se-containing compounds are involved in fundamental biological processes ranging from apoptosis to immunity [5], including cellular antioxidant defence as well as the protection and repair of DNA [6]. Selenoproteins are collectively essential for life and several of them, such as members of the thioredoxin reductase (TrxR) and glutathione peroxidase (GPx) families, function as antioxidant enzymes [7]. However, the physiological roles of most of the other selenoproteins have not yet been fully elucidated [8].

Redox regulation has emerged as an essential process of many pathways in cell biology [9]. However, disruption of the intracellular redox balance leads to a state of oxidative stress, during which proteins, nucleic acids, lipids and other macromolecules can accumulate damage. This can be accelerated by low dietary Se intake, resulting in the organism being prone to oxidative stress-related conditions, reduced fertility and immunity, and to increased risk of cancer [10]. Several mechanisms of action have been proposed to account for this effect, including the stimulation of apoptosis [11,12], the induction of cell-cycle arrest [13], activation of the p53 tumour-suppressor protein [14], induction of DNA repair genes, enhancement of immune functions and influencing estrogen- and androgen-receptor expression [15].

DNA damage caused by reactive oxygen species (ROS) includes a variety of lesions, ranging from base modification, to DNA breaks and DNA-protein cross-links. More than 60 different ROS-induced base lesions have been identified, the best characterized being 8-oxo-7,8-dihydroguanine (8-oxoGua). If not repaired, this oxidized guanine results in GC to TA mutations [16]. 8-oxoGua is repaired *via* the base excision repair (BER) process initiated by a specific DNA glycosylase and an AP endonuclease. The actions of these two enzymes result in the generation of a single strand break (SSB) that requires further processing before gap filling and ligation. Interestingly, BER enzymes are sensitive to the redox status of cells [17,18].

Se may play a protective role in carcinogenesis by both decreasing the levels of DNA damage/mutagenesis and modulating DNA repair capacities. Thus, we designed the present study to investigate the effect of low concentrations of distinct selenocompounds on the cytotoxic and genotoxic properties of four different DNA damaging agents, as well as on the efficiency of DNA repair systems. The most robust results of the Nutritional Prevention of Cancer trial (NPC Trial) came from the analyses of the complete data for prostate cancer incidence. It showed that dietary supplementation with 200 µg/day of yeast-enriched Se resulted in a 63% reduction in prostate cancer risk [19]. Moreover, because dogs and humans are the only two species in which prostate cancer occurs spontaneously with appreciable frequency, Waters et al. examined the effects of dietary Se supplementation on DNA damage in elderly beagle dogs and found efficient protection [20]. Se-mediated protection of the prostate against cancer has been well-documented [21]. For these reason, we focus our work on cultured prostate cells, in particular LNCaP cell line as these cells were largely used as an *in vitro* tool for assessing the protection afforded by Se against genotoxicity of several stress agents [22]. It is important to mention that LNCaP cells are wild type for both p53 and Rb genes, both of which are mutated in the vast majority of cancers. We showed that LNCaP-supplemented cells with low doses of two different selenocompounds, sodium selenite (SS) and selenomethionine (SM) exhibited reduced oxidative DNA damage and enhanced DNA repair capacity after UVA or H₂O₂ exposure. Therefore, Se could act as double-edged sword against carcinogenesis by both decreasing oxidative genome damage and enhancing their repair. These data lead to further advances in the mechanisms of Se cancer prevention.

Methods

Chemicals

Sodium selenite (SS), selenomethionine (SM), Dulbecco's Phosphate Buffered Saline (PBS) with MgCl_2 and CaCl_2 (PBS Ca^{2+} Mg^{2+}), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), crystal violet, bovine serum albumin (BSA), nuclease P1, DNase II, 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB), Cell Lytic M cell lysis agent, protease inhibitor cocktail and colorimetric assay kit for thioredoxin reductase (TrxR), were obtained from Sigma-Aldrich (St Quentin-Fallavier, France). The protein assay kit was from Uptima Interchim (Montluçon, France). NaOH was purchased from Carlo Erba (Rodano, Italy). RPMI '1640 medium and fetal calf serum (FCS) were from GIBCO (Courbevoie, France). Penicillin and streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). Microtitration plates were purchased from Nunc (Dutscher, Brimath, France). Formamidopyrimidine DNA glycosylase (Fpg), T4 endonuclease V (T4 Endo V) and Endonuclease III (Endo III) were Trevigen (Interchim, Montluçon, France). Phosphodiesterase II, phosphodiesterase I and alkaline phosphatase were obtained from Roche Molecular Biochemicals (Indianapolis, USA).

Cell culture

LNCaP cells, which are wild type for both the p53 and Rb genes, were cultured in RPMI 1640 medium with 10% FCS and 1% penicillin/streptomycin in culture flasks at 37°C in 5% CO_2 . For Se treatment, the medium was supplemented with either 30 nM SS or 10 μM SM for 72 h. A no treatment control (NT) was included in each experiment. Using inductively plasma mass spectrometry (ICP-MS), we measured Se concentration in our FCS, and it was about 248.25 ± 45 nM. By using 10% level of serum, the LNCaP cells were grown in 24.825 ± 4.5 nM of Se.

Cytotoxicity assay

LNCaP cells were plated in 35 mm plates or in 96-well microtitration plates (in 2 mL or 200 μL of cell culture medium, respectively) and were incubated at 37°C for 72 h, in the presence of SS and SM. Then, four different DNA damaging treatments were tested: UVA or UVC radiation; hydrogen peroxide (H_2O_2 , added to the medium for 30 min on ice) or methyl-methane sulfonate (MMS, added to the medium for 15 min at 37°C). MMS and H_2O_2 were diluted in PBS Ca^{2+} Mg^{2+} whereas control cells were treated with PBS Ca^{2+} Mg^{2+} alone. Finally, cell viability was determined by the MTT assay 24 h after exposure to the treatment [22].

Clonogenic assay

To study the long-term toxicity of our stress agents, we performed a clonogenic assay or a colony formation assay which is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony after 2 weeks. LNCaP cells were plated in 35-mm plates (10,000 cells/mL) and incubated at 37°C for 72 h with SS and SM. The cells were then stressed as described for the cell viability assay and immediately re-plated in a 60-mm plate at a density of 50 cells/mL. The culture medium was changed every 3 days, and at the 12th day, crystal violet 0.5% in $\text{H}_2\text{O}/\text{MeOH}$ (1:1) was added to each plate. The plates were rinsed with water, dried and the colonies were counted.

Determination of selenoenzyme activities

Cells were removed by trypsinization and were lysed using CellLytic™ M Cell Lysis agent (Sigma) with a protease inhibitor cocktail, and then centrifuged at $1600 \times g$ at 4°C for 10

min. After a second centrifugation at $4800 \times g$ at 4°C for 20 min, enzyme activities were measured in the supernatant.

Cytosolic GPx (GPx1) activity was measured in a coupled reaction with glutathione reductase as described by Flohé et al. [23]. Cytosolic TrxR activity was determined colorimetrically using the Thioredoxin Reductase Assay kit (Sigma), as described previously [24]. The protein content of the samples was determined by bicinchoninic acid assay (BCA) using a protein assay kit (Pierce) [25].

Alkaline single-cell gel electrophoresis (Comet Assay): measure of DNA damage

To evaluate the extent of DNA damage by the Comet assay, LNCaP cells were pre-treated with 30 nM SS or 10 μM SM for 72 h at 37°C and exposed to $50 \text{ J}/\text{cm}^2$ UVA, $10 \text{ J}/\text{m}^2$ UVC, 200 μM H_2O_2 or 500 μM MMS. Control conditions were NT, SS and SM cells sham-treated with PBS. Immediately after treatment, cells were collected, counted and then suspended in freezing medium (20% FCS and 10% DMSO) at a density of 1×10^6 cell/mL, gently frozen to -80°C and stored in liquid nitrogen until use.

Frozen pellets of LNCaP cells (NT, SS, SM treated and the controls) were centrifuged at $300 \times g$ for 5 min at 4°C . The cell suspension was added to low melting point-agarose at 37°C , and 100 μL of the mixture was placed on a microscope slide coated with one dried layer of 1% normal agarose in PBS. After gelling on ice, the slides were immersed overnight in cold lysis buffer (2.5 M NaCl, 0.1 M $\text{Na}_2\text{-EDTA}$, 10 mM Tris, 1% N-lauroylsarcosine sodium salt, 1% Triton X-100, 10% DMSO, pH 10) at 4°C in the dark. Slides were washed three times for 5 min with reaction buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM $\text{Na}_2\text{-EDTA}$, 0.2 mg/mL BSA). Digestion with lesion-specific endonucleases (100 μL per slide) was performed for 30 min at 37°C . Cells on control slides were incubated only with enzyme buffer. After incubation, slides were immersed in pre-cooled electrophoresis buffer (1 mM $\text{Na}_2\text{-EDTA}$ and 300 mM NaOH) for 30 min in order to allow the unwinding of the DNA and then electrophoresis was applied at 25 V/300 mA for 30 min. After electrophoresis, slides were rinsed three times for 5 min with neutralization buffer (0.4 M Tris-HCl, pH 7.4), stained with ethidium bromide (100 μL per slide, 2.5 $\mu\text{g}/\text{mL}$) and covered with a cover slip prior to analysis.

A fluorescence microscope (Carl Zeiss, Germany) connected to a charge-coupled device (CCD) camera and a computer-based analysis system (Comet assay IV software, Perceptive Instruments Ltd) was used to determine the extent of DNA damage after electrophoresis of DNA fragments in the agarose gel. The extent of damage was evaluated as the average of the triplicate values of the tail DNA intensity (% of DNA in the tail of the comet).

Assessment of cellular DNA repair capacity in protein extracts

Substrate preparation—Cells were exposed to an oxidative agent (Riboflavin 1 μM + UVA $10 \text{ J}/\text{cm}^2$) inducing 8-oxoGua, an alkylating agent (MMS, 500 μM) and a pyrimidine dimer-forming agent (UVC $10 \text{ J}/\text{m}^2$), collected by trypsinization and suspended in freezing medium slowly frozen to -80°C and stored in liquid nitrogen.

Preparation of whole cell extracts—Liquid nitrogen-stored frozen aliquots of LNCaP cells (NT, SS, SM conditions) were centrifuged at $300 \times g$, 4°C for 5 min. A dry pellet was subsequently frozen in liquid nitrogen and re-suspended in 33 μL of extraction buffer (45 mM HEPES, 0.4 M KCl, 1 mM EDTA, 10% glycerol, 0.1 mM dithiothreitol [DTT], 0.25% Triton-X 100, pH 7.8). The mixture was then vortexed for 30 s, incubated for 5 min on ice and centrifuged at $14000 \times g$ for 5 min at 4°C . The supernatant was then combined in a 1:5 ratio with reaction buffer. Extracts (50 μL) were placed on each slide covered with a cover

glass and incubated for 30 min at 37°C. Samples digested with specific purified repair enzymes were used as positive controls while negative controls were incubated only with a 50 µL mix of extraction and reaction buffers. After digestion, the slides were transferred to an electrophoresis tank and processed using the Comet assay protocol described above.

High pressure liquid chromatography-tandem mass spectroscopy detection of 8-oxoGua

LNCaP cells (NT, SS and SM conditions) were irradiated in PBS in plastic petri dishes. DNA was extracted from cells using a sodium iodide (NaI) precipitation based chaotropic method [26], and solubilized in 50 µL of deferoxamine. DNA samples were hydrolysed by incubation with nuclease P1, phosphodiesterase II, DNase II, phosphodiesterase I and alkaline phosphatase [27]. Samples were then analysed by high performance liquid chromatography associated with tandem mass spectrometry (HPLC-MS/MS) with electrospray ionization. The apparatus consisted of SCIEX API 3000 electrospray triple quadrupole spectrometer associated with a series 1100 Agilent chromatographic system. Quantification of DNA damage was performed in the multiple reactions monitoring mode as previously described [28].

Statistical analysis

The data were expressed as the mean $\pm$ standard deviation (SD). Statistical significances of differences among treatment groups were determined by Student's t-test. A *p*-value of < 0.05 was considered as statistically significant.

Results

Selenoenzyme activities

The modulation of the activity of the GPx1 and TrxR selenoenzymes by low concentrations of Se (in the form of SS and SM) in LNCaP cells are summarized in Table I. Se supplementation of LNCaP cells with either SS or SM significantly ($p < 0.05$) increased the activities of TrxR (~3-fold) and GPx1 (≥ 2 -fold).

Low-dose of Se supplementation increases resistance to UVA and H₂O₂-induced cytotoxicity

For all cytotoxicity assays, cells were pre-treated with SS or SM during 72 h. Then, they were treated with several DNA-damaging agents and 24 h after that, we measured cell viability. Moreover, to understand whether Se supplementation was fundamental to prevent cellular death or it was able to ameliorate cell survival also after treatment, we carried out Se post-treatment. That is, LNCaP cells were plated for 72 h, then cells were stressed and immediately post-treated or not with SS and SM for 24 h. Figure 1A shows the % viability of the cells irradiated with increasing doses of UVA. Cells pre-treated with Se exhibited a better survival than controls after UVA irradiation ($p < 0.01$). After exposure to 25 J/cm² of UVA, SS and SM pre-treated cells were fully viable whereas viability of NT cells was only 30%. In contrast, post-treatment with selenocompounds had no effect on cell survival. Similar observations were made with H₂O₂ (Figure 1B). Pre-treatment with SS and SM resulted in greater cell survival when compared to NT ($p < 0.05$). The LC₅₀ of H₂O₂ in NT cells was 200 µM whereas SS and SM pre-treated cells had an LC₅₀ of 5 mM of H₂O₂.

The next type of stress was a monofunctional alkylating agent, namely MMS. Compared to the previous treatments, the MMS incubation did not highlight any effects of SS and SM treatment on cell survival (Figure 1C). In contrast to oxidative stresses, there was no protective effect of SS and SM against cell death after exposure to UVC (Figure 1D). UVC radiation cause only limited ROS formation and oxidative stress to cells. On the other hand, the main lesions induced by UVC are bulky adducts (CPDs and 6-4 PP).

The next step was to confirm the Se-mediated modulation of the cellular response to stress by a clonogenic assay. This cell survival assay is based on the ability of a single cell to grow into a colony and includes long term effects of exposure (12 days). The clonogenic assay was assessed following treatment with UVA or H₂O₂ for which Se protection was shown in short-term survival. Again, we showed that Se supplementation increases long-term resistance to UVA and or H₂O₂ stresses (Figure 2).

Se protects specifically against oxidative DNA damage

The standard version of the Comet assay, when performed under alkaline conditions, provides data for evaluating initial DNA strand breaks and alkali-labile sites (ALS) induced by exposure to different types of genotoxicity-inducing agents. In order to obtain information on specific categories of DNA base lesions, several glycosylases were used to convert base lesions to strand breaks. These three glycosylases are Fpg, Endo III or T4 Endo V, which removed respectively oxidized purines, oxidized pyrimidines and cyclobutane pyrimidines dimers. Figure 3A shows an example of undamaged and damaged DNA visualized by the Comet assay. The presence of strand breaks into DNA makes it more flexible. The growing tail intensity is proportional to DNA strand breaks. The incubation with Fpg, or with another glycosylases, allows to excise the Fpg-sensitive sites or other enzyme-sensitive site and thus to convert an oxidized purine or another base damage into strand breaks which can be detected by the increase of tail intensity. The tail intensities measured for controls (NT, SS and SM) were very small and were not significantly increased upon incubation with the three repair enzymes. The four stress agents chosen produced significant levels of DNA breaks as evidenced by increase of tail intensity. In Figure 3B we can observe an increase of tail intensity of approximately six-fold after exposure to 50 J/cm² of UVA and ~10-fold after treatment with 200 µM of H₂O₂ compared to control cells. Exposure to UVA and H₂O₂ produced significant increase in the level of oxidized bases as evidenced by increase in tail intensity after Fpg incubation. Treatment with either 30 nM SS or 10 µM SM reduced the levels of DNA strand breaks induced by UVA or H₂O₂ as evidenced by significant decreases in tail intensity. These results indicate that Se supplementation reduces DNA damage induced by oxidative stress agents.

The increase of strand breaks frequency was of ~four-fold after incubation with 500 µM of MMS (Figure 3C). Exposure to the monofunctional alkylating agent MMS produced significant alkylated bases as evidenced by increase in tail intensity after Fpg and EndoIII digestion (~4.5- and ~2.7-fold respectively). In contrast to oxidative stress agents, no significant differences in tail intensity was observed after SS or SM treatments. Moreover, the increase of strand breaks frequency was of ~4-fold after exposure to 10 J/m² of UVC (Figure 3D) compared to control cells. This UVC dose corresponds to 80% of cell viability obtained by MTT assay. UVC exposure produced cyclobutane pyrimidine dimer (excised by T4 EndoV) as evidenced by the nine-fold increase in tail intensity as compared to control. Same to MMS, no significant differences in tail intensity was observed after SS or SM treatments. These results indicate that Se supplementation has no effect on DNA damage induced by non-oxidative stress agents.

Formation of 8-oxoGua upon UVA irradiation

As a complementary approach to the assessment of oxidative DNA lesions by the Fpg/Comet assay, the formation of 8-oxoGua after exposure to different doses of UVA radiation was quantified by HPLC-MS/MS analysis in NT, SS and SM cells (Figure 4). SM but not SS exposure significantly decreased the induction of 8-oxoGua as compared to NT at 200 J/cm² ($p < 0.05$).

Se supplementation increases 8-oxoGua excision capacity

To study the effect of Se supplementation on DNA repair, we used a Comet assay-based approach, which assessed the ability of the repair enzymes present in cellular extracts to incise the DNA substrate and cause single-strand breaks. The initial steps of the repair processes include the sensing of the DNA damage, followed by the excision of the damage, resulting in single-strand breaks. The increase in the tail intensity after incubation of the damaged DNA with the cell extracts is an indication of enhanced repair capacity of the enzymes involved. The specificity of the assay was provided by the nature of the lesions present in the substrate. Using substrates with distinct classes of damage, both BER and nucleotide excision repair (NER) can be assessed.

DNA tail intensities for the oxidized substrate, measured following incubation with all three cell extracts (NT, SS, SM) were significantly higher compared to incubation in buffer alone ($p < 0.05$) (Figure 5A). The respective ratios were of 1.3-, 2.0- and 1.9-fold. In addition, protein extracts from cells pre-treated with SS and SM exhibited a significantly higher excision potential (30.5 ± 4.3 and 29.9 ± 3.3 respectively) than the NT LNCaP cells (20.1 ± 3.3 , $p < 0.05$), which provides evidence for a beneficial effect of Se supplementation on the repair capacity of oxidative DNA damage. As a positive control, we evaluated the Fpg sensitive-sites values (46.5 ± 4.0) and they were significantly higher than the three cell extracts values.

A second DNA substrate from MMS-treated cells was also used in the repair assay. Unlike the results obtained with UVA/Riboflavin damaged DNA substrate, there were no significant differences in repair capacities for the protein extracts obtained from the SS and SM treated cells as compared to the NT control cells (Figure 5B). It is interesting to note that the activities of the positive controls (Endo III and Fpg) were lower compared to those of cell extracts. A similar trend was obtained for UVC-irradiated substrates (Figure 5C). The DNA tail intensities (%) for all three cell extracts (NT, SS, SM) were significantly higher compared to the tail intensity of the substrate treated with the buffer. However, no differences were found among the activities of the extracts. The efficiency of T4 Endo V activity was 2.5-fold higher than the activity achieved using the cell extracts.

Discussion

In the present study, the protection offered by supplementation with Se, in both organic (SM) and inorganic (SS) forms, against several DNA damaging agents was evaluated in a human prostate cancer derived LNCaP cells. These cells were selected for study as these are commonly used for studies investigating the biochemical and biological response to Se exposure [29] and the effects of altering selenoprotein levels on DNA damage [30]. Four types of agents were chosen in order to generate different types of DNA lesions, the repair of which are carried out by different DNA repair systems. DNA damage induced by UVA is thought to occur *via* an indirect mechanism, involving the generation of ROS, which leads to the formation of 8-oxoGua [31]. H_2O_2 treatment induces several types of lesions in DNA, including single or double-strand breaks and a variety of oxidized purines and pyrimidines [32]. MMS is an alkylating agent and its exposure results in the predominant adducts in double-stranded DNA being methylated purines [33], each of these kinds of lesions are repaired by the BER system. The primary DNA lesions induced by UVC light are cyclobutane pyrimidine dimers (CPD) and 6-4 PP, both of which are repaired by NER [34]. The doses of Se used for media supplementation was based on our previous dose-response studies conducted in LNCaP and another cell type by us and others, which indicated that the selected doses optimize GPx1 activity in cells that carry the common alleles for that enzyme when these cells are cultured in media containing 10% FBS [35,36]. It is important to mention that the Se concentration in our FCS was about 248.25 ± 45 nM. By using 10%

level of serum, the LNCaP cells were grown in 24.825 ± 4.5 nM of Se which could be regarded as being Se deficient condition.

The selenocompounds used in the study were not cytotoxic at the concentrations tested and were both able to enhance viability resulting after UVA and H₂O₂ exposure and afforded greater clonal survival with a greater efficiency of SS over SM [37], which could be due to their difference in entry points in Se metabolism. It should be emphasized that addition of SS or SM immediately after agent exposure (post-treatment) had no effects on the studied endpoints. This immediate effect rules out the involvement of *de novo* synthesized selenoproteins in protection. This finding is consistent with the observation of significant increases in the activities of GPx1 (2.5-fold) and TrxR (3.5-fold) selenoproteins. The induction of GPx1 activity with Se supplementation was previously demonstrated in several tissues and cell lines, and was reported to be due to the enhanced translation, and not transcription [21]. However, the selenocompounds tested were not protective against MMS and UVC-induced cytotoxicity, indicating that Se supplementation supplied protection only against ROS-induced cytotoxicity.

The extent of DNA damage was also investigated by two complementary approaches: the Comet assay and HPLC-MS/MS assessment of 8-oxoGua levels. Although the Comet assay is sensitive and qualitative, it is not adequate to quantitatively assess specific base damage, which was obtained by HPLC-MS/MS analysis. We observed that Se can modulate the induction of damage after exposure to DNA damaging agents. This was demonstrated for the induction of strand breaks induced by UVA and H₂O₂, as well as 8-oxoGua resulting from UVA irradiation. The significant decrease of 8-oxoGua formation in Se-treated cells after UVA irradiation was confirmed only after HPLC-MS/MS analysis. These results are therefore consistent with the cell survival data indicating that Se compounds are efficient at protecting cells from oxidative damage but not from agents that induce other types of deleterious lesions. The interesting cytotoxicity results have highlighted that a pre-treatment with SS or SM confers a significant increase of cell survival in response to an oxidative stress. Even more interesting is the important decrease of DNA damage by Se supplementation still after oxidative stress. We believe that the action of Se is mediated by the activity of antioxidant enzymes leading to a reduction of ROS. More complex cellular signalling mechanisms and biological responses could also be involved.

In the second part of the work, we examined the effect of Se on DNA repair capacity using a modified Comet assay to measure excision activity of the protein extracts from NT, SS and SM cells. Extracts of cells pre-treated with Se recognized and removed greater amounts of oxidized DNA bases than the NT extracts. Moreover, a clear bias towards improved response afforded by Se was observed for oxidative stress. In contrast, the repair capacity of cell extracts (NT, SS, SM) on MMS- and UVC-treated substrates were similar. The pathways involved in the repair of lesions produced by the three stress-inducers are quite different. Since UVC-induced photoproducts are repaired by the NER pathway, our results indicated that the pathways involved in NER were probably not affected by Se supplementation. However, Smith et al. have shown that supplementation with 10 μ M for 15 h of Se in the form of SM stimulates the DNA repair activity of UV lesions in human lung cancer [18], mouse embryonic fibroblasts [38] cells in a p53-dependent manner. The primary DNA lesions induced by UV are cyclobutane pyrimidine dimers and 6-4 PPs, both of which are repaired by NER pathway. In our conditions, we were not able to observe any modulation in the NER capacity after Se supplementation in the LNCaP p53-proficient cells. This contradiction could be due to the different pre-treatment conditions (72 h vs. 15 h) and different cell lines [39]. The improved repair of oxidized lesions obtained by pre-treatment of cells with SS and SM is likely to be linked to the redox properties of enzymes whose levels are increased by Se supplementation (Table I). The AP site is the major product of

OGG1 and its lyase activity is the rate-limiting. Human APE1 significantly stimulates OGG1 activity *in vitro*. Moreover, APE1 is modulated post-transcriptionally *via* redox modification by thioredoxin (Trx). In turn, the redox activity of Trx is maintained by the TrxR selenoprotein. Trx has a critical role in regulating cellular redox status and it modulates APE1 redox activity. Therefore, the translocation of Trx from cytoplasm to nucleus, which is stimulated by the different Se compounds, indicates that cellular redox regulation entails a complex cascade of events [40]. Moreover, hOGG1 possesses critical redox-sensitive residues and being in the reduced state is important for 8-oxoGua DNA glycosylase activity. In particular, molecular epidemiologic case-control studies indicated that a polymorphic variant of hOGG1 (serine to cysteine amino acid substitution at position 326) is associated with a higher risk of developing several types of cancer [41]. The hOGG1-Cys variant is particularly sensitive to the cellular redox status, for example, hOGG1-Cys enzymatic activity decreases after a pro-oxidant treatment [42]. These findings affirm the central importance of oxidative stress in carcinogenesis since it not only induces DNA damage but can also inhibit the cellular protection against such stress. The redox sensitivity of the hOGG1 pathway may explain how Se supplementation and its subsequent stimulation antioxidant defences contribute to the maintenance of a high hOGG1 activity explaining the basis of the enhanced excision activity achieved in Se-treated cell extracts for oxidative damage but not for agents whose exposure results in DNA alkylation. The improved activity of hOGG1 could be due to the enhancement of the activity of GPX1 (Table I), which is an important actor of the enzymatic antioxidant defence by detoxifying hydrogen and lipid peroxides. The cellular redox status may also influence repair activity by modulating specific signal transduction pathways. In that respect, p53 appears as a potential target for Se-mediated protection. This may be important because p53 mediates cellular responses fundamental to carcinogenesis including cell cycle arrest, apoptosis and DNA repair. Seo et al. reported that p53 activity can be augmented by antioxidant mechanisms, thereby protecting cells from DNA damage. SM modulated p53 activity by causing redox regulation of key p53 cysteine residues (275/277) [18]. The resulting conformational shift leads to the induction of p53 DNA-binding activity and the stimulation of DNA repair [14,38]. Smith et al. showed that SS not only caused the reduction of cysteines in p53 as did SM, but also the phosphorylation of p53 at serines 20, 37 and 46 positions which are known to mediate apoptosis [43]. These results contrast with our study as we have shown that SS protected cells against the oxidative stress. However, it should be emphasized that the concentration of SS used by Smith et al. was 300-fold higher than the present study. This difference reflects the well-known biphasic effect of Se where it is toxic in high concentrations but not in physiological levels. Indeed, there is an intriguing U-shaped dose-response relationship between Se status and the extent of DNA damage within the prostate [44]. The same research group demonstrated that dietary Se supplementation decreased DNA damage and increased epithelial cell apoptosis within the aging canine prostate tissue [20]. These findings emphasize that the baseline in nutrient status in the suboptimal range should be considered as a required entry criterion for prevention trials with Se in order to avoid the potential deleterious effects of over-supplementation. In October 2008, the large human prostate cancer prevention trial SELECT was stopped in its seventh year, and the results indicated that there were no beneficial effects of Se against cancer incidence [45]. However, this trial did not test different formulations or doses of Se and there has not been any information as to whether Se might be effective among those with the lowest baseline Se levels [46].

In conclusion, the overall results of this study demonstrated that low doses of Se in two chemical forms, under conditions that stimulate selenoprotein synthesis such as GPX1 and TrxR, protect against toxicity and DNA strand breaks induced by both H₂O₂ and UVA, but not MMS and UVC. Furthermore, Se-treated cells exhibited increased specifically oxidative DNA repair activity (especially repair of 8-oxoGua) that indicated a new mechanism of Se

action. Therefore, the beneficial role of Se may be explained by a combination of antioxidant activity and the maintenance of DNA repair capacity. It is important to mention that SS was found to be 300 times more efficient than SM which indicate that the protective efficiency of Se derivatives strongly depends on its chemical form of Se. The absence of protection against other types of stress, either alkylating or radiating (UVC), emphasizes that SS and SM were only able to protect against oxidative stress and may only be regulators of intracellular redox status. These results confirm the data obtained from our previous studies in which we showed that Se in both SS and SM forms protected LNCaP cells and Leydig against phthalate-induced oxidative stress [47]. In the future, further studies should consider including other cell types (e.g. primary cells) and further work for better understanding of the regulation of the DNA repair by Se.

Abbreviations

6-4 PP	6-4 photoproducts
8-oxoGua	7,8-dihydroguanine
ALS	alkali-labile sites
AP	apurinic sites
APE1	AP endonuclease 1
BER	base excision repair
CPD	cyclobutane pyrimidine dimer
DTT	dithiothreitol
Endo III	endonuclease III
Fpg	formamidopyrimidine-DNA glycosylase
GPx1	glutathione peroxidase 1
GR	glutathione reductase
H₂Se	hydrogen selenide
MMS	methylmethane sulfonate
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NER	nucleotide excision repair
hOGG1	human 8-oxoguanine-DNA glycosylase
ROS	reactive oxygen species
Se	selenium
Sec	selenocysteine
SM	selenomethionine
SS	sodium selenite
T4 Endo V	T4 endonuclease V
TrxR	thioredoxin reductase

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Figure 1.

Se supplementation increases resistance to oxidative stress. Cell viability was determined by the MTT assay 24 h after exposure and data were presented relative to the control cells. LNCaP cells were pre-treated or post-treated with 30 nM SS or 10 μ M SM for 72 h or 24 h. The doses of Se used for media supplementation was chosen in order to optimize GPx1 activity in LNCaP cells. (A) Cells (30 min on ice). (C) Cells were irradiated with increasing doses of UVA. (B) Cells were incubated with several concentrations of H₂O₂ were incubated with several concentrations of MMS (15 min at 37°C). (D) Cells were irradiated with increasing doses of UVC. Values are given as mean $\pm$ SD of n = 3 independent experiments and triplicate measurements, and we used for statistical significance using Student's t-test. * p < 0.05 and ** p < 0.01 vs. NT.

Figure 2.

Se supplementation increases long-term resistance to oxidative stress. Cell viability was assessed by the clonogenic assay (or colony formation assay) which is an in vitro cell survival assay based on the ability of a single cell to grow into a colony after two weeks. LNCaP cells were plated in 35 mm petri dishes and incubated with SS or SM for 72 h at 37°C and then irradiated with growing dose of (A) UVA or (B) incubated with several concentrations of H₂O₂ (30 min on ice) and immediately re-plated in 60 mm petri dishes at a density of 50 cells/mL for 12 days. Cells were then fixed with ethanol, stained with crystal violet in order to count the number of colonies. Results are expressed as the mean percentage $\pm$ SD of colonies of 3 independent experiments and triplicate measurements, and we used for statistical significance using Student's t-test. * $p < 0.05$ and ** $p < 0.01$ vs. NT.

Figure 3.

Se protects against oxidative DNA damage, assessed by alkaline comet assay after UVA, H₂O₂, MMS and UVC-treated LNCaP cells. To evaluate the extent of DNA damage by the Comet assay, LNCaP cells (50,000 cells/Petri dish 60 mm) were pre-treated with 30 nM SS or 10 μ M SM for 72 h at 37°C and exposed to either 50 J/cm² UVA, 200 μ M H₂O₂ 10 J/m² UVC, or 500 μ M MMS. Control conditions were NT, SS and SM cells sham-treated with PBS. Immediately after treatment, cells were collected and lysed in order to evaluate DNA damage following Comet assay protocol. (A) Typical images of comets in UVA-treated LNCaP cells and effects of Se supplementation are presented. LNCaP cell line was cultured with or without 30 nM SS or 10 μ M SM for 72 h; each group was treated (200 μ M for 30 min on ice), (C) MMS (500 μ M for 15 min at 37°C) and (D) UVC with a specific dose of (B) UVA (50 J/cm²), H₂O₂ (10 J/m²). Immediately after stress, cells were collected and comet assay was performed. Values are given as mean $\pm$ SD of n = 3 independent experiments and triplicate measurements. The mean of the tail intensity (n = 3) was calculated and analyzed for statistical significance using Student's t-test. (**p* < 0.05 vs NT UVA and NT H₂O₂; Δ *p* < 0.05 vs NT UVA Fpg and NT H₂O₂ Fpg).

Figure 4.

Dose-response curve for the formation of 8-oxoGua after UVA irradiation detected by HPLC-MS/MS analysis. HPLC coupled with tandem mass spectrometry was used to quantify 8-oxoGua lesion amount in LNCaP cells after UVA exposure. Cells were pre-treated with SS or SM for 72 h and then irradiated with growing dose of UVA (0, 50, 100 and 200 J/cm²). Immediately after irradiation cells were collected and DNA samples were extracted and hydrolyzed. We observed that cells pre-treated with SM significantly reduced 8-oxoGua formation compared to other conditions (NT and SS). Values are given as mean of 8-oxoGua per 10⁶ normal bases $\pm$ SD of $n = 3$ independent experiments and triplicate measurements and analyzed for statistical significance using Student's t-test. * $p < 0.05$ vs. NT.

Figure 5.

Se supplementation increases 8oxoGua excision capacity of cell extracts. Comet assay-based approach was used to evaluate the activity of several glycosylases involved in the recognition and excision of the lesions induced on our genomic substrates. Total protein cell extracts (NT, SS, and SM) were prepared and their excision capacities were tested into different DNA damaged substrate during 30 min at 37°C, using a modified version of comet assay. Three different DNA damaged substrate were used (A) 1 μ M Riboflavin + 10 J/cm² UVA, (B) 500 μ M MMS, (C) 10 J/m² UVC. For each substrate we used an undamaged substrate as a control. Incubation with several repair enzymes was used as positive control (Fpg, Endo III, and T4 Endo V). The SS and SM protein extracts are more efficient for the excision of the lesion generated on substrate (A) compared to (B) and (C). This oxidative substrate is enriched with 8-oxoGua. Three biological replicates were tested in triplicate. The mean of % of excision of each cell extract (n = 3) was calculated and statistical significance was assessed using Student's t-test. ($p < 0.05$ vs. buffer; $*p < 0.05$ vs. NT cell extract, $^{\#}p < 0.05$ cell extract).

Table I

Antioxidant selenoenzyme activities in LNCaP cells and effects of Se supplementation.

	NT	SS	SM
TrxR (U/mg protein)	0.025 ± 0.005	0.081 ± 0.009*	0.078 ± 0.002*
GPx1 (U/mg protein)	9.0 ± 2.1	18.7 ± 5.6*	25.7 ± 2.4*

NT, no treatment control; SS, sodium selenite; SM, sodium methionite; TrxR, thioredoxin eductase; GPx1, glutathione peroxidase.

* $p < 0.05$ vs. NT.