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The role of thioredoxin reductase activity in selenium-induced cytotoxicity

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Abstract

The selenoprotein thioredoxin reductase is a key enzyme in selenium metabolism, reducing selenium compounds and thereby providing selenide to synthesis of all selenoproteins. We evaluated the importance of active TrxR1 in selenium-induced cytotoxicity using transfected TrxR1 over-expressing stable Human Embryo Kidney (HEK-293) cells and modulation of activity by pretreatment with low concentration of selenite. Treatment with sodium selenite induced cytotoxity in a dose-dependent manner in both TrxR1 over-expressing and control cells. However, TrxR1 over-expressing cells, which were preincubated for 72 h with 0.1 μM selenite, were significantly more resistant to selenite cytotoxicity than control cells. To demonstrate the early effects of selenite on behaviour of HEK-293 cells, we also investigated the influence of this compound on cell motility. We observed inhibition of cell motility by 50 μM selenite immediately after administration. Moreover, TrxR1 over-expressing cells preincubated with a low concentration of selenite were more resistant to the inhibitory effect of 50 μM selenite than those not preincubated. It was also observed that the TrxR over-expressing cells showed higher TrxR1 activity than control cells and the preincubation of over-expressing cells with 0.1 μM selenite induced further significant increase in the activity of TrxR1. On the other hand, we demonstrated that TrxR1 over-expressing cells showed decreased glutathione peroxidase activity compared to control cells. These data strongly suggest that TrxR1 may be a crucial enzyme responsible for cell resistance against selenium cytotoxicity.

Keywords: Selenium; Thioredoxin reductase; Cytotoxicity; Glutathione peroxidase; Cell motility; Oxidative stress

1. Introduction

The effects of selenium compounds on cells are strictly concentration dependent. At low levels, selenium is an essential trace element inducing the expression of selenoproteins. Since many of these proteins are peroxidases, selenium in this concentration interval function as an antioxidant. Higher concentrations are toxic leading to DNA-fragmentation and cell death [1,2]. The tolerable levels differ among cell types and cells with high levels of thiols or drug resistant cells with a high rate of proliferation are generally more sensitive [3].

Since selenium compounds have well-documented inhibitory effects on the growth of tumor cells and also show cancer preventive effects, there is now a focus on this element in cancer research [1,4–6]. Several mechanisms behind these effects have been postulated, i.e. causing oxidative stress, inhibition of DNA-synthesis and induction of apoptosis [7–9]. Some selenium compounds efficiently react with thiols and oxygen resulting in oxidative stress due to depletion of thiols and the production of reactive oxygen species [10,11]. For this reason, oxidative stress is believed to be a major mechanism in selenium-induced cytotoxicity.

Abbreviations: Trx, thioredoxin; TrxR1, thioredoxin reductase 1; HEK-293, human embryo kidney cells; GR, glutathione reductase; GPx, glutathione peroxidase; DTNB, 5,5′dithiobis(nitrobenzoic acid)

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The thioredoxin system, comprising the 12 kDa protein thioredoxin (Trx), the selenoenzyme thioredoxin reductase (TrxR) and NADPH, function as a general protein disulfide reductase system [12]. Mammalian thioredoxin reductases are homodimeric flavoenzymes with a FAD, a catalytic dithiol/disulfide and a selenocysteine residue adjacent to a cysteine in each subunit of 58 kDa. Three mammalian TrxRs have so far been isolated and characterized namely the classical cytosolic (TrxR1), the mitochondrial (TrxR2) [13–15] and a third (TrxR3/TGR) [16] isolated from mouse testis. All three enzymes are selenoproteins, the selenocysteine residue being essential for catalytic activity. TrxR1 has a broad substrate specificity reducing thioredoxins from a variety of species and several different low molecular weight compounds including selenium compounds, hydroperoxides and ubiquinone [10,11,17,18]. By reduction of selenium compounds, TrxR1 provides active selenide not only to its own synthesis but also to the synthesis of all other selenoproteins. TrxR1 is thus a key enzyme in selenium metabolism and interaction of selenium compounds with TrxR1 may provide mechanisms for the physiological effects of selenium.

This study was undertaken to explore the importance of active TrxR1 in selenium-induced cytotoxicity using transfected TrxR1 over-expressing stable HEK-293 cells and modulation of activity by pretreatment with low concentrations of selenite.

2. Experimental procedures

2.1. Chemicals

Tris–HCl, EDTA, HEPES, phosphate buffered saline (PBS), sodium azide (NaN_3), H_2O_2, NaOH, HCl, ethanol, 2-propanol (analytical grade), 5,5′dithiobis(nitrobenzoic acid) (DTNB), guanidine-HCl, bovine serum albumin, sodium deoxycholate (NaDOC), fluorescein diacetate, ethidium bromide, sodium selenite, trypan blue, fetal calf serum (FCS), glutathione (GSH), glutathione peroxidase (GPx) from bovine erythrocytes, insulin from bovine pancreas and NADPH were obtained from Sigma; Dulbecco's modified Eagle's medium with F-12 nutrient mixture (DMEM/F-12), geneticin (G-418), gentamicin and trypsin from Gibco Invitrogen; Baker's yeast glutathione reductase from Fluka, *E. coli* thioredoxin (Trx) from Promega and calf thymus thioredoxin reductase from IMCO.

2.2. Cell culture

The stable Human Embryo Kidney (HEK-293) cells over-expressing TrxR1 were established as described previously [18,19].

Cells were cultured in Dulbecco's modified Eagle's medium and F-12 nutrient mixture (ratio 1:1) supplemented with 10% fetal calf serum (FCS) and 100 μg/ml gentamicin in humidified atmosphere with 5% CO_2 at 37 °C. The cells were preincubated with or without 0.1 μM sodium selenite for 72 h prior to experiments. This selenite concentration has been shown to be the optimal for TrxR activity in these cell lines according to titration experiments described elsewhere [18,19].

2.3. Cell viability

To determine the effect of sodium selenite on cell viability, the investigated cells preincubated with or without 0.1 μM sodium selenite for 48 h were plated to 12-wells plates at the density 100,000 cells per well in culture medium and cultured for next 24 h in the same medium (i.e. the total time of preincubation with 0.1 μM was 72 h). Then cells were incubated for another 24 h in the presence of sodium selenite at the final concentrations between 2.5 and 100 μM. Thereafter, the cells attached to the plate were harvested with trypsin treatment and the number of viable cells was determined using trypan blue exclusion test. In some experiments, the fluorescein diacetate and ethidium bromide tests [20] were applied to examine the viability of HEK-293 cells. Cells were prepared for the tests as described above and then the percentage of viable cells was determined. In this assay, both adherent and non-adherent cells were collected for analysis.

2.4. Motility of individual cell monitored by time-lapse technique

Observations of cell motility were carried out on an inverted Leica DM IRBE microscope. All experiments were done at 37 °C. The investigated cells were plated into Corning flask at a density of 25,000 cells/cm^2 and incubated in cultured medium for 24 h at 37 °C. Then the cell movement was recorded for 4 h, followed by the addition of 50 μM sodium selenite and further recording for another 4 h. To examine the effect of preincubation with selenite on cellular motility, cells were grown in culture medium supplemented with 0.1 μM sodium selenite for 72 h.

Cell images were recorded with a Hamamatsu camera and processed with the Open Lab program (Improvision, Image Processing &Vision Company Limited). The cell trajectories were constructed from 48 successive cell centroid positions recorded over 4 h with a time intervals of 5 min. Descriptions of motile activity for specific experimental conditions represent summed observation of 46–161 individual cells.

The cell locomotion was characterized by the ''augmented diffusion constant'' (D^*) computed from the plot of the mean square displacement against time, given by the equation:

$$\langle L^2 \rangle = 4D^* \left\{ t - t^* \left[1 - \exp\left(\frac{-t}{t^*} \right) \right] \right\}$$

where D^* and t^* are constants, L is the length of cell displacement from the starting point to the subsequent position and t is time [21–24].

2.5. Enzyme assays

TrxR activity was determined in cell extracts by the ability to reduce insulin disulfides according to the method described by Holmgren and Björnstedt [25]. Briefly, investigated cells were preincubated for 3 days with or without 0.1 μM sodium selenite and after that treated with 10 μM selenite for 24 h. Then cells were harvested by scraping and washed twice with PBS. After centrifugation (100 × *g*, 7 min, 4 °C), the cell pellets were frozen for at least 30 min (−135 °C) and then the pellets were homogenized by sonication in 50 mM Tris–HCl, (pH 7.6) and 1 mM EDTA. The homogenates thus obtained were centrifuged at 25,200 × *g* for 7 min and the resulting supernatants were used for biochemical measurements.

The GPx enzyme activity was determined by the method described by Lawrence and Burk [26], but determined in 96-well plate.

2.6. Data analysis

The statistical significance was determined by the Student's *t*-test. Values of $p < 0.01$ were considered to be significant. Statistical significance tests for evaluating changes in D^* were determined according to the method described by Gail and Boone [27].

3. Results

3.1. Effects of selenite on cell viability

The cytotoxic effect of sodium selenite on control HEK-IRES and over-expressing TrxR1 (HEK-TrxR15 and HEK-TrxR11) cells are shown in Fig. 1A–C. In each experiment, the cells adhered to the substratum were collected 24 h after addition of different concentrations of sodium selenite and the number of viable cells was determined using trypan blue exclusion test. It was found that sodium selenite treatment caused a dose-dependent decrease in the number of viable, adhered HEK-IRES, HEK-TrxR15 and HEK-TrxR11 cells. Cells with high TrxR1 activity were less sensitive to selenite compared to control. The differences in the sensitivity of the control and the over-expressing cells were obvious after preincubation for 72 h with 0.1 μM selenite (IC_{50}: 7.5, 40 and 70 μM for HEK-IRES, HEK-TrxR15 and HEK-TrxR11 cells, respectively). The preincubated HEK-TrxR15 and HEK-TrxR11 cells were also less sensitive for selenite treatment than their non-preincubated equivalents. The sensitivity of HEK-IRES cells to the selenite treatment, however, did not alter after preincubation with 0.1 μM selenite.

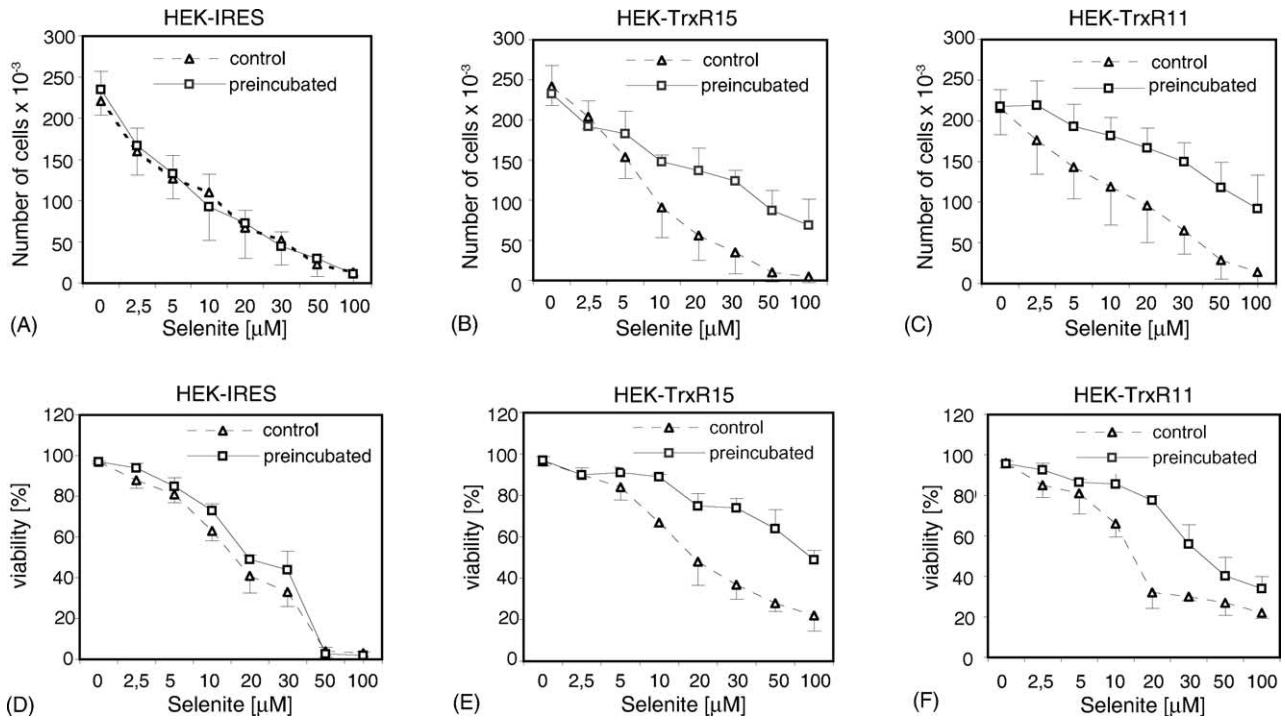

Fig. 1. Effects of selenite on the viability of HEK-IRES, HEK-TrxR15 and HEK-TrxR11 cells preincubated with or without 0.1 μM sodium selenite for 3 days. (A–C) Number of viable, adherent cells 24 h after addition of different concentrations of sodium selenite. (D–F) Percentage of viable cells (in this assay both adherent and non-adherent cells were collected and proportion of viable cells was calculated on the basis of fluorescein diacetate and ethidium bromide test). Each experiment was repeated at least three times in triplicate. Each experimental point represents mean (±S.D.) of all experiments.

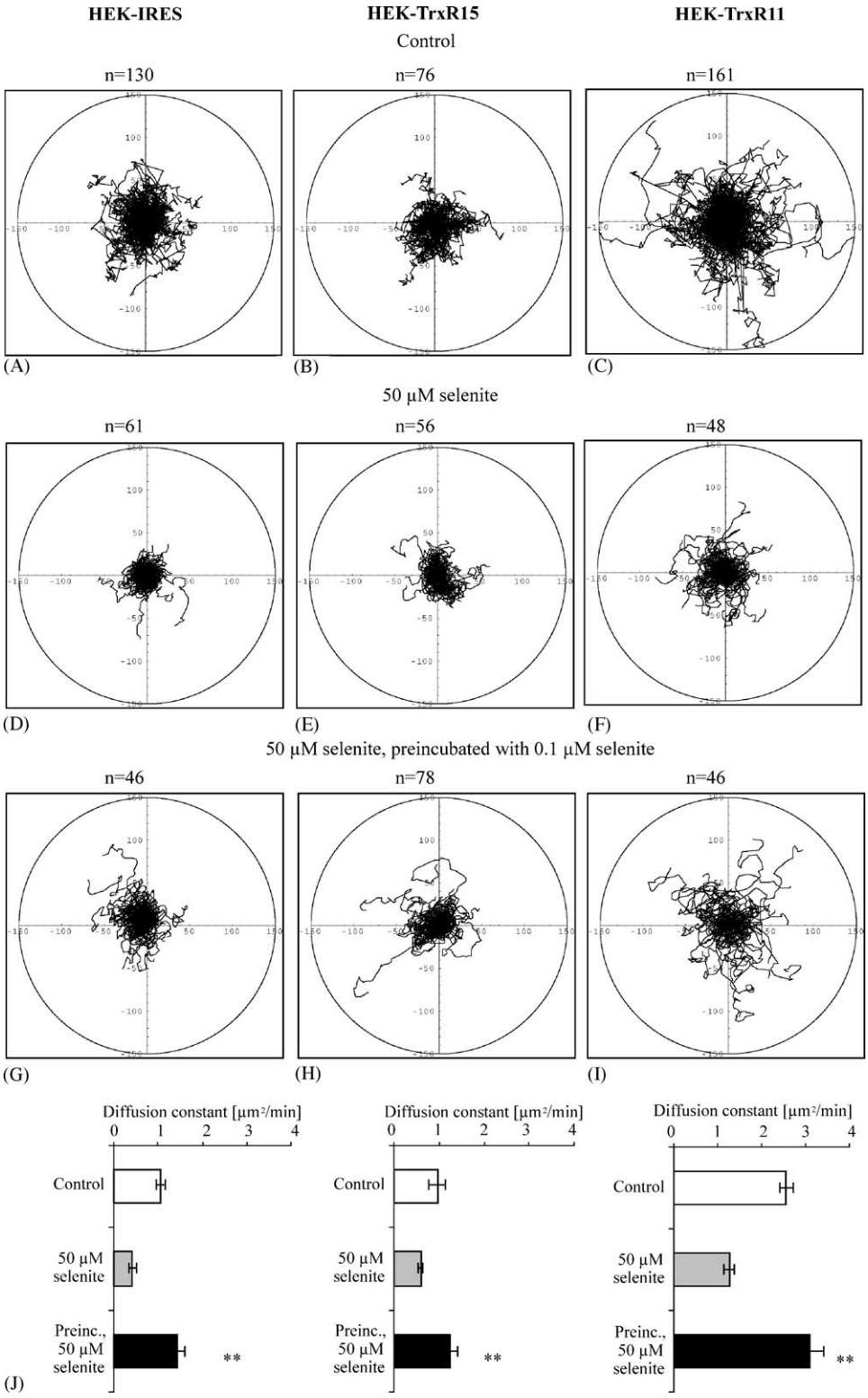

Fig. 2. Effects of selenite on the motility of HEK-IRES, HEK-TrxR15 and HEK-TrxR11 cells. Composite trajectories of HEK-IRES (A, D, G), HEK-TrxR15 (B, E, H) and HEK-TrxR11 (C, F, I) cells are displayed in circular diagrams drawn with the initial point of each trajectory placed at the origin of the plot. Each panel shows the trajectories of 46–161 individual cells. The plots show the effect of sodium selenite at concentration 50 μM on the motility of cells preincubated with or without 0.1 μM sodium selenite for 3 days. (J) Summary of quantitative data showing the effect of selenite on motility of HEK-IRES, HEK-TrxR15 and HEK-TrxR11. Error bars represent the standard error for diffusion constant parameter estimated during fitting procedure. Axis scale in μm. **Statistically significant vs. 50 μM selenite treated cells ($p < 0.01$).

Since detachment of some cells from the plastic substratum was observed at higher concentrations of sodium selenite, both adherent and non-adherent cells were collected for analysis in the further experiments. To determine the percentage of viable cells after incubation with different concentrations of sodium selenite, we used a fluorescein diacetate and ethidium bromide test. This test allows to discriminate between necrotic cells, which have the cell membrane totally compromised (ethidium bromide staining) and viable cells in which esterases are able to convert fluorescein diacetate to the fluoroescein. Treatment with sodium selenite resulted in a dose-dependent cytotoxicity for all investigated cells. These results also showed that the cells over-expressing TrxR1 preincubated for 3 days in medium containing 0.1 μM sodium selenite were more resistant for the acute cytotoxic effects of higher concentrations of sodium selenite (Fig. 1D–F).

3.2. Effect of selenite on cell motility

The effect of selenite on cell viability was investigated after prolonged incubation of cells. The motility of cells after cytotoxic selenite treatment enabled us to observe the early effects of this compound on different cell lines with or without preincubation with a low concentration of selenite. To determine the influence of selenite on the locomotory properties of HEK-293 cells, we analysed the behaviour of HEK-IRES, HEK-TrxR15 and HEK-TrxR11 cells migrating on plastic substratum for 4 h after the addition of selenite to a final concentration of 50 μM. The tracks used for analysis of cell migration under selenite stress revealed significant differences in their properties (Fig. 2). The cell migration was characterized by the ''augmented diffusion constant'' (D^*) computed from the plot of the mean square displacement against time (Fig. 2J). The inhibition of cell movements by 50 μM selenite was observed immediately after its administration.

Similar to the results on cell viability, the over-expressing cells preincubated with a low concentration of selenite were more resistant to the inhibitory effects of 50 μM selenite than their non-preincubated equivalents. Furthermore, we observed that the inhibition of motility was more pronounced in control (60%) than in the over-expressing cells (39 and 51% for HEK-TrxR15 and HEK-TrxR11, respectively). The 72 h preincubation of cells with 0.1 μM selenite had not statistically significant effect on cell motility (data not shown).

3.3. Effect of selenite on cellular TrxR1 activity

The effect described above strongly suggests that TrxR1 is an enzyme, which is responsible for cellular resistance against selenium toxicity. The investigations of TrxR1 and GPx activities were performed after 24 h incubation of cells with 10 μM selenite.

The results presented in Fig. 3 show that both HEK-TrxR15 (Fig. 3B) and HEK-TrxR11 (Fig. 3C) have much higher TrxR1 activity than the control HEK-IRES cells (Fig. 3A), as previously shown [18,19]. Moreover, further increase in TrxR1 activity was observed in the over-expressing cells after preincubation with 0.1 μM selenite. The result corresponds to the observation that preincubated HEK-TrxR15 and HEK-TrxR11 cells are more resistant to toxic selenite treatments as described above. The treatment of cells with 10 μM selenite did not significantly affect TrxR1 activity in any of the cell lines.

3.4. Effect of selenite on cellular GPx activity

GPx activity increased in both control and over-expressing cells as a result of preincubation with 0.1 μM selenite (Fig. 4). Interestingly, it was observed that TrxR1 over-expressing cells showed decreased GPx activity compared to control cells. There was no decrease of the GPx activity observed when cells were treated with 10 μM selenite. The

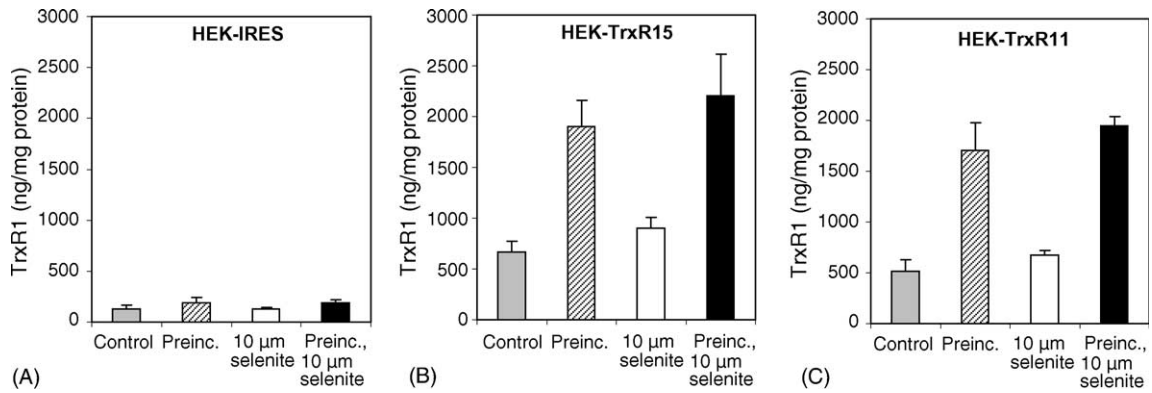

Fig. 3. The effects of sodium selenite on TrxR1 activity in 25,200 × *g* supernatants from HEK-IRES (A), HEK-TrxR15 (B) and HEK-TrxR11 (C) cells. The cells were grown for 72 h in culture medium without or with 0.1 μM sodium selenite and then treated for the next 24 h with 10 μM sodium selenite. Control (gray bar); control preincubated with 0.1 μM selenite (striped bar); 10 μM selenite (white bar); 10 μM selenite, preincubated with 0.1 μM selenite (black bar). Data represent the mean of three experiments. Error bars represent S.D.

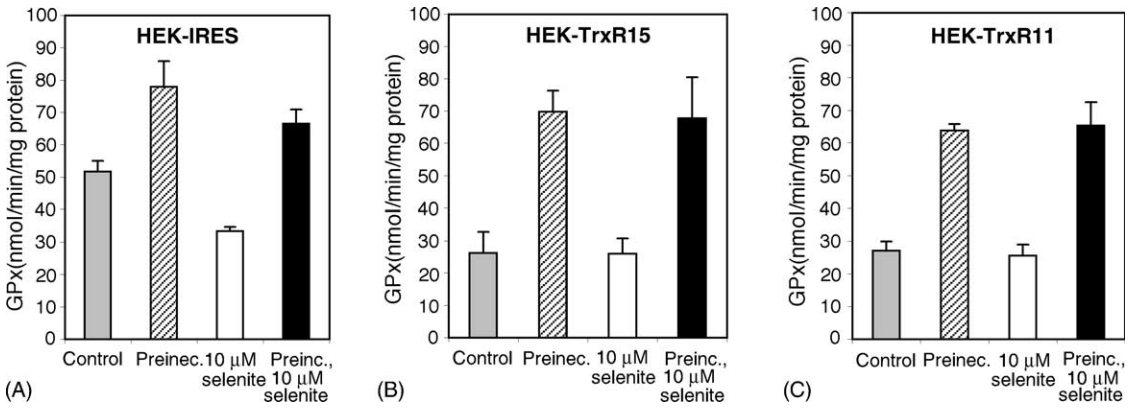

Fig. 4. The effects of sodium selenite on GPx activity in 25,200 × *g* supernatants from HEK-IRES (A), HEK-TrxR15 (B) and HEK-TrxR11 (C) cells. The cells were grown for 72 h in culture medium without or with 0.1 μM sodium selenite and then treated for the next 24 h with 10 μM sodium selenite. Control (gray bar); control preincubated with 0.1 μM selenite (striped bar); 10 μM selenite (white bar); 10 μM selenite, preincubated with 0.1 μM selenite (black bar). Data represent the mean of three experiments. Error bars represent S.D.

present results confirm the hypothesis that TrxR1 can be one of the most important enzymes in defence of the cell against selenium-induced cytotoxicity.

4. Discussion

In the present study, an elevated resistance to selenium cytotoxicity was demonstrated as increased viability in HEK-TrxR11 and HEK-TrxR15 compared to control HEK-IRES cells after incubation with increasing concentrations of selenite for 24 h. The effects were especially prominent after preincubation with a low (0.1 μM) concentration of selenite. This concentration optimises the synthesis of active, selenium saturated, TrxR1 and probably prevents the accumulation of truncated TrxR which may induce apoptosis [28].

The protective effect of active TrxR1 was also demonstrated in assays measuring changes in cell motility and displacement. Changes in cell motility may be early signs of cytotoxicity [29,30], and also in this respect, high level of active TrxR1 protected cells from toxic effects of selenite. Interestingly, in contrary to the other assays, the inhibitory effect of selenite on the motility of HEK-IRES was reduced by preincubation with a low concentration of sodium selenite. We suggest that in the case of cell migration also the relatively low level of TrxR and GPx induction is important in cell resistance against noxious agents although also other enzymes may be important.

Selenium is an essential element with vital antioxidant properties in low concentrations. In this low concentration span, selenium compounds induce the expression of selenoproteins of which several are antioxidant enzymes, e.g. TrxR1 and GPx. In moderate doses, selenium compounds inhibit cell growth and give prooxidant effects especially in rapidly dividing cells with a high intracellular redox state [3]. High concentrations of selenium lead to acute toxicity with DNA-strand breaks and necrosis.

The prooxidant effects of selenium compounds are explained by the fact that several selenium compounds efficiently react with thiols and that selenide formed in these reactions may redox-cycle with oxygen leading to a non-stoichiometric oxidation of intracellular thiols under the formation of reactive oxygen species [10,11]. These reactive oxygen species contribute to the oxidation of additional thiols and the initiation of lipid peroxidation.

The results of our experiments with TrxR1 over-expressing cells preincubated with 0.1 μM selenite strongly suggest that active selenium saturated TrxR1 is one of the most important enzymes in protection from selenium cytotoxicity. The enzyme may protect cells against selenium cytotoxicity by at least three different mechanisms. One mechanism is the direct reduction and detoxification of hydroperoxides including lipid-hydroperoxides and hydrogen peroxide [17]. The second mechanism involves reduction of thioredoxin and regeneration of antioxidants like ubiquinone [18]. The third and maybe most important mechanism is to restore intracellular thiols lost by oxidation and also to reduce selenite to elemental selenium with a comparably low toxicity [10,11].

In an other study, we observed that TrxR1 over-expressing cells are less resistant to hydrogen peroxide treatment than control cells, and the cells only recovered basic resistance after preincubation with 0.1 μM selenite (Sroka J., Madeja Z., Nyström C., Björkhem-Bergman L., Nordman T., Damdimopoulos A.E., Nalvarte I., Eriksson L.C., Spyrou G., Björnstedt M. and Olsson J.M. The effects of triethyllead and hydrogen peroxide on cells with different expression of thioredoxin reductase and glutathione peroxidase, manuscript in preparation). These data suggest that all observations on differential selenium-induced cytotoxicity in the over-expressing cells compared to control cells is not a general effect of noxious agents but a specific effect of selenite. Furthermore, these data indicate that the detoxification of hydroperoxides does not seem to be the major mechanism in the protective effect of TrxR, but indicates

that the third mechanism mentioned above is a more likely explanation.

Measurements of the activities of TrxR1 and GPx revealed differential responses after preincubation with a low concentration of selenite and incubation with toxic levels in the different cell lines. In the TrxR1 over-expressing cells, the basal level of GPx activity was repressed indicating a competition of selenium and that TrxR1 was prioritised in a situation of selenium shortage. However, addition of 0.1 μM selenite restored the GPx activity.

This study demonstrates increased resistance to selenium cytotoxicity in cells with high levels of active thioredoxin reductase. The results are supported by recently published data concerning the effects of selenite on doxorubicin-resistant and sensitive human small cell carcinoma of the lung cell lines [3,7]. In these studies, doxorubicin-sensitive cells responded by a four-fold increase in TrxR activity after exposure to selenite. These cells had a four-fold higher IC_{50} for selenite compared to the doxorubicin-resistant cells that only marginally increased their activity of TrxR after selenite treatment. We therefore conclude that high level of active TrxR or a capacity to respond by inducing the expression of TrxR is a crucial mechanism for cells to survive exposure to sub-toxic/toxic levels of selenium compounds.

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