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Dependence of mixed disulfide formation in alveolar macrophages upon production of oxidized glutathione: effect of selenium depletion*†

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In unstressed cells, oxidized glutathione (GSSG‡) is normally less than 1% of the total glutathione in the cells [1, 2]. Formation of GSSG in cells is a consequence of the reduction of peroxides and hydroperoxides by glutathione peroxidase (glutathione H_2O_2 oxidoreductase, EC 1.11.1.9). During this process, reduced glutathione (GSH) is consumed. Removal of GSSG is possible by reduction of GSSG to GSH by glutathione reductase, or by extrusion from the cell. It has also been proposed that formation of glutathione–protein conjugates in hepatocytes is dependent on the formation of GSSG [3], so that protein acts as an additional supply of reducing equivalents:

$$GSSG + \text{protein-SH} \rightarrow GSH + \text{protein-S-S-G}$$

However, protein disulfides could be formed during oxidant stress followed by conjugation of GSH to those proteins through disulfide exchange. Whether GSSG production is essential to the formation of mixed disulfides induced by oxidant stress was, therefore, investigated.

Since glutathione peroxidase is a selenium containing enzyme, selenium deficiency could decrease GSSG formation. Using selenium deficiency with *t*-butyl hydroperoxide (tBOOH) as the oxidant stress, we examined the relationships among glutathione peroxidase activity, loss of free intracellular glutathione (the sum of GSH + GSSG), and formation of glutathione–protein conjugates in rat alveolar macrophages.

Methods

Alveolar macrophages were obtained by lavage from rats by a modification of the method of Myrvik *et al.* [4] as described in Forman *et al.* [5]. Alveolar macrophages used in the selenium studies were obtained from rats purchased at 60 g and fed *ad lib.* for 6 weeks either a selenium-deficient torula yeast diet (−Se), or the identical diet with 0.1 ppm added sodium selenite (+Se) (ICN Biochemicals, Cleveland, OH), as described in Forman *et al.* [6]. The alveolar macrophages were then resuspended in Krebs–Ringer phosphate buffer containing 10 mM HEPES, and 5 mM glucose, pH 7.4 (KRP), at 2.5×10^6 cells/ml. Aliquots were added to wellplates, and the cells were allowed to adhere for 60 min at 37°. The KRP medium was removed and replaced with fresh medium ± tBOOH (Sigma Chemical Co., St Louis, MO) as indicated. After a 30-min incubation, the medium was removed, and 0.5 ml of 0.1% Triton X-100 in KRP buffer was added. Both the original medium and the detergent-containing samples were stored at −70°. Comparison with freshly assayed medium revealed no effect of the storage method.

The samples were assayed for glutathione content by the recycling assay, which measures both GSH and GSSG [7]. The protein content of these samples was determined by the BioRad protein assay (BioRad Laboratories, Richmond, CA). Using a modification of the method of DeLucia *et al.* [8], the intracellular pool of glutathione–protein conjugates was measured as the increase in glutathione after reduction with sodium borohydride. In these experiments, 5×10^6 cells were plated in 1 ml, and the supernatant fraction and the adhered cells were removed separately as described above. The measurements obtained from the borohydride reduced samples were considered to contain the total intracellular glutathione: GSH + GSSG + the glutathione–protein conjugates.

Release of lactate dehydrogenase (LDH) was measured in medium immediately after incubation as previously described in Forman *et al.* [9].

Results and discussion

We first determined the glutathione peroxidase activity in the alveolar macrophages of rats raised on the ±Se diets. In the alveolar macrophages of rats raised on the selenium-deficient diet, the glutathione peroxidase activity was reduced to 14 ± 3% of the activity found in the alveolar macrophages of rats raised on the selenium-supplemented diet [6]. The effect of diminished glutathione peroxidase activity on the GSH + GSSG pool in alveolar macrophages

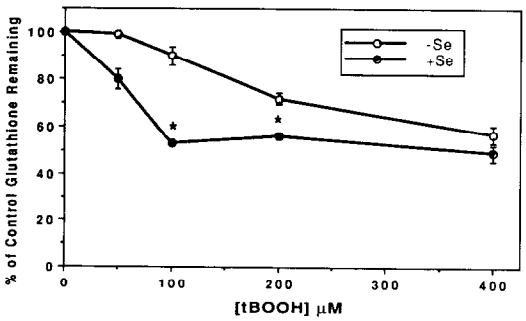

Fig. 1. Glutathione remaining in alveolar macrophages from rats fed either a selenium-deficient diet or the identical diet with selenium added, after a 30-min exposure to tBOOH. The indicated concentration of tBOOH was added to fresh medium, and the alveolar macrophages were incubated for 30 min. the medium containing the tBOOH was removed, and fresh medium containing 0.1% Triton X-100 was added to remove the viable cells. Each data point is the mean ± SE of three determinations. The control value was 9.38 ± 0.45 nmol glutathione/mg protein. Key: (*) $P < 0.05$ (Student's *t*-test).

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‡ Abbreviations: GSSG, oxidized glutathione; Se, torula yeast diet with 0.1 ppm added sodium selenite; −Se, torula yeast diet without sodium selenite; GSH, reduced glutathione; KRP, Krebs–Ringer phosphate buffer containing 10 mM *N*-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid (HEPES), and 5 mM glucose, pH 7.4; LDH, lactate dehydrogenase; and tBOOH, *t*-butyl hydroperoxide.

was determined by incubating the ±Se cells with 50–400 μM tBOOH for 30 min and then measuring the GSH + GSSG pool remaining (Fig. 1). As indicated in Fig. 1, in the +Se cells, the GSH + GSSG pool decreased with 50 μM tBOOH. The levels plateaued at nearly a 50% loss of the GSH + GSSG pool at ≥100 μM tBOOH. Even with 10 mM tBOOH no further decrease in the GSH + GSSG pool occurred (see below), suggesting that the decrease in the GSH + GSSG pool was limited by the available protein sulfhydryl groups accessible for conjugation with GSSG. In contrast, in the −Se cells, the levels of the GSH + GSSG pool did not decrease at 50 μM tBOOH and were not significantly below control until 200 μM. Nevertheless, at 400 μM tBOOH, the loss from the GSH + GSSG pool in the −Se cells was the same as with the +Se cells.

To determine whether the loss in the GSH + GSSG pool could be accounted for by formation of glutathione–protein conjugates, we measured the GSH + GSSG pool in the medium and in the cells of control and 10 mM tBOOH-treated cells from +Se rats before and after borohydride reduction. Sodium borohydride converts all of the GSSG and glutathione conjugates in mixed disulfides to GSH. Within the cells, the GSH + GSSG pool decreased to 58 ± 5% of control (Table 1) in cells incubated with 10 mM tBOOH. However, in the samples where sodium borohydride reduction of glutathione protein conjugates was performed to determine if there were changes in total glutathione (GSH + GSSG + glutathione–protein conjugates) in the cells, there was no effect of 10 mM tBOOH (Table 1). No change in the levels of glutathione in the medium occurred (Table 2). These data suggest that the entire loss from the GSH + GSSG pool was to glutathione–protein conjugates rather than to degradation or loss into the medium. Since formation of glutathione–protein mixed disulfides in the selenium-deficient cells required a greater oxidant stress than in the control cells, the formation of GSSG appears to be essential to the formation of glutathione–protein mixed disulfides. In other systems, GSSG can be transported out of cells [10–15], but in alveolar macrophages the formation of mixed disulfides is apparently the only way in which GSSG can be decreased.

No release of LDH from alveolar macrophages occurred within the first 30 min of exposure to any tBOOH concentration used, although prolonged incubation with 10 mM tBOOH results in a small but significant rate of LDH release after 60 min [9]. We found no difference in protein content/10^6 cells between alveolar macrophages obtained from either selenium adequate or selenium deficient diets.

The GSH/GSSG ratio has often been suggested as a measure of intracellular oxidant stress. The data presented here and by others [3] suggest that intracellular GSSG levels are kept low, not only by reduction via glutathione reductase, or by excretion, but also by conversion to glutathione–protein conjugates. Formation of such conjugates has been proposed as a mechanism of altered enzymatic function [16–19]. In selenium deficiency, however, less glutathione–protein mixed disulfide formation occurred upon oxidant stress than in controls. Since selenium deficiency excacerbated oxidant stress, these results suggest that glutathione conjugation to proteins may not be a major mechanism of toxicity.

Previous work has demonstrated an increase in glutathione–protein conjugates in oxidant stress. Whether this production of mixed disulfides is dependent upon or concident with oxidation of GSH to oxidized glutathione was not well established. In this study, depletion of glutathione peroxidase activity to 14% of control was accomplished by a selenium-depleted diet, while controls were fed identical diets supplemented with selenium. Formation of oxidized glutathione by glutathione peroxidase was then stimulated by incubation of alveolar macrophages with *t*-butyl hydroperoxide (50–400 μM). Our results suggest that the formation of glutathione–protein conjugates in alveolar macrophages appears to be dependent upon the prior formation of oxidized glutathione via glutathione peroxidase rather than a direct conjugation of reduced glutathione to protein.

Table 1. Determination of distribution of glutathione pools by reduction of glutathione mixed disulfides with $NaBH_4$ after treatment with 10 mM *t*-butyl hydroperoxide

	Glutathione (nmol/mg protein)	% of Control
Non-reduced samples		
Control	9.38 ± 0.45	
10 mM tBOOH	5.36 ± 0.37*	58 ± 5
Sodium borohydride reduced samples		
Control	10.15 ± 0.06	
10 mM tBOOH	10.05 ± 0.46	99 ± 5

Alveolar macrophages obtained for these studies were from selenium-sufficient rats. After 30 min of incubation the medium was removed, and a fresh aliquot of medium containing 0.1% Triton X-100 was added and the contents were removed. The samples were divided, and one was reduced by $NaBH_4$ while the other was treated identically except for the addition of buffer only. Perchloric acid precipitation was used to remove any excess $NaBH_4$ remaining, and to precipitate remaining proteins before assaying for glutathione. Values are means ± SE, N = 4 for each group.

* Significantly different from control, $P < 0.01$ (Student's *t*-test).

Table 2. Glutathione content in medium from alveolar macrophages incubated with tBOOH for 30 min

	N	Glutathione (pmol/200 μl medium)
0 min	10	243 ± 12
30 min		
Control	5	343 ± 35
10 mM tBOOH	5	255 ± 27

Alveolar macrophages obtained for these studies were from selenium-sufficient rats. Cells were plated and allowed to adhere. The medium was replaced with fresh medium containing ± 10 mM tBOOH as indicated, and the cells were incubated for 30 min. The medium was then removed, and the GSH + GSSG content in the medium was determined as described. Values are means ± SE. There was no significant difference between the groups by ANOVA. There was no release of LDH found in these samples after a 30-min incubation with 10 mM tBOOH, as we have reported previously [9].

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