The inhibition of radiation-induced mutagenesis by the combined effects of selenium and the aminothiol WR-1065

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*Abbreviations: GPx, glutathione peroxidase; CHO, Chinese Hamster Ovary; WR-1065, 2-[((aminopropyl)amino)ethanethiol; FBS, fetal bovine serum; WR-2721, S-2-(3-aminopropylamino)ethylphosphorothioic acid; GSH, glutathione; BSO, buthionine sulfoximine; hprt, hypoxanthine-guanine phosphoribosyl transferase.
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In order to evaluate the anti-mutagenic effects of the potential chemoprotective compounds selenium and S-2-(3-aminopropylamino)ethyl phosphorothioic acid (WR-1065), CHO AA8 cells were exposed to both compounds either individually or in combination prior to irradiation. Mutation frequency following exposure to 8 Gy was evaluated by quantitation of the mutations detected at the hprt locus of these cells. Protection against radiation-induced mutation was observed for both 30 nM sodium selenite or 4 mM WR-1065. In addition, the protection against mutation induction provided by the combination of these agents appeared additive. In contrast, sodium selenite did not provide protection against radiation toxicity when provided either alone or in conjunction with WR-1065. In order to evaluate the possible mechanisms of the anti-mutagenic effects observed in these cells, glutathione peroxidase (GPx) activity was evaluated following exposure to the chemopreventative compounds. The addition of sodium selenite to the culture media resulted in a 5-fold increase in GPx activity, which was unaltered by the presence of the WR-1065. Northern analysis of RNA derived from these cells indicated that selenium supplementation resulted in a marginal increase in the mRNA for the cytosolic GPx (GSHPx-1) which was insufficient to account for the stimulation of GPx activity observed in cellular extracts. These results suggest that selenium and WR-1065 offer protection via independent mechanisms and that GPx stimulation remains a possible mechanism of the anti-mutagenic effect of selenium.

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Introduction

A large body of experimental evidence has demonstrated the chemopreventative role of selenium in carcinogenesis. Animal studies have indicated that dietary supplementation with low, non-toxic levels of selenium can inhibit tumor formation following insult with a wide variety of carcinogens (1-3). These include chemicals having diverse modes of action, exposure to different qualities of radiation, and oncogenic viruses (1-3). Protection can be observed for most organ systems with a typical reduction in tumor incidence ranging from 40%-80%. Numerous in vitro studies have likewise indicated the protective effects of selenium with regard to both mutagenesis and toxicity (4-8). Collectively, scientific data indicate the promise of selenium as both a chemoprotective and chemopreventative agent and provide the impetus to study and understand the mechanism of action of selenium-containing compounds.

In protein, selenium exists predominately in the form of selenocysteine. This amino acid is encoded by the UGA codon, which more typically signals the termination of translation (9). In mammalian cells, several dozen selenoproteins have been observed, only a few of which have been characterized in detail (10). The best studied of these is the glutathione peroxidase (GPx*) family of proteins. These important anti-oxidant enzymes contain a single UGA-encoded selenocysteine moiety and utilize reducing equivalents from glutathione to detoxify both hydrogen and lipid peroxides (11). The cytosolic GPx, GSHPx-1, is the best characterized of these proteins. It is expressed at varying levels in most tissue types and, unlike catalase, is not sequestered in any particular subcellular compartment. It is also distinguished from catalase in its ability to detoxify both hydrogen and lipid peroxides. Given the role for GSHPx-1 in the elimination of reactive oxygen species, it might follow that the
chemopreventative properties described above for selenium may, at least in part, be mediated via its effects on GPx proteins. However, this is often regarded as not to be the case as older studies have indicated that chemopreventative doses of selenium did not cause a measurable change in GPx activity (12,13). In addition to GPx effects, it has been speculated that selenium may exert its protective effects by altering carcinogen metabolism (14), influencing the cell cycle (15) and the induction or inhibition of apoptosis (16,17).

The radioprotective compound WR-2721 has been shown to be both anti-mutagenic and anti-carcinogenic in animal model systems (18-21). It is activated in biological systems by dephosphorylation to its thiol form, referred to as WR-1065. This activated form has been shown to be protective against cytotoxicity when administered to cells just prior to exposure to radiation or chemotherapeutic drugs (22-25). In contrast, the anti-mutagenic properties of WR-1065 can be exhibited when the drug is added after exposure to these toxic agents, suggesting that the effects on toxicity and mutagenesis may occur by distinct mechanisms (21,24). Possible mechanisms proposed include free radical scavenging, modifications of repair processes and alterations of chromatin structure (26).

Given the similarities between the properties of selenium and WR-1065, we investigated their combined in vitro effects on cells exposed to ionizing radiation. It is reported that both sodium selenite and WR-1065 are effective individually in reducing mutation frequency at the hprt locus and that this effect is additive when both reagents are provided simultaneously. Only WR-1065 was protective against radiation toxicity. It is also observed that the low levels of sodium selenite which provide protection against radiation-induced mutation significantly induced glutathione peroxidase enzyme indicating that the activity of GPx 's may contribute to the radioprotection offered by selenium supplementation.
Material and methods

Cells and culture conditions

The Chinese hamster ovary (CHO) cell line CHO-AA8 was grown in a-minimal essential medium (a-MEM; Gibco, Grand Island, NY) with 10% fetal bovine serum (Biologos, Naperville, IL) in a humidified atmosphere containing 5% CO2 and 95% air at 37°C. WR-1065 was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment (National Cancer Institute) and was dissolved in phosphate-buffered saline (PBS) (8.1 mM Na2HPO4. 1.5 mM KH2PO4, 0.14 M NaCl, 2.6 mM KCl) at a 1.0 M concentration and sterilized by filtration prior to use. Sodium selenite (Sigma Chemical Co., St. Louis, MO) was prepared in H2O as a 3 μM stock solution sterilized by filtration. For studies including selenium in the culture medium, cells were maintained in 30 nM sodium selenite for at least three days prior experimental manipulation and maintained at this concentration throughout all manipulations. Cells were exposed to 4 mM WR-1065 for thirty minutes prior to irradiation after which the WR-1065 was removed by resuspension in standard medium.

Irradiation Conditions

Cell suspensions were irradiated at ice bath temperatures. Cells were irradiated with 60Co γ-rays from a γ-beam 650 irradiator (Atomic Energy of Canada).

Survival and mutation analysis

All cell cultures were grown in a-MEM medium containing hypoxanthine (10 μg/ml), aminopterin (0.4 μl/ml) and thymidine (5 μg/ml) for 24 h to reduce the background of spontaneous hprt mutants. The mutation frequency at the hprt locus was determined by seeding at least 10^6 surviving cells per experiment. Cells were grown in non-selective medium for 8 days to allow for the expression of the induced mutations. 8 X 10^4 cells were seeded into each of 20 plates and exposed to 5 μg/ml 6-thioguanine (Sigma Chemical Co., St. Louis, MO) in standard growth medium for 7 days and then stained with 0.5% methylene blue. Mutation frequency is expressed as mutants/10^6 viable cells. Radiation induced mutation frequencies were corrected for the
appropriate background and experiments were performed in triplicate. For survival studies, cells were exposed to g-rays as described above and survival was determined by plating the appropriate numbers of cells to give between 80 and 200 colonies/dish after 10 days incubation.

**GPx assay**

GPx activity was measured by a standard assay which spectrophotometrically measures the oxidation of NADPH in a coupled system containing reduced glutathione, glutathione reductase, cellular extracts and hydrogen peroxide as the substrate as described elsewhere (27). The rate of oxidation of NADPH is measured at 339 nm in a Beckman DU 540 spectrophotometer at 30 second intervals over 5 minutes. The background rate of oxidation is determined without lysate. GPx activity is determined as:

\[
\Delta A_{339} - \text{Background} \times 1000 \\
0.00625 \times \text{pg protein}
\]

and is expressed as the nmoles of NADPH oxidized per minute per μg protein. At least 4 assays were performed on independent cultures.

**Northern Blot analysis**

Total RNA was isolated using the RNeasy Total RNA Kit (Qiagen) and electrophoresed in 1% formaldehyde/agarose gels and transferred to Gene Screen Plus hybridization membranes (DuPont) as described by the vendor. ³²P-labelled bovine GSHPx-1 cDNA was prepared by random oligo-labeling. Filters were washed with 0.2X SSC and 1% SDS at 65°C twice for 1 hour each at which time the filters were dried and exposed to X-ray film at -85°C using an intensifying screen. Filters were rehybridized with a chicken 7S RNA gene (28) probe as an indicator of equal loading of RNA onto the gels.

**Results**

Mutation induction at the *hprt* locus is a generally used method for assessing potentially carcinogenic DNA damage that arises after exposure of mammalian cells to a variety of insults (29). Because dietary supplementation with low levels of selenium have been reported to inhibit carcinogenesis and DNA damage (3-8,30), we
assessed whether exposure to sodium selenite could reduce the incidence of radiation-induced mutations at the \textit{hprt} locus. CHO-AA8 cells were incubated in 30 nM sodium selenite for at least three days prior to exposure to 8 Gy of $^{60}$Co $\gamma$-rays and compared to cells grown under identical conditions without selenite supplementation. As seen in Figure 1, exposure of AA8 cells to this amount of radiation resulted in an average mutation frequency of $116 \pm 10$ mutations per $10^6$ surviving cells as determined by the ability of cells to grow in 6-thioguanine. As presented in the Figure, the mutation frequency in the absence of exposure to radiation was negligible (< 2 ± 0.6 mutations/10$^6$ cells). When CHO-AA8 cells were preincubated with low levels of sodium selenite, a 37% decline in mutation frequency was observed (73±6 mutations/10$^6$ cells), indicating the efficacy of sodium selenite as an anti-mutagenic agent in this system. Similar results were obtained when cells were exposed to 40 mM WR-1065 for 30 minutes just prior to irradiation (64±4 mutations/10$^6$ cells). Under these conditions, the mutation frequency was reduced by 45%. In order to assess the combined effects of selenium and WR-1065 with regard to the protection from radiation-induced mutation, cells were treated with both agents and the mutation frequency at the \textit{hprt} locus was determined. Under these conditions, a decline in mutation frequency of 68% to 38±4 mutations/10$^6$ cells was observed (Figure 1). Thus, the exposure of AA8 cells to both selenium and WR-1065 resulted in levels of protection against radiation-induced mutation that were similar to the sum of the protection offered by the two individual agents.

The effects of selenium and WR-1065 on radiation toxicity were also examined. Exposure of CHO-AA8 to 8 Gy of $\gamma$-rays resulted in a surviving fraction of under 10%. These results and the survival of these cells incubated with 30 nM sodium selenite
and/or 4 mM WR-1065 are presented in Figure 2. As previously reported, exposure to WR-1065 significantly increased the survival of cells exposed to this dose of radiation (31). In contrast to the data obtained regarding radiation-induced mutations, incubation of these cells with sodium selenite did not significantly increase the survival of exposed cells. Similarly, incubation of CHO-AA8 cells with both WR-1065 and sodium selenite did not result in an increase in survival above that seen with WR-1065 alone. Therefore, selenium supplied in the form of sodium selenite was able to protect irradiated cells from mutation but not from toxicity in this system.

In order to evaluate possible mechanisms of the anti-mutagenic effects observed for selenium in CHO-AA8, the effects of sodium selenite supplementation on GPx enzyme activity were investigated. CHO-AA8 cells were incubated with sodium selenite and WR-1065 either alone or in combination as described above. The resulting GPx activities were compared to control cells incubated in the standard medium. GPx activity was determined by a coupled spectrophotometric assay which quantitates the amount of this enzyme from sonicated extracts (27). These results are presented in Table 1 and indicate that low-level supplementation of the culture medium with sodium selenite significantly induced GPx activity by approximately 5-fold. Incubation of these cells with WR-1065 did not result in any stimulation of GPx activity and incubation with both sodium selenite and WR-1065 did not result in stimulation above that seen with the sodium selenite alone. Stimulation of GPx activity by selenium supplementation has been reported to occur by transcriptional and/or post-transcription mechanisms (32-36). In order to evaluate the mechanism of GPx stimulation in CHO-AA8 cells, the cells were treated as described above with sodium selenite, WR-1065 or both. Total RNA was prepared and analyzed by Northern analysis using a cDNA for the cytosolic glutathione peroxidase GSHPx-1. As presented in Figure 3, GSHPx-1 mRNA levels are somewhat stimulated by exposure to sodium selenite, but to insufficient levels to account for the observed
increment in enzyme activity. As in the case of the GPx enzyme assays, no increase in steady state levels of GSHPx-1 mRNA were observed when cells were incubated with WR-1065.

Discussion

In this manuscript, the effects of selenium and WR-1065 on radiation-induced mutation induction and toxicity were examined. Both selenium and WR-1065 were effective to a similar degree in reducing the frequency of γ-ray induced mutations at the hprt locus, as indicated by survival in 6-thioguanine. When CHO-AA8 cells were incubated in both selenium and WR-1065, the protection observed was significantly more than either agent alone. In contrast, only WR-1065 protected these same cells against radiation toxicity. When the effects of radioprotective doses of selenium on GPx activity were examined, there was an approximately 5-fold increase in the activity of this anti-oxidant enzyme in selenium-supplemented cells. This observed stimulation of GPx enzyme activity could not be accounted for by increased GSHPx-1 transcription as indicated by only marginal increment in the steady state levels of GSHPx-1 mRNA. While it is possible that the elevation in GPx activity observed following media supplementation is due to the stimulation of GPx genes other than GSHPx-1, the post-transcriptional effects of selenium on GSHPx-1 enzyme activity has been documented for several cell types (32-36).

While there has been a large number of reported studies indicating the efficacy of selenium in the reduction of chemical carcinogenesis, only a few manuscripts have evaluated the radioprotective effects of selenium. Borek et al. have demonstrated a significant reduction in the X-ray-induced transformation of C3H/10T½ cells when the culture media is supplemented with non-toxic levels (2.5 μM) of sodium selenite (30). This result is consistent with data presented in this manuscript indicating low
levels of selenium are capable of reducing the mutation frequency at the *hprt* locus of γ-irradiated cells. In contrast, others have failed to detect a protective effect for selenium following irradiation of animals or cells in culture (37,38). The mechanism by which selenium protects against mutation in this study remains unknown. The stimulation of GPx activity following selenium supplementation indicates the possibility that the anti-oxidant function of this enzyme directly reduces the levels of oxidative DNA damage associated with radiation exposure. However, Se75-labelling experiments have indicated that there are several selenium-containing proteins in a mammalian cell (10), any of which might contribute to radioprotection. In addition, selenoprotein-independent mechanisms of mutation reduction are also possible (14-17).

As seen here and in previously published reports, the aminothiol WR-1065 was effective in the protection of CHO-AA8 cells from both the mutagenic and toxic effects of radiation (31). Possible mechanisms of action of this compound have included effects on cell cycle, endogenous nuclease activity, and topoisomerase I and II activities (32). Significantly, the radioprotective effects of WR-1065 are apparent even when administered at low doses up to three hours following radiation exposure (21-25). This observation argues against a simple model of radioprotection involving only free radical scavenging. Recently, it has been demonstrated that the protein synthesis inhibitor, cyclohexamide, can eliminate the post-exposure radioprotective effects of WR-1065 (26). These results are consistent with a model of WR-1065 action functioning by enhancing the fidelity of an error-prone inducible DNA repair process.

The additive effect of selenium and WR-1065 in the prevention of γ-ray induced mutation suggests that these two agents are functioning by distinct mechanisms. The thiol has been shown previously to significantly increase the levels
of glutathione (GSH) in exposed cells grown under in vitro conditions by stimulating the conversion of cystine in the media to cysteine and subsequent transport into the cell where it stimulates GSH biosynthesis (26,39). However, this cellular response to WR-1065 has been shown not to contribute to the radioprotective properties as pretreatment of the cells with BSO, a GSH synthesis inhibitor, had no effect on either enhanced survival or reduced mutagenesis (26). Although WR-1065 exposure did not result in enhanced GPx activity in cellular extracts, it remains possible the elevation of the synthesis of GSH, the hydrogen donor for GPx-mediated detoxification, influences the efficacy of GPx enzyme activity in vivo. The coupled assay used in this study only quantitates the amount of GPx protein in the extract as it is performed under conditions where GPx is limiting and the other components are in excess (27). Therefore, elevated GSH may have a stimulatory effect on GPx enzyme kinetics.

In this study, selenium was shown to neither protect CHO-AA8 from radiation toxicity nor potentiate the protection provided by WR-1065. This in contrast to a previous report indicating that selenium could enhance the radioprotective effects of the WR-1065 precursor, WR-2721, with regard to survival-enhancing and hematopoietic-regenerating effects when these compounds were provided to mice prior to exposure to 60Co radiation (40). The difference between these experiments may be due to the form of the thiol or tissue specific consequences of selenium supplementation.

In conclusion, the data presented indicated that low level supplementation of culture media with selenium in the form of sodium selenite could significantly protect CHO-AA8 cells from radiation-induced mutagenesis and that this is associated with a significant elevation of GPx activity. The additive radioprotective effects of selenium and WR-1065 suggest that these agents may function by independent mechanisms and further suggest that the combined administration of non-toxic levels of these compounds may be useful in enhancing their chemopreventative effectiveness.
Acknowledgements

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References


mouse splenocytes when administered prior to or following irradiation. Carcin., 13, 811-814.


Table 1. GPx activity in CHO-AA8 cells exposed to selenium and/or WR-1065

<table>
<thead>
<tr>
<th>Cells</th>
<th>30 nM NaSelenite</th>
<th>4 mM WR-1065</th>
<th>GPx Activity*</th>
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<tr>
<td>CHO-AA8</td>
<td>-</td>
<td>-</td>
<td>20.7±9.5</td>
</tr>
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<td>+</td>
<td>-</td>
<td>84.8±16.7</td>
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<tr>
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<td>-</td>
<td>+</td>
<td>19.9±7.5</td>
</tr>
<tr>
<td>CHO-AA8</td>
<td>+</td>
<td>+</td>
<td>84.5±9.4</td>
</tr>
</tbody>
</table>

*Activity expressed in nmoles NADPH oxidized/min/mg protein and presented ± S.D. Data obtained from four independent experiments.
**Figure Legends**

**Fig. 1.** Effects of 30 nM sodium selenite and 40 mM WR-1065 or the combination of both agents on mutation induction at the \textit{hprt} locus in CHO AA8 cells exposed to 8 Gy $^{60}$Co $\gamma$-rays. The data presented is from three independent experiments and the error bars represent the standard error of the mean (SEM).

**Fig. 2.** Effects of 30 nM sodium selenite and 40 mM WR-1065 or the combination of both agents on the survival of CHO AA8 exposed to 8 Gy $^{60}$Co $\gamma$-rays. The data presented is from three independent experiments and the errors represent the SEM.

**Fig. 3.** Northern analysis of GSHPx-1 expression following exposure of CHO AA8 cells to 30 nM sodium selenite and WR-1065 or the combination of both agents. Total RNA was prepared and electrophoresed on a 1% agarose/formaldehyde gel. The gel was transferred to a Gene Screen Plus hybridization membrane and probed with a $^{32}$-labelled bovine GSHPx-1 cDNA and exposed overnight to X-ray film at -85°C. MCF-7 human breast carcinoma total RNA (which does not express detectable levels of GSHPx-1 transcripts) and RNA from an adriamycin resistant derivative were included as negative and positive hybridization controls. The same filter was re-hybridized with a mouse 7S RNA probe as a control for equal loading of RNA onto the gel. The lines to the right of the autoradiograph indicate the mobility of the 18S and 28S ribosomal RNAs.