Selenium spares ascorbate and α-tocopherol in cultured liver cell lines under oxidant stress

Xia Li, Kristina E. Hill, Raymond F. Burk, James M. May*

Department of Medicine, 715 Medical Research Building II, Vanderbilt University School of Medicine, Nashville, TN 37232-6303, USA

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Abstract The selenoenzyme thioredoxin reductase (TR) can recycle ascorbic acid, which in turn can recycle α-tocopherol. Therefore, we evaluated the role of selenium in ascorbic acid recycling and in protection against oxidant-induced loss of α-tocopherol in cultured liver cells. Treatment of HepG2 or H4IIE cultured liver cells for 48 h with sodium selenite (0–116 nmol/l) tripled the activity of the selenoenzyme TR, measured as aurothioglucose-sensitive dehydroascorbic acid (DHA) reduction. However, selenium did not increase the ability of H4IIE cells to take up and reduce 2 mM DHA, despite a 25% increase in ascorbate-dependent ferricyanide reduction (which reflects cellular ascorbate recycling). Nonetheless, selenium supplements both spared ascorbate in overnight cultures of H4IIE cells, and prevented loss of cellular α-tocopherol in response to an oxidant stress induced by either ferricyanide or diazobenzene sulfonate. Whereas TR contributes little to ascorbate recycling in H4IIE cells, selenium spares ascorbate in culture and α-tocopherol in response to an oxidant stress. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Ascorbate; Ascorbate free radical; Selenium; Oxidant stress

1. Introduction

Liver is the site of ascorbic acid biosynthesis in most mammals. Liver is also capable of recycling the vitamin from its two-electron oxidized form, dehydroascorbic acid (DHA). Since humans lack the ability to synthesize ascorbic acid, such recycling may help to supplement stores of the vitamin supplied by diet. The capacity for DHA reduction by hepatocytes is substantial. For example, uptake and reduction of DHA by cultured liver cell lines of both rat and human origin is complete in several minutes [1]. In response to DHA loading in these cell lines, ascorbate acutely reaches concentrations of 5–20 mM [1,2], although the steady-state ascorbate concentration in rat liver is about 1 mM [3].

In recent studies, we found that DHA reduction in cultured liver cell lines is largely GSH-dependent [2]. GSH can reduce DHA directly [4], or act as a co-factor for DHA reduction by enzymes such as glutaredoxin and protein disulfide isomerase [5,6]. NADPH-dependent DHA reduction has also been described in liver, and is mediated by 3α-hydroxysteroid dehydrogenase [7] and the selenoenzyme thioredoxin reductase (TR) [3]. In extracts from liver, we found that more than two-thirds of NADPH-dependent DHA reduction could be attributed to TR, based on specific inhibition by aurothioglucose [3]. TR can also reduce the ascorbate free radical (AFR) in liver [8], which brings up the question as to whether the TR system in liver contributes significantly to the recycling of ascorbate from its oxidized forms in this organ.

One approach to answering the above question is to modify the endogenous activity of TR and determine whether that affects DHA reduction, either directly or indirectly through effects on ascorbate recycling. Since TR is a selenoenzyme, and is inactive without selenium, its activity can be decreased by lowering the amount of selenium available to an animal or to cultured cells. In rats deficient in selenium due to dietary deprivation, we found that dialyzed liver extracts had only 25% of the TR-dependent DHA reducing capacity of extracts from control animals [3]. Moreover, the liver ascorbate content of selenium-deficient rats was decreased by 20–30% [3]. The selenium content in most tissue culture media is inadequate to maintain optimal activities of selenoenzymes such as glutathione peroxidase [9], phospholipid hydroperoxide glutathione peroxidase, and TR [10]. Therefore, in the present studies we have modified the activity of TR by supplementing selenium in culture and then assessed how this affects the ability of the cells to reduce DHA and to withstand an oxidant stress. Whereas we were unable to show that selenium deficiency affects the ability of the cells to reduce DHA to ascorbate, we did find that selenium supplements spare ascorbate and α-tocopherol in the cells.

2. Materials and methods

2.1. Materials

Sodium selenite was obtained from Sigma/Aldrich Chemical Co. (St. Louis, MO, USA). DHA was prepared from ascorbate just before use by bromine oxidation, as previously described [11].

2.2. Cell culture and modification of cell selenium content

HepG2 cells, which are derived from a human hepatocellular carcinoma, were grown in six-well plates in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum. H4IIE cells, which are derived from a rat hepatoma, were cultured in six-well plates in Dulbecco's modified Eagle's medium containing 2.5% (v/v) fetal calf serum and 2.5% newborn calf serum. The cell content of selenium was modified as previously described [9]. Briefly, cells cultured to confluence in six-well plates were cultured for 48 h in serum-free medium. Selenium was added to the indicated concentration (0–116 nmol/l),

*Corresponding author. Fax: (1)-615-936 1667.
E-mail address: james.may@mcmail.vanderbilt.edu (J.M. May).

Abbreviations: AFR, ascorbate free radical; DABS, diazobenzene sulfonic acid; DHA, dehydroascorbic acid; PBS, phosphate-buffered saline; TR, thioredoxin reductase

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and the incubation was continued for another 48 h in serum-free medium before starting an experiment.

2.3. Measurement of TR-dependent DHA reduction

TR activity in overnight-dialyzed liver cell extracts was measured following overnight dialysis of cell extracts. Cells were scraped from four wells of a six-well plate in 1.5 ml of phosphate-buffered saline (PBS) and transferred to a microfuge tube. PBS was prepared using de-ionized water to contain 12.5 mM sodium phosphate, 140 mM sodium chloride, pH 7.4. The suspension was frozen in dry ice–acetone and allowed to thaw on ice. The extract was microfuged at 3°C for 5 min at 13 000 × *g*. The supernatant was removed and centrifuged at 3°C for 1 h at 100 000 × *g* in a Beckman T-100 ultracentrifuge. The resulting supernatant was dialyzed overnight against three changes of Tris–EDTA buffer in dialysis tubing with a molecular weight cut-off of 3500. The dialysate was then taken for assay of DHA reduction as previously described [3].

2.4. Measurement of DHA uptake and reduction, and cell ascorbate and of α-tocopherol

For measurement of DHA uptake and reduction, cells cultured in six-well plates were rinsed three times in 2 ml of PBS at 37°C. Freshly prepared DHA was added to a concentration of 2 mM in PBS containing 5 mM D-glucose. The cells were incubated for 30 min at 37°C, followed by rapid removal of the medium and one rinse in 2 ml of PBS before assay of ascorbate in the cells.

The ascorbate content of rinsed cell monolayers in six-well plates was determined as follows. Cells were lysed by addition of 0.5 ml of ice cold 80% methanol (v/v) that contained 1 mM EDTA. After scraping the cells from the dish using a rubber spatula, the lysate from each well was removed and saved. Each well was rinsed again with 0.5 ml of methanol/EDTA, and the methanolic solutions were combined in a microfuge tube. Cell debris was pelleted in a microfuge at 13 000 × *g* for 5 min at 3°C. Aliquots of the supernatant were taken for assay of ascorbate as previously described [12] using ion pair high-performance liquid chromatography (HPLC) with electrochemical detection. Ascorbate was expressed as a concentration inside the cells, based on a measured intracellular water space of 2.8 μl per mg protein, or 1 μl per well of cells [2].

The α-tocopherol content of cultured liver cells was measured by adding 0.1 ml of a 5 mg/ml solution of pyrogallol to each well containing cells from which buffer had been removed, followed by 0.4 ml of 3% (w/v) sodium dodecyl sulfate. The solution was mixed and the cells were scraped from the plate with a rubber spatula. The suspension was removed from the plate to a glass culture tube on ice. Another aliquot of 0.2 ml of 3% sodium dodecyl sulfate was added, and the remaining cell debris was transferred to the glass tube. The well was rinsed with 0.7 ml of reagent alcohol (95 parts ethanol, five parts methanol), and this was added to the cell extract and mixed by vortexing. Heptane (0.6 ml) was added to each tube, and mixed by vortexing for 2 min. The layers were allowed to separate and an aliquot of the clear upper heptane phase was taken for assay of α-tocopherol by HPLC using electrochemical detection [12].

2.5. Measurement of ferricyanide reduction

Cells that had been cultured for 48 h in the absence or presence of 116 nmol/l sodium selenite were rinsed twice with PBS and incubated at 37°C in PBS that contained 5 mM D-glucose and 1 mM DHA as noted. After 15 min, ferricyanide was added to a concentration of 2 mM and the incubation was continued for an additional 30 min at 37°C before aliquots of the medium were removed for determination of ferrocyanide by the method of Avron and Shavit [13].

2.6. Data analysis and statistical methods

Data are expressed as mean ± S.D. from the indicated number of experiments. Differences between two treatments were analyzed by paired *t*-testing, and between multiple treatments by one-way repeated measures analysis of variance with post-hoc analysis by Tukey's test, using the statistical software SigmaStat 2.0 (Jandel Scientific, San Rafael, CA, USA).

3. Results

To determine whether TR might contribute to DHA reduction in cultured liver cells, it was first necessary to establish that the activity of TR was increased by the selenium content of the culture medium. This was done by treating either H4IIE cells (Fig. 1A) or HepG2 cells (Fig. 1B) with varying amounts of selenium in serum-free culture medium for 48 h, then measuring TR activity as the ability of dialyzed extracts to reduce DHA to ascorbate [3]. To enhance the activity of TR and the specificity of the assay for the enzyme, dialysates were treated with both thioredoxin and selenocystine [8]. Total activity was doubled by increasing medium concentrations of selenium in both cell lines. TR activity, measured as that sensitive to 10 μM aurothioglucose [14], was increased by selenium in both cell types by about three-fold over basal (lower curves in each panel of Fig. 1). Further, most of the increase due to selenium supplementation of the medium occurred in the aurothioglucose-sensitive fraction. In both cell types NADPH-dependent DHA reductase activity was saturable, and was half-maximal at medium selenium concentrations of 6–12 nmol/l. These results show that DHA reductase activity in these cells is selenium-dependent, and is sensitive to the amount of selenium available. Aurothioglucose-sensitive DHA reductase activity was significantly higher in H4IIE cells than in HepG2 cells across the range of selenium concentrations supplemented ($P < 0.05$). Glutathione peroxidase activity also showed a saturable increase in activity with increasing medium selenium, and was half-maximal at 6–12 nmol/l selenium in both cell lines (results not shown). Because of the

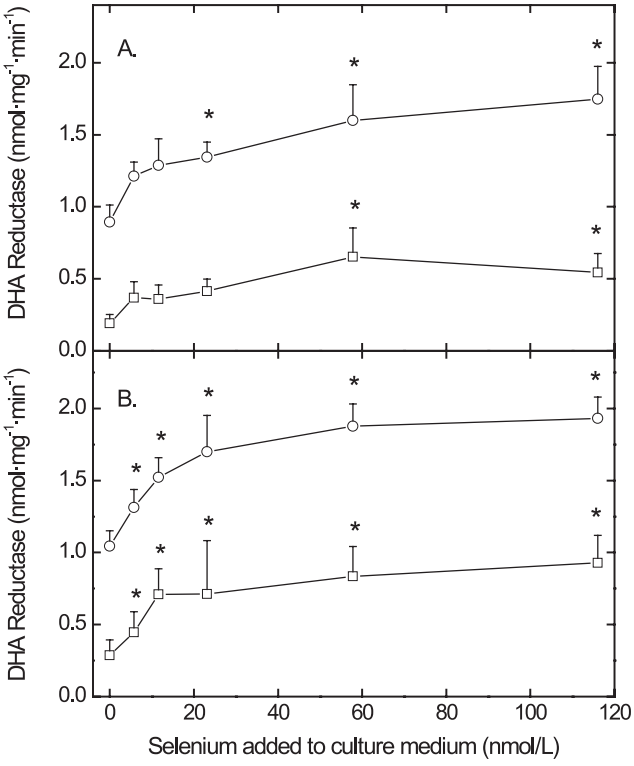

Fig. 1. Concentration dependence of TR-dependent DHA reductase activity on medium selenium supplements. HepG2 cells (A) or H4IIE cells (B) were cultured for 48 h with the indicated concentration of selenium as described under Section 2. NADPH-dependent DHA reductase activity was measured in dialyzed extracts prepared from the cells and expressed as total activity (circles), or activity that could be inhibited by 10 μM aurothioglucose (squares). Results are shown as mean ± S.E.M. from six experiments with each cell type, with an asterisk indicating $P < 0.05$ compared to the zero selenium treatment.

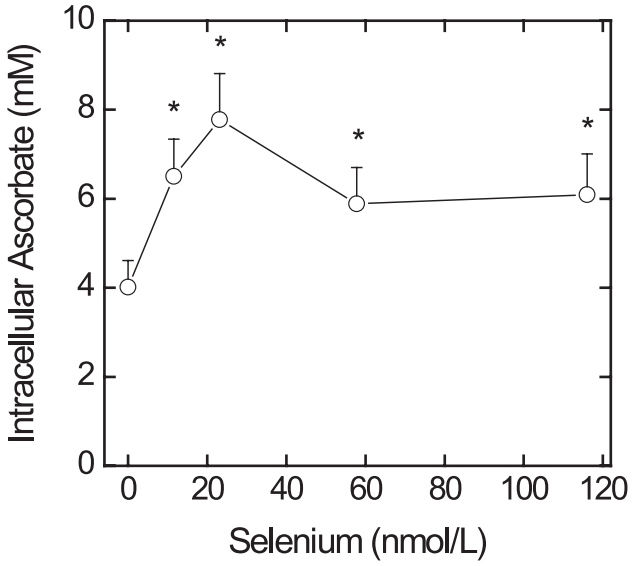

Fig. 2. Preservation of ascorbate in H4IIE cells by selenium treatment in culture. H4IIE cells were treated with the indicated selenium concentration for 48 h as described under Section 2. On the day before the experiment, cells were treated with 100 μM ascorbate and cultured for an additional 16–18 h. The cells were rinsed three times in 1 ml of PBS to remove the medium and taken for assay of ascorbate. Data are shown from nine experiments as mean ± S.E.M., with an asterisk indicating $P < 0.05$ compared to the zero selenium treatment.

greater increase in DHA reductase activity with selenium in H4IIE cells, these cells were used in further studies.

Having established that TR activity can be modified by changes in the concentration of selenium in the cell culture medium, we next tested the ability of the cells in culture to reduce DHA to ascorbate. Despite use of conditions likely to saturate DHA reduction (a 5 min time of uptake at 2 mM DHA), there was no effect of medium selenium on the ability of H4IIE cells to take up and reduce DHA to ascorbate (results not shown). To determine whether a more subtle effect of selenium might be apparent on ascorbate action in the cell, we studied the effects of selenium addition to the culture medium on the ability of H4IIE cells to reduce extracellular ferricyanide. Ferricyanide was used as an oxidant stress that would derive electrons from intracellular ascorbate and provide an index of maximal intracellular ascorbate recycling [15]. Basal rates of ferricyanide reduction by H4IIE cells were increased six-fold by incubation with DHA, from 1.7 ± 0.2 nmol/min/mg protein, to 10.8 ± 0.9 nmol/min/mg protein ($n = 7$ experiments). Culture of the cells in serum-free medium containing 116 nmol/l sodium selenite for 48 h did not affect basal rates of ferricyanide reduction, which were 2.2 ± 0.4 nmol/min/mg protein. However, when cells were loaded with ascorbate by treatment with DHA, H4IIE cells cultured in selenium-replete medium showed a significant 25% increase in their ability to reduce ferricyanide compared to selenium-deficient cells (13.5 ± 1.0 nmol/min/mg protein, $P < 0.05$ compared to 10.7 ± 0.8 nmol/min/mg protein in control cells, $n = 7$ experiments).

Despite the failure of selenium to increase acute reduction of DHA by H4IIE cells, selenium treatment did increase the ability of the cells to maintain intracellular ascorbate following an overnight incubation with 100 μM ascorbate. As shown in Fig. 2, the intracellular ascorbate concentration reached 4 mM after ascorbate treatment, and was increased by 50–80% above control across the range of selenium concentrations. This increase in intracellular ascorbate could have been due to increased recycling capacity, but also due to an enhanced ability of the cells to defend against oxidant stress in culture. To explore the latter possibility, the effect of selenium supplements to maintain α-tocopherol in H4IIE cells was evaluated. In these cells, endogenous α-tocopherol concentrations are very low, but can be supplemented by adding 100 μM α-tocopherol to the culture medium the day before the experiment. This increased the endogenous α-tocopherol concentration in H4IIE cells over 100-fold, from 0.22 ± 0.03 nmol/mg protein ($n = 6$ determinations) to between 35 and 38 nmol/mg protein, as shown in Table 1. Supplementing cells with 23 nmol/l selenium had no effect on either endogenous (0.22 ± 0.08 nmol/mg protein) or added α-tocopherol (37 ± 2 nmol/mg protein) in H4IIE cells. To determine whether selenium supplementation can prevent loss of α-tocopherol in the face of an acute oxidant stress, cells were treated with either ferricyanide or diazobenzene sulfonic acid (DABS). These reagents were used because both ferricyanide [16] and DABS [17] have been shown to oxidize α-tocopherol and increase lipid peroxidation measured as F_2-isoprostanes in human erythrocytes. In cells loaded with 100 μM α-tocopherol, acute treatment with either ferricyanide or DABS decreased the supplemented α-tocopherol contents by about 45%, an effect that was nearly reversed in cells also treated with selenium (Table 1). These results show that addition of selenium to the culture medium improves the ability of H4IIE cells to retain loaded ascorbate and α-tocopherol, the latter in response to an acute oxidant stress.

4. Discussion

Selenium is necessary for full expression of selenium-dependent enzymes and proteins in cultured cells [9,18–20]. In this work we show that adding increasing amounts of selenium as selenite to cultures of H4IIE and HepG2 liver cells increases the activity of TR, measured as its ability to reduce DHA to ascorbate (Fig. 1). Despite increased TR activity, selenium

Table 1
Effects of oxidants on the α-tocopherol content of H4IIE cells

Treatment	Ferricyanide ($n = 10$)	DABS ($n = 7$)
(−) Selenium, (−) oxidant	38 ± 2	35 ± 3
(−) Selenium, (+) oxidant	21 ± 2*	19 ± 3*
(+) Selenium, (+) oxidant	31 ± 2	33 ± 4

H4IIE cells were treated with or without selenium (23 nmol/l) for 48 h, and incubated with 100 μM α-tocopherol for 16 h. The cells were rinsed three times with 2 ml of PBS and incubated for 30 min at 37°C in PBS that contained 5 mM D-glucose and either 1 mM ferricyanide or 0.1 mM DABS, as indicated. At the end of the incubation, the cells were washed three times with 2 ml of PBS and taken for assay of α-tocopherol, which is expressed as nmol per mg protein. Results are shown as mean ± S.E.M. for the indicated number of experiments (n), with an asterisk (*) indicating $P < 0.05$ compared to both control and selenium treatment.

supplements did not increase the ability of either cell type to take up and reduce a high concentration of DHA to ascorbate over a period of 5 min. Previous results from this laboratory indicate that under these conditions transport is not limiting for DHA uptake and reduction [2]. Although TR may account for most of the NADPH-dependent DHA reduction in liver [3], our failure to see an effect of selenium on DHA reduction suggests that TR plays little role in the initial reduction of DHA in these cells. The current results agree with our previous conclusion that DHA reduction is mediated largely by GSH in H4IIE cells [2]. However, we did find that selenium treatment increased in the ability of H4IIE cells to reduce ferricyanide in DHA-treated cells by 25%. Ascorbate-dependent ferricyanide reduction has been shown to reflect the ability of cells to recycle ascorbate from its oxidized forms [15,21]. Since DHA reduction is not increased, the increased ferricyanide reduction may be due to recycling from the AFR, which is also reduced by the TR system [8].

The effect of selenium to increase ascorbate recycling, albeit small, may contribute to the ability of selenium to spare ascorbate in overnight cultures of H4IIE cells (Fig. 2). Whereas this effect could in part be due to TR, it could also be due to enhanced ability of other selenoenzymes such as glutathione peroxidase [9,18–20], and phospholipid hydroperoxide glutathione peroxidase [19,20] to scavenge hydroperoxides generated by the cells in culture. This would in turn spare ascorbic acid from destruction and result in higher intracellular ascorbate concentrations.

In addition to preserving ascorbate, selenium supplementation of H4IIE cells also spared α-tocopherol in response to an acute oxidant stress induced by ferricyanide and DABS (Table 1). Most cells in culture are probably deficient in α-tocopherol [20,22], and we found this to apply to H4IIE cells, in which α-tocopherol was increased by two orders of magnitude by an overnight treatment with 100 μM α-tocopherol. Acute treatment of α-tocopherol-supplemented cells with ferricyanide and DABS lowered α-tocopherol by almost 50%. In short-term incubations with human erythrocytes, both ferricyanide [23] and DABS [17] are limited to the extracellular space, probably because of their size and charge. Therefore, the observed sparing of α-tocopherol probably reflects that present in the plasma membrane. Others have shown that selenium supplements of a variety of cell types can prevent cell lysis and death following treatment with hydroperoxides [18,19,24]. Our finding that selenium can spare α-tocopherol and ascorbate in H4IIE cells may provide an additional mechanism by which selenium can prevent oxidant-induced cell damage in cells that contain physiologic amounts of the vitamins.

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