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Selenium supplementation acting through the induction of thioredoxin reductase and glutathione peroxidase protects the human endothelial cell line EAhy926 from damage by lipid hydroperoxides

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Abstract

The human endothelial cell line EAhy926 was used to determine the importance of selenium in preventing oxidative damage induced by *tert*-butyl hydroperoxide (*tert*-BuOOH) or oxidised low density lipoprotein (LDLox). In cells grown in a low selenium medium, *tert*-BuOOH and LDLox killed cells in a dose-dependent manner. At 555 mg/l LDLox or 300 μM *tert*-BuOOH, >80% of cells were killed after 20 h. No significant cell kill was achieved by these agents if cells were pre-incubated for 48 h with 40 nM sodium selenite, a concentration that maximally induced the activities of cytoplasmic glutathione peroxidase (cyGPX; 5.1-fold), phospholipid hydroperoxide glutathione peroxidase (PHGPX;1.9-fold) and thioredoxin reductase (TR; 3.1-fold). Selenium-deficient cells pre-treated with 1 μM gold thioglucose (GTG) (a concentration that inhibited 25% of TR activity but had no inhibitory effect on cyGPX or PHGPX activity) were significantly ($P < 0.05$) more susceptible to *tert*-BuOOH toxicity (LC$_{50}$ 110 μM) than selenium-deficient cells (LC$_{50}$ 175 μM). This was also the case for LDLox. In contrast, cells pre-treated with 40 nM selenite prior to exposure to GTG were significantly more resistant to damage from *tert*-BuOOH and LDLox than Se-deficient cells. Treatment with GTG or selenite had no significant effect on intracellular total glutathione concentrations. These results suggest that selenium supplementation, acting through induction of TR and GPX, has the potential to protect the human endothelium from oxidative damage.
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Keywords: Selenium; Endothelial cell; Thioredoxin reductase; Glutathione peroxidase; Low density lipoprotein; Human; EAhy926

1. Introduction

Endothelial cells are continually exposed to a pro-oxidant environment in the vasculature, and to the possibility of

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Abbreviations: BAEC, bovine aortic endothelial cells; DMEM, Dulbecco's modified Eagle's medium; EBSS, Earle's balanced salt solution; EC, endothelial cells; FBS, foetal bovine serum; GSH, glutathione; GTG, gold thioglucose; cyGPX, cytoplasmic glutathione peroxidase; HCAEC, human coronary artery endothelial cells; HUVEC, human umbilical vein endothelial cells; LDH, lactate dehydrogenase; LDLox, oxidised low density lipoprotein; PHGPX, phospholipid hydroperoxide glutathione peroxidase; Se, selenium; *tert*-BuOOH, tertiary-butyl hydroperoxide; TR, thioredoxin reductase

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damage by reactive oxygen species, hydrogen peroxide and lipid peroxides, etc. [1–3]. Oxidised low density lipoprotein (LDLox) is an important mediator of oxidative damage to the endothelium [4–6], and is directly cytotoxic to human endothelial and smooth muscle cells [7–11]. Lipid peroxidation of LDL by metal ions or by cells in vitro gives rise to a large variety of primary and secondary products from the lipid constituents. Some of these products react with the lysine groups of apoprotein B, resulting in recognition by the scavenger receptor [7]. Other products are cytotoxic [11].

Oxidative damage to the endothelium by LDLox may be one of the principal mechanisms in the pathogenesis of atherosclerosis [1–3]. Protection against oxidative damage is achieved through numerous enzymatic and non-enzymatic systems [12,13]. It has been suggested that the development and progression of atherosclerosis may be

inhibited by selenium (Se). This essential trace metal exerts antioxidant actions through increased expression of selenoenzymes, such as the family of glutathione peroxidases (GPX) [14–17]. More recent evidence has suggested that the selenoenzyme thioredoxin reductase (TR; EC 1.6.4.5) may be particularly important in providing an antioxidant role in the human endothelium [17,18].

Studies using cultured animal endothelial cells have provided convincing evidence that Se is essential to provide maximum protection from oxidative damage. For example, bovine aortic endothelial cells (BAEC) in culture are more resistant to cytotoxic damage by photo-generated LDLox or *tert*-butyl hydroperoxide (*tert*-BuOOH), when pre-treated with sodium selenite compared with unsupplemented controls [16]. This resistance was ascribed to increased expression of cytoplasmic GPX (cyGPX; EC 1.11.1.9) and phospholipid hydroperoxide GPX (PHGPX; EC 1.11.1.12). The involvement of other selenoenzymes, such as TR, in such a protective role was not considered.

TR is a homodimeric selenoenzyme belonging to the flavoprotein family of pyridine nucleotide-disulfide oxidoreductases, which include lipoamide dehydrogenase and glutathione reductase [19]. Each subunit of TR has a selenocysteine residue as the penultimate amino acid residue at the carboxyl terminal that is essential for catalytic activity of the enzyme [19]. Three isoforms of TR have been identified in humans, one mitochondrial and two cytoplasmic. They share considerable homology (reviewed in Ref. [20]). The predominant isoform of TR is the ubiquitous cytoplasmic form, TR1 [20]. TR is a multifunctional selenoprotein that, in conjunction with thioredoxin (Trx) and NADPH, forms a powerful dithiol-disulfide oxidoreductase system. TR1 can reduce and detoxify lipid hydroperoxides, hydrogen peroxide, and organic hydroperoxides directly using NADPH as a cofactor [19–21]. In addition, TR1 restores bioactivity in some enzymes inactivated by oxidative stress [22]. It can regenerate ascorbic acid from dehyroascorbate [23]. The TRs may also exert antioxidant action through their ability to reduce thioredoxin [19,20].

The GPXs have antioxidant function in the cell [24], with cyGPX catalysing the reduction of hydrogen peroxide and a variety of hydroperoxides [25]. PHGPX is capable of catalysing the reduction of phospholipid hydroperoxides and cholesterol hydroperoxides, many of which are not substrates for cyGPX [26]. TR can detoxify hydrogen peroxide and lipid peroxides more efficiently than the GPXs under certain conditions [21]. This, together with the high expression of TR in human EC [17,18], suggests that the selenoenzyme may be more efficient than the GPXs in defence against oxidative damage. Thus, it is possible that decreased expression and activity of these selenoproteins as a result of Se deficiency in man, increases the susceptibility of the endothelium to oxidative damage by LDLox or other lipid hydroperoxides.

Gold thioglucose (GTG) interacts with Se residues in selenoenzymes [27], and thus inhibits their activity. Sele-

noenzymes show marked variation in their sensitivity to inhibition by GTG. The GPXs are relatively resistant to inhibition by gold compounds. TR is very sensitive, having an $IC_{50} \sim 1000$-fold lower than that of the GPXs [28]. Gold compounds can be administered to animals in specific doses that can inhibit TR activity without modifying the activity of the GPX [29]. Thus, the use of different concentrations of GTG may offer a convenient tool to elucidate the relative importance of selenoenzymes in antioxidant defence of endothelial cells.

Sodium selenite pre-treatment affords protection against the harmful effects of *tert*-BuOOH in human endothelial cells from different sources [17]. However, no studies in human endothelial cells have investigated the possibility that Se can protect such cells from the harmful effects of LDLox. The human endothelial cell line EAhy926, established by hybridising primary human umbilical vein endothelial cells (HUVEC) with A549 human lung tumour cells [30], has been used in a number of functional studies. EAhy926 retain many of the differentiated functions common to primary endothelial cells beyond 100 passages. These functions include the expression of von Willebrand Factor [30], prostacyclin formation [31], and expression of endothelin-1 [32]. This cell line is susceptible to damage from LDLox [10]. We have used this as a model system to determine if human endothelial cells can be protected from the harmful effects of LDLox by selenite. In addition, we have used GTG at selective concentrations that predominantly inhibit TR, to examine whether the enzyme plays a significant antioxidant role in human endothelial cells.

2. Materials and methods

2.1. Chemicals and cell culture reagents

GTG, sodium selenite, 5,5′-dithiobis(2-nitrobenzoate) (DTNB), hydrogen peroxide, hypoxanthine, glutathione, aminopterin, thymidine media supplement (HAT), lactate dehydrogenase (LDH) assay kit, NADPH, and *tert*-BuOOH were supplied by Sigma Aldrich, Poole, Dorset, UK.

Dulbecco's modified Eagle's medium (DMEM) with (25 mM HEPES) and 4500 mg/l glucose, phosphate-buffered saline (PBS), Earle's balanced salt solution (EBSS), foetal bovine serum (FBS) were supplied by Gibco, Life Technologies, Paisley, UK.

Cell culture plastics were supplied by Iwaki, Japan.

2.2. Cell culture

The human endothelial cell line EAhy926, derived from umbilical vein endothelial cells, was a kind donation from Professor C.-J.S. Edgell, University of North Carolina, Chapel Hill, NC, USA. The cell line was maintained in high glucose (4.5 g/l) DMEM containing 10% FBS, 5 mM hypoxanthine, 0.02 mM aminopterin, and 0.8 mM thymi-

dine, in a humidified atmosphere of 5% CO_2, 95% air at 37 °C. The cells were passaged weekly using 0.25% trypsin–0.02% EDTA solution. EAhy926 cells displayed the characteristic morphology of endothelial cells in culture and stained positively for von Willebrand Factor.

The selenium content of the basal medium (without FBS), determined by acid digestion followed by fluorimetric analysis [33,34], was 0.35 nM and was classified as 'selenium-deficient medium'.

2.3. Determination of cellular integrity by measurement of LDH retention

Cell viability was assessed, in 24-well plates, as the percentage retention of LDH by the cell layer after 20 h exposure to *tert*-BuOOH or LDLox. Intracellular LDH activity in cells and in the culture medium was determined using a kit method (Sigma Diagnostics), modified for use on the Cobas Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, UK). The culture medium (1 ml) was removed for analysis, and the cells washed twice with 1 ml EBSS. The cells were then lysed in 0.5 ml of 0.5% Triton X-100 (in PBS, pH 7.4). After 15 min, the cell lysates were collected and the wells were washed with a further 0.5 ml EBSS and the washings combined with the respective lysates. Cell debris in the culture medium and cell lysates was removed by centrifugation at $11,500 \times g$ for 10 min prior to assay.

The LDH activity was also measured in culture media that had not been in contact with cells as a measure of endogenous LDH in the culture medium (blank). All results were blank-corrected. Results were expressed as percent LDH activity retained.

2.4. Total protein measurement

The Bradford assay [35] was used to measure total protein in the native LDL, oxLDL and cells using BSA as the standard. All enzyme activity results were corrected for total protein measured using this method.

2.5. Measurement of TR activity

TR activity was measured by an optimised method based on the method of Hill et al. [36] using DTNB (5 mM) as substrate in the presence and absence of 720 nM GTG (final concentration). This method was adapted for use on the Cobas FARA centrifugal analyser (Roche Diagnostics). All samples were measured in duplicate. Results were corrected for total protein content and one unit of TR activity was defined as 1 μmol of DTNB reduced per minute.

2.6. Measurement of cytoplasmic GPX, phospholipid GPX and total glutathione

cyGPX activity was measured by monitoring the rate of oxidation of NADPH at 340 nm in the presence of H_2O_2 (2.2 mM), using a Unicam UV/Vis spectrometer (UV4) linked to a computer installed with 'Vision' software [37]. All samples were measured in triplicate and results were corrected for total protein concentration. PHGPX activity was determined using the same assay system as for cyGPX, but with phosphatidyl choline hydroperoxide as the substrate. Total glutathione was measured according to Tietz [38], and adapted for use on a Cobas FARA centrifugal analyser. All samples were measured in triplicate and results corrected for total protein [35]. One unit of GPX activity was defined as 1 μmol of NADPH oxidised per minute.

2.7. Preparation of LDLox

LDL was isolated from 300 to 450 ml of human citrate plasma (Blood Transfusion Service, Edinburgh) by ultracentrifugation using a Ti 45 rotor in a Beckman L8.55 ultracentrifuge (Beckman, Glenrothes, UK). Plasma (45 ml) was overlayered with 15 ml of a buffered saline solution (ρ 1.019 g/ml) containing EDTA (10 mg/l) and centrifuged for 18 h at $186,000 \times g$ max at 4 °C. The very low density lipoproteins were discarded. The density of the infranatant (40 ml) was adjusted to 1.063 g/ml by addition of 14.7 ml of buffered saline (ρ 1.182 g/ml), overlayered with 5.3 ml of saline solution (ρ 1.063 g/ml) and recentrifuged for 18 h at $186,000 \times g$. The LDL fraction was removed and transferred into 30 cm dialysis tubing (7.5 mm diameter, Spectra/Por, MW cut-off 300,000; Medicel, London) and dialysed overnight against 5 l of PBS (pH 7.4, 0.2 g/l chelex resin). The combined dialysates of known protein concentration were divided, one part was kept as native LDL (control for experiments) and the other was used to prepare LDLox as follows. Approximately 25 ml native LDL was oxidised at 37 °C using $CuCl_2$ (Cu–protein ratio 0.16 μmol/mg protein), and the formation of conjugated dienes monitored at 234 nm. At peak absorbance (usually 60–90 min after initiation), 10% excess EDTA was added to stop lipid peroxidation and the volume of the lipoprotein fraction was reduced to ~ 5–10 ml using a 30,000 MW cut-off polyethersulfone filter and an Amicon 52 filtration unit (Amicon Millipore, Bedford, MA, USA). Traces of Cu^{2+} were then removed by chromatography over a Sephadex G25 column (PD-10; Amersham Pharmacia Bio-Technics, Uppsala, Sweden) using PBS as the eluant. The tube containing LDLox was flushed with a 0.22 μm filtered stream of argon, and the LDL stored at 4 °C until required (within 2–3 weeks). Native LDL was treated in an identical manner (filtration, chromatography, storage under argon) except the fraction was not exposed to Cu^{2+}.

2.8. Statistical analysis

Comparison of all data was performed using ANOVA and the Student's *t*-test (with Welch correction as appropriate) for unpaired data. Enzyme activities in Table 1 were

Table 1
The effect of sodium selenite supplementation on the activity of TR, cyGPX, and PHGPX in EAhy926 cells [mean (U/g protein) ± S.E.]

Selenite (nM)	TR activity (U/g protein)	cyGPX activity (U/g protein)	PHGPX activity (U/g protein)
0	2.0 ± 0.5	27.3 ± 1.6	5.7 ± 1.2
1	3.2 ± 0.1[†]	40.8 ± 3.9[†]	5.4 ± 0.8
10	5.3 ± 2.6*	115.4 ± 10.2[‡]	8.8 ± 2.0[†]
50	6.1 ± 0.4[‡]	138.5 ± 5.9[‡]	11.1 ± 1.1[‡]
100	5.1 ± 0.3*	182.5 ± 26.9[‡]	10.5 ± 2.5[‡]
200	4.7 ± 0.3*	166.2 ± 29.4[‡]	10.5 ± 1.1[‡]
1000	7.4 ± 1.1[‡]	153.7 ± 13.6[‡]	12.6 ± 0.7[‡]

Cells were cultured in 75-cm^2 flasks with selenite for 48 h. All measurements are for triplicate flasks, except the controls for which $n = 4$.
One unit of GPX activity was defined as 1 μmol of NADPH oxidised per minute.
One unit of TR activity was defined as 1 μmol of DTNB reduced per minute.

 * $P < 0.01$ cf. control cells (ANOVA with Fisher's test for least significant difference).
 † $P < 0.05$ cf. control cells (ANOVA with Fisher's test for least significant difference).
 ‡ $P < 0.001$ cf. control cells (ANOVA with Fisher's test for least significant difference).

compared using ANOVA with Fisher's test for least significant difference.

3. Protocols

3.1. The ability of selenite to prevent tert-BuOOH and LDLox toxicity

EAhy926 cells were passaged into 24-well plates at a density of 5×10^5 cells/cm^2 and left to grow in high glucose (4.5 g/l) DMEM containing 10% FBS, 5 mM hypoxanthine, 0.02 mM aminopterin, and 0.8 mM thymidine for 48 h.

To assess the optimal concentration of sodium selenite for protection of EAhy926 cells from tert-BuOOH-mediated cytotoxicity, cells were pre-incubated with a range of selenite concentrations (0–1000 nM) for 48 h before being washed twice with 1 ml of EBSS. For each experiment, a concentration of tert-BuOOH was found that would kill approximately 90% of Se-deficient cells and this was determined from a pilot experiment that was performed while the cells to be used for the main experiment were being preincubated with selenite. This concentration varied slightly between experiments ranging from approximately 200 to 300 μM tert-BuOOH. Cell damage was produced by incubating cells with tert-BuOOH for 20 h. LDH retention was measured as described above, all determinations being carried out in triplicate wells.

The ability of EAhy926 cells to resist LDLox toxicity in the presence or absence of 40 nM selenite was determined as for tert-BUOOH above. Medium containing either native or oxidised LDL (prepared from the same blood donation, and diluted to a common protein value in medium) was added to the cells at the specified concentrations and cells left to incubate for 20 h. LDH activity was then measured in the medium and cell lysates as described above, and percentage LDH retention calculated. All determinations were carried out in triplicate wells.

3.2. Induction of TR, cyGPX, and PHGPX activities in EAhy926 cells by selenite

EAhy926 cells were passaged into 75-cm^2 flasks and grown to 70% confluence. The cells then received medium containing 0, 1, 10, 40, 50, 100, 200 or 1000 nM sodium selenite (triplicate flasks for each selenite concentration, and quadruplicate flasks for the control) for 48 h. Following this incubation, the cells were washed twice with 10 ml EBSS, and harvested by scraping into 20 ml EBSS. Efficiency of harvesting was determined by light microscopy. The cells were then pelleted by centrifugation at $500 \times g$ for 10 min. The EBSS was aspirated, and the pellets frozen at -70 °C until enzyme assays were carried out. Prior to enzyme activity determinations, the cell pellets were thawed and lysed by sonication (three pulses of 10 s using a Soniprep 150) on ice in 0.125 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 0.1% Triton X-100 (peroxide- and carbonyl-free). The activities of the selenoenzymes were then determined as detailed above.

3.3. Optimisation of the GTG concentration

EAhy926 cells were passaged into 75-cm^2 flasks and grown to 70% confluence. The cells then received medium containing 0, 1, 10, or 100 μM GTG (triplicate flasks for each GTG concentration, and quadruplicate flasks for the control) for 48 h. Following the incubation, the cells were washed twice with 10 ml EBSS, and harvested via scraping into 20 ml EBSS. Efficiency of harvesting was checked by light microscopy. The cells were then pelleted by centrifugation at $500 \times g$ for 10 min. The EBSS was aspirated, and the pellets frozen at -70 °C until enzyme assays were carried out. Prior to enzyme activity determinations, the cell pellets were thawed, lysed and the activities of the selenoenzymes were then determined as described above.

3.4. tert-BuOOH and LDLox toxicity in the presence of GTG

EAhy926 cells were passaged into 24-well plates in either normal medium or medium containing 40 nM selenite, and incubated for 48 h. After this time, the cells were washed twice with 1 ml EBSS. Cells then received normal unsupplemented medium, or the same medium containing either 1 or 10 μM GTG for 48 h. When the incubation was

finished, the cells were again washed, and fresh medium containing *tert*-BuOOH (0–250 μM) added for a 20h incubation. LDH retention was then measured as described above. All determinations were carried out in triplicate wells.

The effect of 1μM GTG on modifying the susceptibility of EAhy926 cells to LDLox was carried out in an identical manner to that described above using an LDLox protein concentration of 220 mg/l.

4. Results

4.1. Effect of selenite on tert-BuOOH and LDLox-induced cell damage

Pre-incubation of EAhy926 cells with sodium selenite for 48 h showed that selenite provided optimal protection from the cytotoxic effects of 300 μM *tert*-BuOOH at concentrations ranging from 10 to 50 nM ($P < 0.0005$, Fig. 1). Selenite when added at a concentration of 1000 nM was significantly toxic to EAhy926 cells in the absence of *tert*-BuOOH ($P < 0.05$).

There was a concentration-dependent cytotoxicity of LDLox (Fig. 2). Native LDL was not cytotoxic to EAhy926 cells at any of the concentrations tested. In the presence of 555 mg/l LDLox, only $19.8 \pm 1.8\%$ of cells survived in the absence of selenite. In contrast, $92.7 \pm 0.4\%$ of cells pre-incubated with 40 nM selenite for 48 h survived when

exposed to (555 mg/l) LDLox ($P < 0.0005$). This concentration of 40 nM selenite was chosen as this was the lowest concentration of selenite that gave optimal protection from *tert*-BuOOH (Fig. 1).

4.2. Effect of selenite on GPX and TR activity

Incubation of EAhy926 cells with 50 nM sodium selenite resulted in maximal expression of TR and PHGPX while maximal expression of cyGPX was achieved at a selenite concentration of 100 nM. Significant induction of cyGPX ($P < 0.05$), and TR ($P < 0.01$) was first achieved with a concentration of 1 nM selenite, and for PHGPX ($P < 0.05$) with 10 nM selenite (Table 1).

4.3. Effect of GTG on GPX and TR activity

At a concentration of 1 μM GTG, $75 \pm 7.0\%$ of TR activity was retained ($P < 0.05$ cf. control cells). There was no significant loss of cyGPX or PHGPX activity (Table 2). Using 10 μM GTG, $15 \pm 10\%$ of TR activity, $40 \pm 4\%$ of cyGPX, and $65 \pm 3\%$ of PHGPX activity were retained compared to control cells ($P < 0.01$, $P < 0.05$, and $P < 0.005$, respectively). When GTG was added at a concentration of 100 μM, marked inhibition of all selenoenzymes was observed such that $0.5 \pm 0.5\%$, $15.0 \pm 1.6\%$, and $54 \pm 7\%$ of enzyme activities were retained for TR, cyGPX and PHGPX, respectively ($P < 0.0005$, < 0.0005, and < 0.01 cf. control cells).
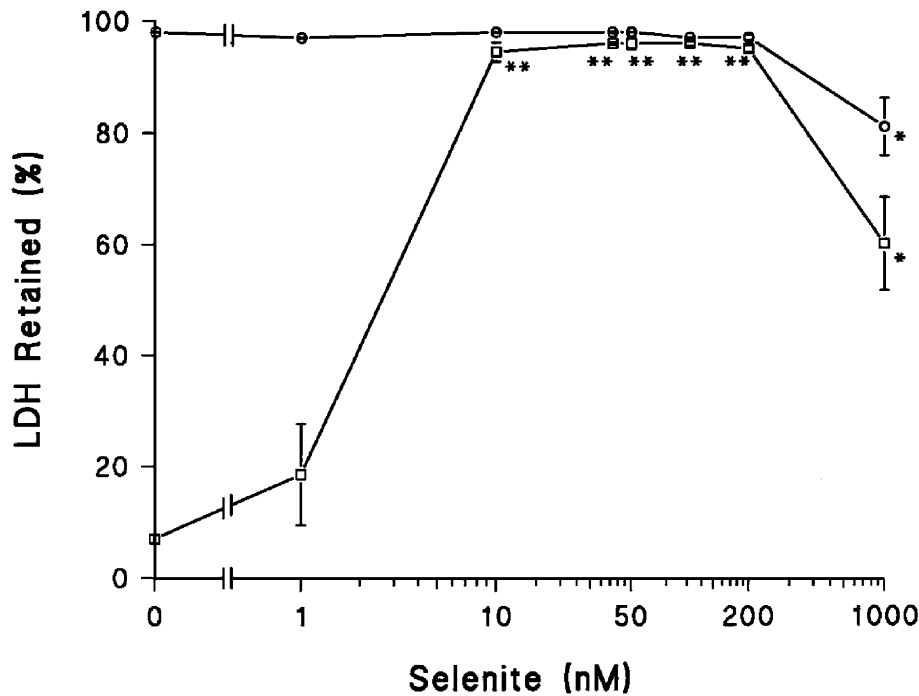

Fig. 1. Cytoprotection of EAhy926 cells from *tert*-BuOOH by sodium selenite pre-incubation. Pre-incubations with sodium selenite were for 48 h, prior to exposure to 0 μM *tert*-BuOOH (O) or 300 μM *tert*-BuOOH (□) for 20 h. Cytotoxicity was determined by LDL retention (%). All determinations are mean ± S.E. for triplicate wells. *$P < 0.05$; **$P < 0.0005$ cf. control cells (*t*-test for unpaired data, with Welch correction).
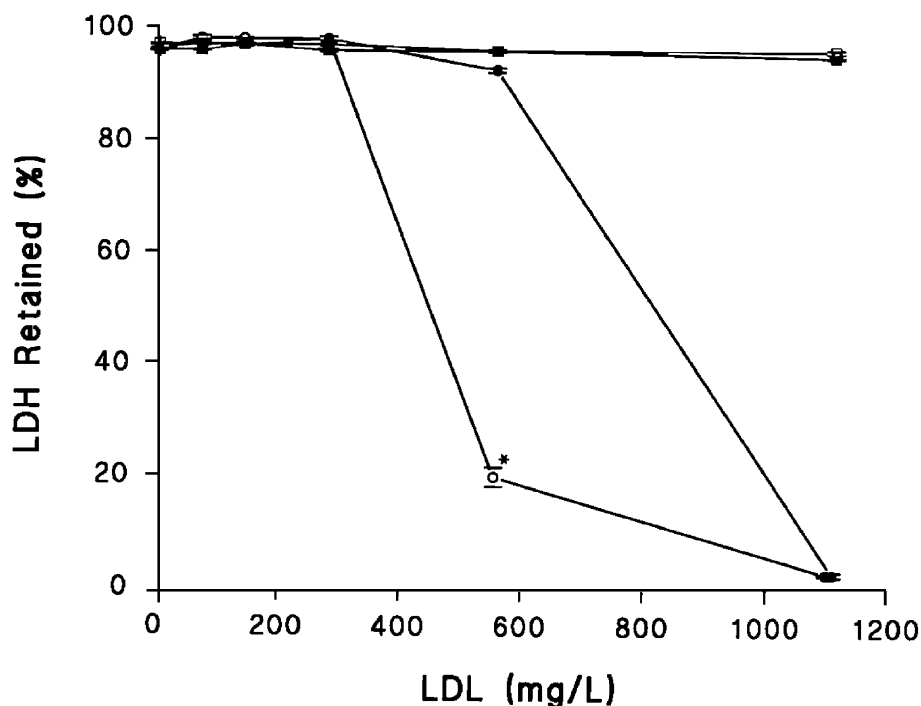
Fig. 2. Cytoprotection of EAhy926 cells from LDLox by preincubation with (40 nM) selenite. Cells were pre-incubated with sodium selenite (40 nM) for 48 h. Exposure of selenium-supplemented cells (closed symbols) or selenium-deficient cells (open symbols) to native LDL (squares) or LDLox (circles) was for 24 h. Cytotoxicity was determined by LDH retention. Results shown are means of triplicate wells ± S.E. *$P < 0.0005$ cf. LDLox, cells plus Se (t-test for unpaired data).

4.4. Effect of GTG and/or selenite on oxidative cell damage

The LC$_{50}$ for tert-BuOOH for Se-deficient cells was 175 μM. The same cells treated with either 1 or 10 μM GTG showed an increase in susceptibility to tert-BuOOH toxicity (LC$_{50}$ = 110 and 75 μM, respectively). Cells pre-treated with 40 nM selenite were more resistant to cytotoxic damage by tert-BuOOH in the presence of 1 μM GTG than the corresponding cells pre-treated with 1 μM GTG alone (LC$_{50}$ 195 cf. 110 μM $P < 0.05$), or Se-deficient cells (Fig.3).

A similar pattern of results was found using LDLox as the cytotoxic agent (Table 3). Cells treated with 1 μM GTG were more susceptible to LDLox toxicity than Se-deficient cells, while preincubation with 40 nM selenite prevented LDL toxicity in cells treated with GTG.

Pretreatment with selenite (40 nM) or GTG (1 μM) had no significant effect on total glutathione concentrations in the cells (data not shown).

Table 2
The effect of gold thioglucose on the activity of TR, cyGPX, and PHGPX in EAhy926 cells not supplemented with selenite [mean (U/g protein) ± S.E.]

GTG (μM)	TR activity (U/g protein)	cyGPX activity (U/g protein)	PHGPX activity (U/g protein)
0	1.6 ± 0.1 (n=7)	39.2 ± 3.0 (n=4)	5.5 ± 0.4 (n=4)
1	1.2 ± 0.1 (n=6)*	39.6 ± 2.0 (n=3)	5.4 ± 0.3 (n=3)
10	0.2 ± 0.1 (n=6)‡	15.6 ± 1.5 (n=3)**	3.6 ± 0.2 (n=3)*
100	0.01 ± 0.01 (n=6)‡	5.9 ± 0.6 (n=3)‡	3.0 ± 0.4 (n=3)†

Results are activity determinations in duplicate from a minimum of triplicate 75-cm² flasks incubated for 48 h.
One unit of GPX activity was defined as 1 μmol of NADPH oxidised per minute.
One unit of TR activity was defined as 1 μmol of DTNB reduced per minute.
  *$P < 0.05$ cf. control cells (t-test for unpaired data, with Welch correction).
  **$P < 0.005$ cf. control cells (t-test for unpaired data, with Welch correction).
  †$P < 0.01$ cf. control cells (t-test for unpaired data, with Welch correction).
  ‡$P < 0.0005$ cf. control cells (t-test for unpaired data, with Welch correction).

Table 3
Cytotoxicity (% LDL retention) of LDLox on EAhy926 cells pre-incubated with GTG and selenite

	Control LDH retention (%)	LDLox (220 mg/l) LDH retention (%)
No pretreatment	100 ± 0.1	70 ± 2.5*
GTG (1 μM)	99 ± 0.8	60 ± 5.1*
GTG (1 μM), selenite (40 nM)	99 ± 0.4	99 ± 1.0
Selenite (40 nM)	98 ± 1.7	99 ± 1.6

The sodium selenite pre-incubation was for 48 h at a concentration of 40 nM. GTG pre-incubation was with 1 μM for 48 h. The cytoxicity of LDLox was tested at 220 mg protein per liter.
Results are mean ± S.E. determinations from triplicate wells.
Cells treated with GTG alone had significantly ($P < 0.05$) more cell death than cells not treated with GTG or selenite.
  * Significant ($P < 0.05$) differences from control cells.

5. Discussion

The endothelial cell line EAhy926 [30], has been used for a number of studies of endothelial function [8–10]. The activities of TR and the GPXs that we have found in these EAhy926 cells (Table 1) are similar to values we have reported in primary cultures of human endothelial cells [17]. Furthermore, the sensitivity of EAhy926 cells to *tert*-BuOOH (Fig. 1) and the concentrations of selenite that confer optimal protection from *tert*-BuOOH toxicity (Fig. 1) are also similar to previous results using HUVEC and human coronary artery endothelial cells (HCAEC) [17]. These data taken together indicate that the EAhy926 cell line provides an ideal and convenient model to study the role of Se in preventing oxidative damage to human endothelial cells.

As in HUVEC and HCAEC, oxidative damage to EAhy926 cells by *tert*-BuOOH can be prevented by pre-incubation with low nanomolar concentrations of selenite (Fig. 1) [17]. Protection was accompanied by significantly increased TR, cyGPX and PHGPX expression, which was optimal with a selenite concentration of approximately 50 nM (Table 1). While *tert*-BuOOH is widely used as a model agent to induce oxidative stress, in vitro, LDLox is considered to be the principal agent that damages the endothelium and promotes atherogenesis. LDLox has also been shown to damage EAhy926 cells in culture [8]. Supplementation of cells with 40 nM selenite provides protection from oxidative damage initiated by LDLox (Fig. 2). Cells preincubated

with (1 μM) GTG (a concentration that inhibited TR, but not the GPXs), were more susceptible to toxicity from both *tert*-BuOOH and LDLox (Fig. 3, Table 3). These data suggest that TR plays an important role in preventing damage to human endothelial cells from oxidised lipids. Although unlikely, these data cannot exclude the possibility that GTG has inhibited another, as yet, unidentified selenoenzyme with an antioxidant role. Cells treated with GTG at a concentration that inhibited both TR and the GPXs (10 μM) were more susceptible to *tert*-BuOOH toxicity than cells in which only TR was inhibited. This suggests that under normal circumstances, both TR and the GPXs are involved in the prevention of oxidative damage to human endothelial cells. These multiple enzyme systems could act in different cellular compartments. While glutathione is important in preventing LDLox-induced damage [13], the doses of Se and GTG used in our experiments produced no significant modification to intracellular glutathione concentrations.

Gold compounds are also administered to humans for the treatment of rheumatoid arthritis, although the mechanism by which these compounds produce a therapeutic effect is unknown. Reglinski et al. [39] have shown that such treatment increases 'oxidative stress'. Our data shows that GTG increases the susceptibility of Se-deficient endothelial cells to oxidative damage and that this damage may be prevented by pre-treatment with selenite at doses that maximally induce the expression of the GPXs and TR (Fig. 3, Table 3). Countries, such as the United Kingdom, have a Se intake
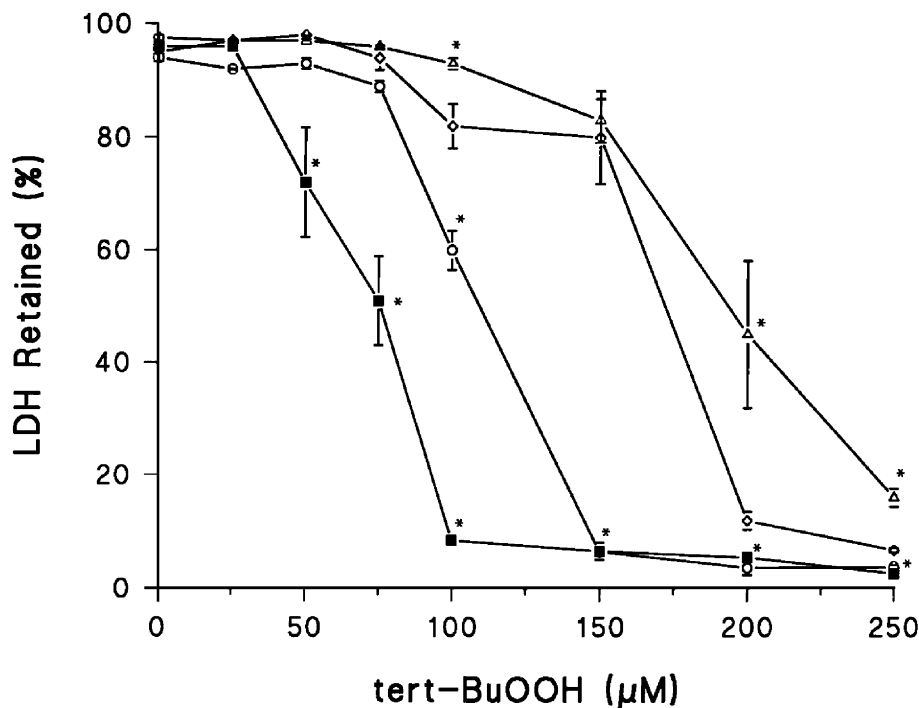

Fig. 3. Cytotoxicity profile of *tert*-BuOOH to EAhy926 cells pre-incubated with various combinations of GTG and selenite. (△) Selenite (40 nM) GTG (1 μM), (◇) No additions, (○) GTG (1 μM), (■) GTG (10 μM). Sodium selenite pre-incubation was for 48 h at a concentration of 40 nM. GTG pre-incubations were for 48 h. Results are mean ± S.E. determinations from triplicate wells. Significant (*P* < 0.05) differences from control cells are shown by (*).

that is insufficient to maximally induce selenoenzyme expression [40]. It could thus be argued that in such populations, GTG treatment may lead to damage to the endothelium. If this were the case, such patients may benefit from selenium supplementation prior to treatment with GTG. However, if the efficacious effects of GTG on rheumatoid arthritis required the production of oxidative stress, it is possible that Se supplementation may prevent the therapeutic action of GTG. Clinical trials would be required to determine the potential benefits of Se supplementation prior to GTG treatment.

Finally, our observations support the view that a low Se status would promote endothelial injury and atherosclerosis initiated by LDLox in humans [41–43]. There are no reports to our knowledge that show in vivo how the Se content of the endothelium responds to Se supplementation. However, our results suggest that selenium supplementation at doses which optimise the expression of the TR and the GPXs may have significant beneficial effects when applied to populations that have an Se intake below that currently recommended. Properly conducted controlled trials of selenium supplementation are urgently required.

Acknowledgements

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