Free Radical Research, September 2006; 40(9): 936–943

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healthcare

Selenoprotein P protects endothelial cells from oxidative damage by stimulation of glutathione peroxidase expression and activity

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Accepted by Professor A. Azzi

(Received 7 March 2006; in revised form 2 May 2006)

Abstract

A major fraction of the essential trace element selenium circulating in human blood plasma is present as selenoprotein P (SeP). As SeP associates with endothelial membranes, the participation of SeP in selenium-mediated protection against oxidative damage was investigated, using the human endothelial cell line Ea.hy926 as a model system. Hepatocyte-derived SeP prevented *tert*-butylhydroperoxide (*t*-BHP)-induced oxidative cell death of Ea.hy926 cells in a similar manner as did sodium selenite, counteracting a *t*-BHP-induced loss of cellular membrane integrity. Protection was detected after at least 10 h of SeP supplementation and it peaked at 24 h. SeP time-dependently stimulated the expression of cytosolic glutathione peroxidase (cGPx) and increased the enzymatic activities of glutathione peroxidase (GPx) and thioredoxin reductase (TR). The cGPx inhibitor mercaptosuccinate as well as the γ-glutamylcysteine synthetase inhibitor buthionine sulfoximine counteracted the SeP-mediated protection, while the TR inhibitors cisplatin and auranofin had no effect. The presented data suggest that selenium supplementation by SeP prevents oxidative damage of human endothelial cells by restoring expression and enzymatic activity of GPx.

Keywords: *Selenoprotein P, endothelial cell, Ea.hy926, oxidative stress, glutathione peroxidase, thioredoxin reductase*

Introduction

During periods of inflammation and infection, the vascular endothelium is exposed to reactive oxygen species (ROS), mainly generated by migrating leukocytes and by activated endothelial cells [1]. These ROS include the superoxide anion, hydrogen peroxide as well as hydroxyl and lipid radicals, which usually serve as signaling molecules in vascular cell function, but can induce cellular damage at higher concentrations. Oxidative stress, resulting from an imbalance of oxidants and antioxidants [2], is implicated in the initiation and progression of cardiovascular diseases such as hypertension, heart failure and atherosclerosis [3]. Therefore, dietary antioxidants providing protection against oxidative damage have been extensively studied. Among the dietary supplements with beneficial effects on endothelial cell function, inflammation markers and cardiovascular diseases, the vitamins C and E, polyphenolic compounds such as flavonoids and the trace element selenium received most attention in recent years [4,5].

Epidemiological studies in Finland and Denmark reported a correlation of low serum selenium levels with a higher risk for ischemic heart disease and atherosclerosis [6,7]. Selenium might exert its anti-atherogenic effect by directly affecting the immune

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2006 Informa UK Ltd.
DOI: 10.1080/10715760600806248

system via modulation of the phagocytic respiratory burst as well as by lowering oxidative stress in the endothelium via regulation of the arachidonic acid cascade and detoxification of hydroperoxides [4]. A number of *in vitro* studies, using endothelial cells of different origin, demonstrated the protective capacity of selenium supplementation against oxidative cell death induced by *tert*-butylhydroperoxide (*t*-BHP) or oxidised low-density lipoproteins (ox-LDL) [8,9]. Selenium was administered in the form of sodium selenite and increased the activity of the selenoenzymes cytosolic glutathione peroxidase (cGPx), phospholipid hydroperoxide (PH)-GPx and/or thioredoxin reductase (TR) [8,9], thought to be responsible for its antioxidative effects.

In vivo, most of the ingested selenium is metabolised first in the liver, where hepatocytes synthesize and secrete selenoprotein P (SeP). SeP was hypothesized to serve as transport protein for the selenium supply of peripheral target tissues [10] and this idea was recently proven by studies with SeP knock-out mice [11]. SeP comprises around 50% of selenium in human blood plasma, where its concentration has been estimated to be 40 nM [12,13]. Measurement of SeP concentration in blood plasma has been used as a marker of selenium nutritional status [14]. The hepatocyte-derived SeP found in human blood plasma is highly glycosylated, contains up to 10 selenocysteine residues and runs as two closely migrating bands with molecular weights between 55 and 72 kDa [12,15]. Immunohistochemical studies demonstrated an association of SeP with endothelial cells in liver, kidney and brain of rats [16]. Association of SeP to endothelial cell membranes occurs through binding of histidine and lysine to cellular heparin sulfates [17,18]. In addition to its role as selenium transport protein, SeP was reported to chelate heavy metals such as mercury and cadmium [19]. Furthermore, there is some evidence for direct antioxidative effects of SeP *in vivo* and *in vitro*: it protects rats from diquat-induced oxidative liver damage [20], reduces phospholipid hydroperoxides in a cell-free *in vitro* system [21] and inhibits oxidation of isolated low-density lipoproteins (LDL) [22]. In human astrocytes, constitutively expressed intracellular SeP appears to be involved in antioxidant protection under selenium-deficient conditions [23]. The current state of knowledge about the role of selenium and SeP in cellular defense systems against excess ROS and its impact on human health was recently reviewed by our group [24].

The aim of the present study was to investigate the protective capacity of the natural selenium supplier SeP against *t*-BHP-induced oxidative cell death of human endothelial cells. The data presented herein provide evidence that SeP is capable to protect peripheral target cells from oxidative stress, predominantly by stimulation of the selenium-dependent antioxidant enzyme cGPx.

Materials and methods

Reagents

Reagents for SDS-polyacrylamide gel electrophoresis were purchased from Roth (Karlsruhe, Germany). If not otherwise stated, all other reagents were obtained from Sigma (Taufkirchen, Germany). The polyclonal primary antibody against human SeP was produced in rabbits as described [25], while the polyclonal antibodies against cGPx and TR1 were from LabFrontier (Seoul, South Korea). The polyclonal antibody against poly (ADP-ribose) polymerase (PARP) was obtained from New England Biolabs (Frankfurt am Main, Germany), while the monoclonal antibody against α-tubulin was from Santa Cruz (Heidelberg, Germany). The secondary anti-rabbit IgG- horseradish peroxidase (HRP)-coupled antibody was obtained from Dianova (Hamburg, Germany) and the secondary anti-mouse IgG- HRP-coupled antibody was from Pierce (Bonn, Germany). The TR inhibitor auranofin was a kind gift from Prof. K. Becker-Brandenburg (Justus-Liebig-University, Giessen).

Cell culture

The human endothelial cell line Ea.hy926, established by fusion of the epithelial A549 cell line with HUVEC and expressing markers of primary endothelial cells [26], was kindly provided by Dr C.S. Edgell (University of North Carolina, Chapel Hill, NC); it was used between passages 10 and 18. The human hepatocytoma cell line HepG2 was a kind gift from Dr J. Bode (Heinrich-Heine-University, Duesseldorf); it was used between passages 15 and 30. Ea.hy926 cells were grown in DMEM (Sigma) and HepG2 cells were cultured in RPMI-1640 (Sigma), both supplemented with 10% fetal calf serum (PAA; Pasching, Austria), 100 U/ml penicillin, 100 µg/ml streptomycin (PAA) and 2 mM glutamax (Invitrogen; Karlsruhe, Germany). The cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Enrichment of selenoprotein P and determination of selenium content

SeP was enriched from supernatants of HepG2 cells as described [23]. Briefly, HepG2 cells were cultured in serum-free RPMI-1640 medium supplemented with 100 nM sodium selenite for 2 days. The supernatant containing the secreted SeP was collected and centrifuged with 1000*g* for 10 min at 4°C to remove cell debris. By ultrafiltration using Vivaspin 15R concentrator columns (Vivascience; Hannover, Germany) with a 30 kDa cut-off Hydrosart membrane, the supernatant was concentrated 40-fold and SeP was assessed by immunoblotting.

The selenium content in the concentrated HepG2 supernatant was determined by a fluorimetric assay [11]. The selenium concentration was calculated according to a standard curve of serial dilutions of sodium selenite in water (1 nM–10 μM), which were treated in parallel with the sample. The SeP concentration was calculated from the selenium content of the sample, assuming that SeP contains the maximal number of 10 selenium atoms per molecule.

Immunoblotting

Cells were washed with ice-cold PBS and lysed at 4°C in 1% SDS. The protein concentration of the cell lysates was determined by DC Protein Assay (Bio-Rad; Muenchen, Germany). Aliquots of cell lysates or supernatants were run on SDS–polyacrylamide gels and the proteins were electroblotted onto Hybond-ECL nitrocellulose membranes (GE Amersham; Freiburg, Germany). Membranes were blocked in Tris-buffered saline (TBS) with 0.1% Tween 20 and 5% non-fat dry milk. Primary antibodies were used at a 1:1000 dilution, while secondary antibodies were diluted 1:20 000 in blocking buffer. Immunodetection was carried out by a chemiluminescence system (SuperSignal West Pico Substrate; Pierce) on Hyperfilm-ECL (GE Amersham). Molecular sizes of the bands were calculated by comparison with a prestained protein marker (Fermentas; St. Leon-Rot, Germany). For quantification of the bands, the developed films were scanned by an image-analysis system and analysed with the AIDA image software (Raytest; Straubenhardt, Germany).

Selenium-mediated protection of endothelial cells from oxidative damage

Ea.hy926 endothelial cells were cultured in 24-well plates. At 90% confluency, growth medium was replaced by serum-free medium, supplemented with 2 nM SeP (from concentrated HepG2 supernatant) or 100 nM sodium selenite for 10 or 24 h. Oxidative damage was caused by exposure to *t*-BHP (50, 100 and 250 μM) for 24 h. The protective capacity of selenium supplementation was examined by comparing cell viability with or without selenium preincubation. Cell viability was determined by measurement of the ability of the cells to metabolise MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) to a purple formazan dye as described [27].

LDH release

Ea.hy926 cells in 24-well plates were cultured in serum-free medium with or without selenium supplementation for 24 h prior to treatment with *t*-BHP (50, 100, 250 μM) for additional 24 h. Supernatants were analysed for enzymatic activity of lactate dehydrogenase (LDH) to assess membrane integrity of the cells. LDH activity was detected as described [28] by combining 14 μl supernatant and 200 μl LDH assay buffer (100 mM Tris/HCl pH 7.2, 200 mM NaCl, 2 mM sodium pyruvate, 0.4 mM NADH) and measuring NADH consumption at 340 nm with a Lambda 25 spectrophotometer (Perkin Elmer; Norwalk, CT). LDH activity was determined using the extinction coefficient of NADH at 340 nm ($\varepsilon = 6.2\,mM^{-1}cm^{-1}$).

Analysis of DNA fragmentation

Ea.hy926 cells in 10 cm-diameter dishes were cultured in serum-free medium with or without selenium supplementation for 24 h. Thereafter, cells were treated with *t*-BHP (100, 250 μM) for 4 h and DNA fragmentation was assessed using the commercially available Apoptotic DNA Ladder Kit (Roche; Mannheim, Germany) according to the manufacturer's protocol.

GPx activity assay

Ea.hy926 cells in 10 cm-diameter dishes were cultured in serum-free medium with or without selenium supplementation for 10 or 24 h. Cells were lysed in GPx assay buffer (100 mM Tris/HCl pH 7.6, 5 mM EDTA, 1 mM sodium azide, 0.1% Triton X-100), sonicated and centrifuged with 5000*g* for 10 min at 4°C. Enzymatic GPx activity in the cell extracts was detected as described [29] using GPx assay buffer supplemented with 3 mM glutathione, 600 mU/ml glutathione reductase and 0.1 mM NADPH. Reaction was started by addition of *t*-BHP to a final concentration of 50 μM and detected by measurement of NADPH consumption at 340 nm with a Lambda 25 spectrophotometer (Perkin Elmer). GPx activity was determined using the extinction coefficient of NADPH at 340 nm ($\varepsilon = 6.2\,mM^{-1}\,cm^{-1}$).

Thioredoxin reductase activity assay

Ea.hy926 cell extracts were prepared as described above. Enzymatic TR activity was measured spectrophotometrically using insulin as substrate [30]. A stock reaction mixture, consisting of 200 μl HEPES buffer (1 M, pH 7.6), 40 μl EDTA (0.2 M), 40 μl NADPH (40 mM) and 500 μl insulin (10 mg/ml), was prepared. For each sample, 40 μl reaction mixture were combined with 70 μl cell lysate and 10 μl thioredoxin (60 μM) and incubated for 1 h at 37°C. The reaction was stopped by addition of 500 μl 1 mM DTNB/ 6 M guanidine hydrochloride in 0.2 M Tris/HCl buffer (pH 8.0) and absorbance of the yellow-coloured

DTNB reaction product 5′-thionitrobenzoic acid (TNB) was measured at 412 nm with a Lambda 25 spectrophotometer (Perkin Elmer). TR activity was determined using the extinction coefficient of TNB at 412 nm ($\varepsilon = 13.6\,mM^{-1}cm^{-1}$).

Statistical analysis

All means were calculated from three independent experiments and the error bars represent standard error of the mean (SEM). Analysis of statistical significance was done by Student's *t*-test with $^{*}P < 0.05$ and $^{**}P < 0.01$ as levels of significance.

Results

SeP-mediated protection of Ea.hy926 endothelial cells from oxidative cell death

The organic hydroperoxide *t*-BHP is used as a model compound for induction of oxidative stress followed by cell death of human endothelial cells such as HUVEC and HUVEC-derived cell lines [8,31]. Exposure of endothelial Ea.hy926 cells to *t*-BHP for 24 h resulted in a dose-dependent lowering of cell viability, as measured by MTT assay: 50 μM *t*-BHP decreased cell viability to 30% compared with mock-treated controls, while 100 μM and 250 μM *t*-BHP induced complete cell death.

SeP from sodium-selenite treated HepG2 hepatocytoma cells efficiently protected Ea.hy926 cells against *t*-BHP-induced toxicity, if the cells were preincubated with 2 nM SeP for at least 10 h (Figure 1A). No protection was observed at shorter preincubation times or when cells were simultaneously treated with SeP and *t*-BHP (data not shown). Maximal protection was achieved after 24 h preincubation with SeP. After 24 h, SeP completely counteracted the cytotoxic effect of 50 μM and 100 μM *t*-BHP and increased the cell viability of Ea.hy926 cells treated with 250 μM *t*-BHP to 50% (Figure 1B). Concentrated supernatants of selenium-deficient HepG2 cells, which do not secrete SeP [23], were used as a negative control and did not protect from *t*-BHP-induced cytotoxicity (data not shown). By contrast, preincubation with 100 nM sodium selenite was used as a positive control [8] and resulted in a similar protective effect as pre-incubation with SeP (Figure 1A and B).

Hydroperoxides were reported to induce apoptotic as well as necrotic cell death in human endothelial cells [32]. Therefore, parameters of apoptotic and necrotic cell death were determined in *t*-BHP-treated Ea.hy926 cells. Leakage of LDH into the cell supernatant was measured as a parameter for necrotic loss of cell membrane integrity. Treatment with *t*-BHP increased the amount of LDH in supernatants of Ea.hy926 cells 3.5-fold, which was independent of the used concentration. Pretreatment of the cells with SeP

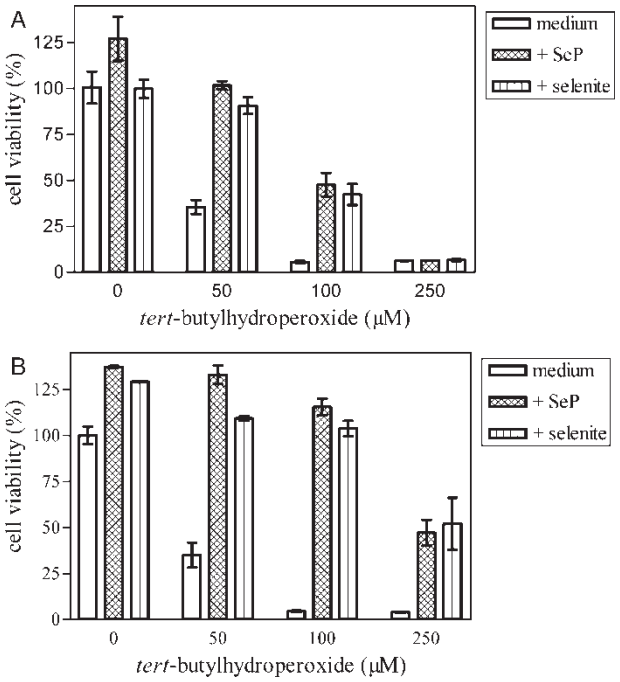

Figure 1. Protection of human endothelial cells from oxidative cell death by SeP. Ea.hy926 cells were cultured in 24-well plates and preincubated with 2 nM SeP or 100 nM sodium selenite (positive control) for 10 h (A) or 24 h (B). The cells were exposed to *t*-BHP at the indicated concentrations for 24 h and an MTT assay was performed. The metabolic activity of mock-treated cells grown in FCS-free medium was set as 100% cell viability. All experiments were done in triplicate.

or sodium selenite for 24 h counteracted the *t*-BHP-induced LDH leakage (Figure 2A). On the other hand, no evidence for *t*-BHP-induced apoptosis was found in Ea.hy926 cells. In that context, *t*-BHP did not cause DNA fragmentation (Figure 2B), cleavage of PARP (Figure 2C), translocation of cytochrome c from mitochondria to cytosol or activation of caspase-3/7 (data not shown).

SeP-stimulated selenoenzyme expression and activity in Ea.hy926 endothelial cells

In many cell types including endothelial cells, the expression and/or activity of selenium-containing isoenzymes of GPx and/or TR is increased upon selenium supplementation [8,9,33]. Therefore, protein expression of cGPx and TR1 was assessed by immunoblotting. Incubation with SeP time-dependently stimulated the expression of cGPx in selenium-deficient Ea.hy926 cells after 10 and 24 h, while TR1 expression was not affected. Likewise, sodium selenite had the same effect on cGPx and TR1 protein expression as SeP (Figure 3). The enzymatic activities of GPx and TR were both time-dependently increased by incubation with SeP or sodium selenite in a similar manner. SeP increased the GPx activity in Ea.hy926 cells 2-fold after 10 h and 3-fold after 24 h and the TR

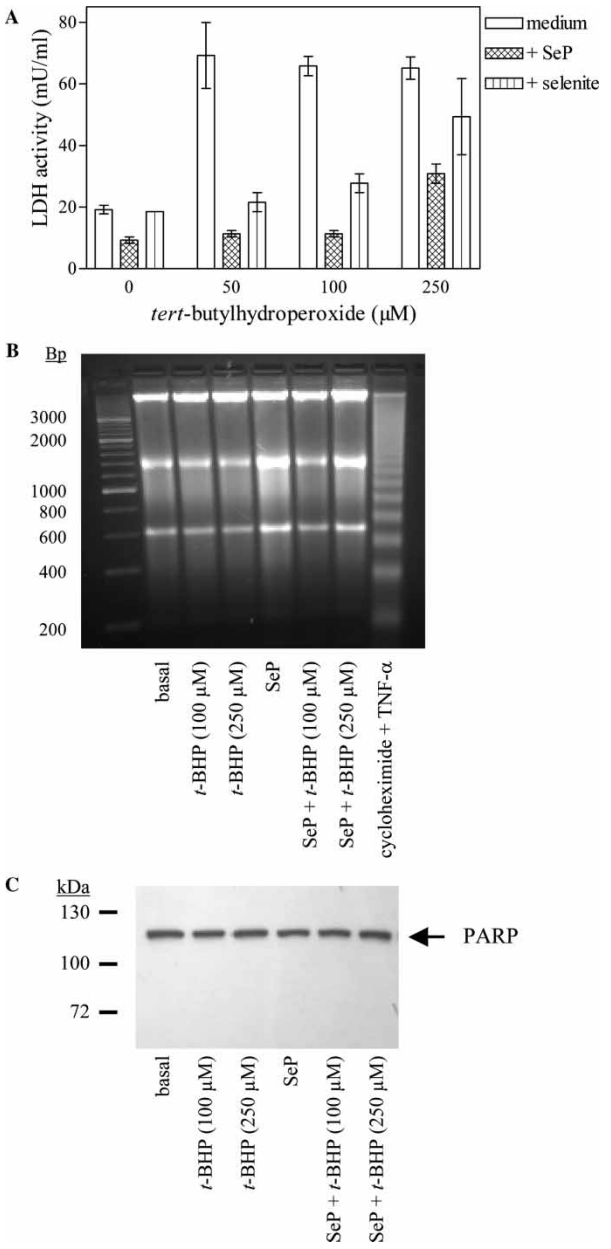

Figure 2. *t*-BHP-induced cell death in Ea.hy926 cells. Ea.hy926 cells were treated as described in Figure 1B and stimulated with *t*-BHP at the indicated concentrations for 24 h. Enzymatic activity of LDH was measured in culture supernatants for estimation of cellular membrane integrity (A). Cells were incubated with or without SeP for 24 h and stimulated with *t*-BHP at the indicated concentrations for 4 h. DNA fragmentation was assessed by Apoptotic DNA Ladder Kit. Treatment with 5 μg/ml cycloheximide + 10 ng/ml TNF-α was used as a technical control for apoptosis induction [48] (B). Cells were treated as in Figure 2B and cell lysates were analysed for protein expression of PARP and cleaved fragments of PARP by immunoblotting. (C). All experiments were performed in triplicate.

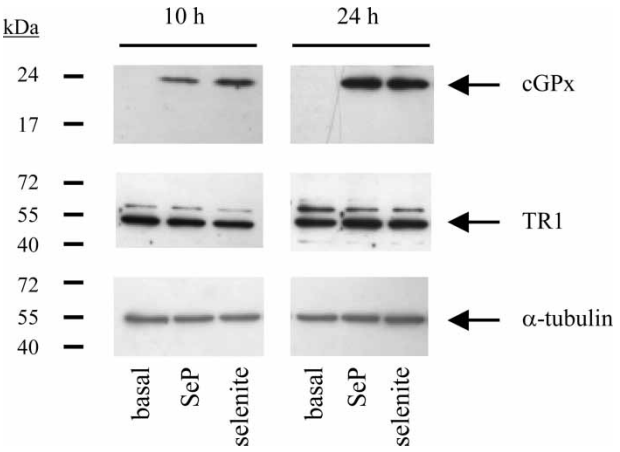

Figure 3. Selenium supplementation induces protein expression of cGPx, but not of TR. Ea.hy926 cells were treated with 2 nM SeP or 100 nM sodium selenite for 10 h or 24 h. Cell lysates were analysed for protein expression of cGPx and TR1 by immunoblotting. α-tubulin was used as loading control. Blots represent 1 out of 3 independent experiments.

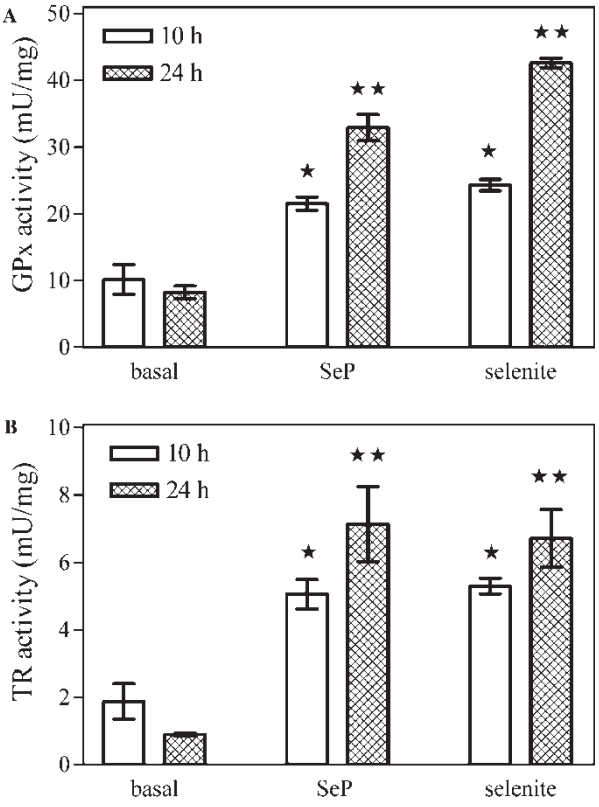

Figure 4. Selenium supplementation stimulates enzymatic activities of GPx and TR. Ea.hy926 cells were incubated with 2 nM SeP or 100 nM sodium selenite for 10 h or 24 h. GPx activities of Ea.hy926 cell extracts were determined with *t*-BHP as substrate (A) and TR activities were measured with insulin as substrate (B). Experiments were performed in triplicate. $\star P < 0.05$ and $\star\star P < 0.01$ vs. basal.

activity 2.2-fold after 10 h and 3.1-fold after 24 h (Figure 4).

In order to elucidate the participation of GPx and TR in SeP-mediated protection from *t*-BHP-induced cell death, a pharmacological approach using different

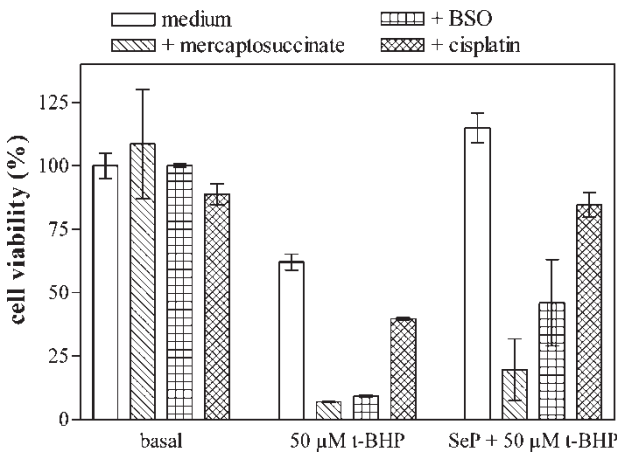

Figure 5. Effect of enzyme inhibitors on the protective effect of selenium against oxidative cell death. Ea.hy926 cells were cultured in 24-well plates. Upon treatment with 2 mM SeP or 100 nM sodium selenite for 24 h, the cells were preincubated with 100 μM mercaptosuccinate, 1 mM BSO or 50 μM cisplatin for another 24 h. Then, cells were treated with 50 μM *t*-BHP for 24 h and an MTT assay was performed. The metabolic activity of mock-treated cells grown in FCS-free medium was set as 100% cell viability. All experiments were done in triplicate.

inhibitors was applied: mercaptosuccinate, a well-known inhibitor of cGPx [34] was recently shown by our group to suppress SeP-mediated antioxidative protection of human astrocytes [23]. Buthionine sulfoximine (BSO) prevents the synthesis of the GPx cofactor glutathione by inhibiting the activity of γ-glutamylcysteine synthetase [35]. Cisplatin and auranofin were described as inhibitors for TR activity [36,37]. Non-toxic concentrations of 100 μM mercaptosuccinate or 1 mM BSO potentiated the cytotoxicity induced by 50 μM *t*-BHP in selenium-deficient Ea.hy926 cells. In SeP-pretreated Ea.hy926 cells, mercaptosuccinate and BSO both counteracted the SeP-mediated cytoprotection from cell death induced by 50 μM *t*-BHP. By contrast, 50 μM cisplatin only slightly affected the *t*-BHP-induced death of Ea.hy926 cells in selenium-deficient as well as in SeP-treated cells (Figure 5). Likewise, auranofin with an IC_{50} value of 20 nM for TR inhibition [37] did not counteract SeP-mediated cytoprotection of Ea.hy926 cells, even when used at concentrations up to 1 μM (data not shown).

Discussion

Low serum selenium levels are considered to be a risk factor in the pathogenesis of cardiovascular diseases [6,7]. Recently, Helmersson et al. [38] found an inverse correlation of serum selenium and levels of urinary prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) as well as 8-*iso*-$PGF_{2\alpha}$ in a longitudinal 27 year follow-up study of Swedish men, indicating that high concentrations of serum selenium are associated with less oxidative stress and less cyclooxygenase-mediated inflammation *in vivo*. The beneficial effects of higher serum selenium levels might be due to direct and/or indirect functions of the selenium carrier SeP in blood plasma. Serum SeP may serve as an antioxidant, protecting LDL as well as plasma proteins against oxidation [22,39]. Ox-LDL induce cell death in endothelial cells [33,40] and therefore, the SeP-mediated inhibition of LDL oxidation might directly contribute to protective selenium effects in the cardiovascular system.

On the other hand, adequate dietary selenium supply indirectly affects the antioxidant capacity of the endothelium *in vivo* by stimulating expression and/or activity of GPx and TR, as recently shown in rats which were fed selenium-deficient or selenium-supplemented diets [41]. This is corroborated by *in vitro* studies with endothelial cells and cell lines of human and bovine origin [8,9,33]. However, those studies used inorganic sodium selenite or ebselen, which might not reflect the *in vivo* situation. The data presented in our report demonstrate for the first time that the selenium carrier SeP is able to protect human endothelial cells from oxidative damage. Herein, the working concentration of SeP for cytoprotection is 2 nM, which is far below the physiological SeP level in human serum estimated to be 40 nM [12]. Recently, it was shown by our group that SeP also protects human astrocytes from *t*-BHP-induced cell death [23]. In endothelial cells as well as in astrocytes, a preincubation period was required for SeP-mediated protection. This is in accordance with protection of endothelial cells from oxidative damage by preincubation with sodium selenite [8] and supports the idea that antioxidative protection by selenium supplementation is mediated via enhanced production of selenium-containing proteins such as GPx and TR.

Numerous studies have demonstrated an involvement of the selenoenzymes cGPx and PH-GPx in selenium-mediated cytoprotective effects on endothelial cells [8,9,33,41]. Expression and activity of cGPx are particularly sensitive to selenium deficiency, which is consistent with its low ranking in the hierarchy of selenoproteins [29,42]. Under selenium-deficient conditions, cGPx rapidly undergoes a loss of expression and activity, which are regained upon selenium supplementation [41,43]. In addition to GPx, TR is also a part of the selenium-mediated defense system against oxidative stress and its enzymatic activity was reported to be stimulated in endothelial cells upon treatment with sodium selenite [8,9]. In the present study, SeP time-dependently stimulated GPx and TR activities in Ea.hy926 cells in a similar manner and furthermore, it induced protein expression of cGPx, while expression of TR1 remained unchanged (Figures 3 and 4). As SeP as well as sodium selenite-likewise stimulated the steady-state mRNA level of cGPx, but did not influence the TR1 mRNA level (data not shown), TR1 expression

seems not to be regulated by selenium in Ea.hy926 cells. However, we cannot exclude that the TR1 in selenium-deficient cells might represent a truncated form of the protein, lacking the last two amino acids including selenocysteine. Therefore, the increase of TR activity in selenium-supplemented cells could either be due to synthesis of selenocysteine-containing TR1 or by provision of selenocysteine from SeP, because free selenocysteine-derived selenocysteine was reported to stimulate the NADPH-dependent degradation of hydroperoxides by TR [44].

In an attempt to determine to what extent GPx and TR contribute to the protection of SeP-preincubated Ea.hy926 cells from *t*-BHP-induced cell death, inhibitors were used to counteract activities of GPx or TR (Figure 5). The cGPx inhibitor mercaptosuccinate as well as the γ-glutamylcysteine synthetase inhibitor BSO suppressed SeP-mediated protection of Ea.hy926 cells, while the TR inhibitors cisplatin and auranofin had only a slight inhibitory effect, suggesting that stimulation of cGPx expression and activity is mainly responsible for the protection provided by SeP supplementation. However, the basal GPx activity was reported to be significantly lower in selenium-deficient Ea.hy926 cells than in HUVEC cells [45]. Therefore, the stimulation of GPx synthesis and activity upon selenium supplementation in comparison to stimulation of TR might be stronger in Ea.hy926 cells than in primary endothelial cells. We cannot exclude that TR might play a more important role in antioxidative defense in primary endothelial cells such as HUVEC.

The mode of endothelial cell death induced by hydroperoxides is currently still under debate. Lorenz et al. [32] described the presence of apoptotic as well as necrotic cells upon *t*-BHP treatment of HUVEC, while another study showed no evidence for *t*-BHP-induced apoptosis in HUVEC and HUVEC-derived cell lines [46]. We investigated several characteristic parameters of apoptosis such as caspase activation, translocation of cytochrome c from mitochondria to cytoplasm, cleavage of PARP and DNA fragmentation, but none of these apoptotic responses were induced in *t*-BHP-treated Ea.hy926 cells. On the other hand, *t*-BHP-treatment disturbed the membrane integrity of Ea.hy926 cells (Figure 2). Therefore, we conclude that Ea.hy926 cells undergo non-apoptotic, probably necrotic cell death upon *t*-BHP-treatment. As the mechanism of cell death also depends on the power of the cellular antioxidant system [47], the *t*-BHP-induced necrosis in Ea.hy926 cells might be related to the low basal activity of GPx. This hypothesis was used to explain that ox-LDL induce necrosis in Ea.hy926 cells, but apoptosis in HUVEC [40]. Furthermore, it is in line with our finding that SeP-treated Ea.hy926 cells, which displayed enhanced GPx expression and activity, were resistant to the *t*-BHP-induced loss of membrane integrity.

Taken together, hepatocyte-derived SeP, the major selenium source in human blood plasma, was shown in this study to protect the human endothelial cell line Ea.hy926 from *t*-BHP-induced loss of membrane integrity and subsequent cell death. SeP exerted its protective effect by supplying the Ea.hy926 cells with selenium, required for synthesis of the glutathione-dependent antioxidant selenoenzyme cGPx.

Acknowledgements

This study was supported by Deutsche Forschungsgemeinschaft, Bonn, Germany (SPP Selenoproteine (Si 255/11–3)). H. Sies is a Fellow of the National Foundation for Cancer Research (NFCR), Bethesda, MD.

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