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Deoxyribonucleoside Triphosphate Pools and Growth of Glutathione-Depleted 3T6 Mouse Fibroblasts

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Buthionine sulfoximine (BSO) selectively blocks g-glutamylcysteine synthetase and thereby depletes cells of glutathione (GSH). In cultures of exponentially growing 3T6 mouse fibroblasts, 0.1 mM BSO rapidly stopped GSH synthesis after treatment for 12 hours. The GSH-depleted cells grew as well as control 3T6 cells with no decrease in DNA synthesis. Furthermore, the pools of deoxyribonucleoside triphosphates (dNTPs), typically tightly regulated in cultured cells, did not change in size. Ribonucleotide reductase catalyzes the reduction of all four ribonucleotides and occupies a key position in dNTP regulation. Our data suggest that the GSH-glutaredoxin (a GSH-dependent disulfide-oxidoreductase) system is not the sole/major hydrogen carrier from NADPH for the reduction of ribonucleoside diphosphates by ribonucleotide reductase.

The four common dNTPs serve as building blocks for DNA replication and are formed by an enzymatic reduction of the corresponding ribonucleotide by the replacement of the hydroxyl group at position C-2′ with a hydrogen. The reaction is catalysed by ribonucleotide reductase which is tightly controlled by elaborate allosteric mechanisms (1,2). Either of the two small proteins (12 KDa) thioredoxin (Trx) or glutaredoxin (Grx) (also known as thioltransferase) in their dithiol form serve as the sources of electrons from NADPH (3) (Fig. 1). Both Trx and Grx contain a redox active dithiol/disulfide in the active site, with sequences Cys-Gly-Pro-Cys in thioredoxin and Cys-Pro-Tyr-Cys in glutaredoxin. Reduced thioredoxin operates together with thioredoxin reductase (TR) and NADPH, the thioredoxin system, which was the first reported hydrogen donor for ribonucleotide reductase (1). The glutaredoxin system was originally discovered in a viable strain of *E. coli* lacking thioredoxin but with a fully active NADPH dependent ribonucleotide reaction (4) and later in mammalian cells (5). The glutaredoxin system is GSH dependent and glutaredoxin couples the oxidation of the monothiol GSH to the reduction of ribonucleotides (Fig. 1).

Maintenance of the proper balance between dNTP pools is important for cell growth. For example, inhibition of ribonucleotide reductase by hydroxyurea (6) leads to cessation of *de novo* synthesis of dNTPs and consequently first slows down and then rapidly stops DNA synthesis. The relative contribution of the thioredoxin and glutaredoxin systems *in vivo* as hydrogen donors for ribonucleotide reductase in mammalian cells is not known.

In this report we show that when 3T6 mouse fibroblasts were depleted of GSH by treatment with BSO, the growth rate or DNA synthesis and the size of the deoxyribonucleoside triphosphate pools were not affected.

MATERIALS AND METHODS

Materials. [*methyl*-^{3}H]Thymidine with a specific activity of 25 Ci/mmol was obtained from Amersham. Polyd(AT), polyd(IC) and *E. coli* DNA polymerase I for pool assays were from Boehringer-Mannheim. Glutathione was purchased from Sigma and buthionine sulfoximine (BSO) was a kind gift from Prof. A. Meister, Cornell University Medical center, New York, USA.

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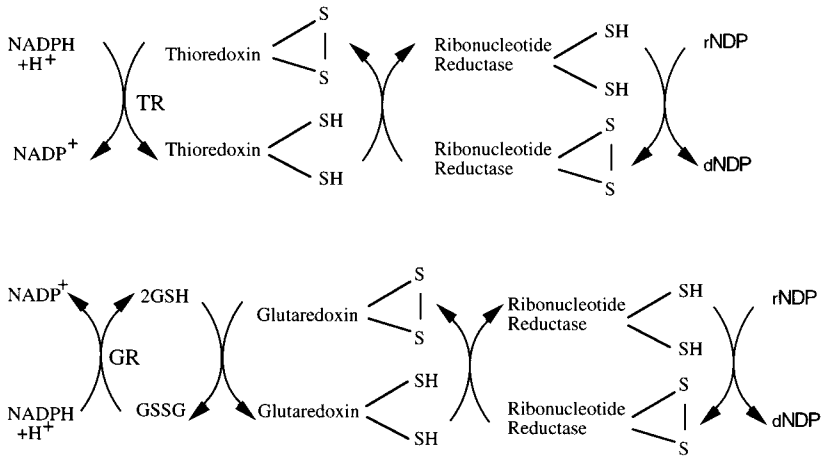

FIG. 1. The alternative hydrogen donor systems for ribonucleotide reduction by ribonucleotide reductase. Thioredoxin reductase and glutathione reductase are indicated by TR and GR, respectively.

Growth and incubation of cells. 3T6 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% inactivated horse serum at 37°C in a 7,5% CO_2 atmosphere. The cells were maintained for at most six passages and for each experiment approximately one hundred thousand cells were distributed in 5 cm cell culture dishes containing 5 ml of medium each. All experiments were done in duplicate. Results were normalised for 10^6 cells.

Analyses of dNTP pools, DNA and glutathione. At the end of the incubation each dish was washed twice rapidly with ice-cold Tris-saline. Ice-cold 60% methanol, 1.5 ml was added to each dish. After 30 min on ice the methanol solution was carefully removed, the cell layer was washed with another 1.5 ml ice cold 60% methanol and the combined methanolic extracts were used to determine the dNTP pools. The size of the dNTP pools was determined enzymatically as described (7,8). The remaining cell layer on the dish was dissolved overnight at room temperature in 2 ml 0.3 M NaOH and isotope incorporation into DNA was determined as described (9). The glutathione levels, both GSH and GSSG in the cells were determined as previously described (10).

RESULTS AND DISCUSSION

Addition of BSO to the medium of rapidly growing 3T6 cells in concentrations above 0,1 mM resulted in a time-dependent loss of intracellular glutathione. After 6 h only 32% of the GSH remained and after 12 h only 4% of GSH was present. At 24 h the levels of glutathione were below detection limit (Fig 2). We also measured the growth of 3T6 cells after treatment with BSO. As seen in Fig 3, concentrations of BSO between 0.1 and 1 mM had little effect on the growth rate of the cells.

The effect of BSO on the rate of DNA synthesis was analysed by examining the incorporation of radioactive thymidine into DNA. Two parallel sets of exponentially growing cultures of 3T6 cells were incubated with labelled thymidine. Isotope incorporation was monitored between 30 and 60 min after addition of the isotope when equilibration of the dTTP pool had occurred and incorporation of the thymidine was linear (data not shown, 9). The incorporation of ^{3}H-dTMP into DNA was not significally changed by the BSO treatment (Table I).

Next we examined the effects of BSO on the size of deoxyribonucleotide triphosphate pools. The cells were treated with three different concentrations for 6, 12 and 24 h. In 3T6 cells, the pyrimidine pools are approximately 5 fold larger than the purine dNTPs with dATP and dGTP being about equal. Addition of BSO did not significantly affect the size of the dNTP pools (Fig. 4).

These data show that under conditions where the glutathione levels are strongly decreased or abolished, cell growth, as monitored by cell number and DNA synthesis, is largely unaffected. In addition, the cells continue to synthesise *de novo* balanced quantities of deoxyribonucleotides.

Since ribonucleotide reductase is the sole enzyme responsible for *de novo* synthesis of deoxyribonucleotides and perturbation of its activity leads to an imbalance of the pools a major impli-

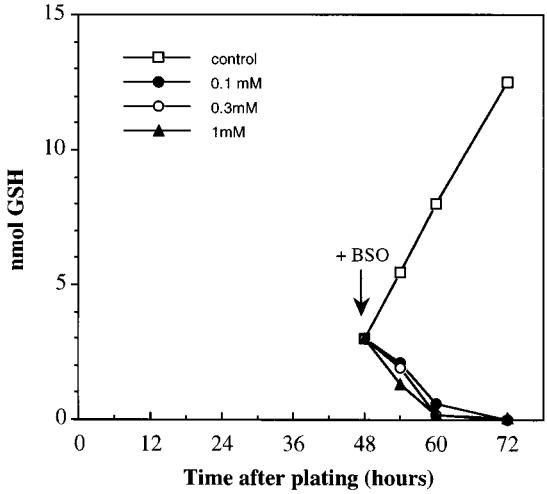

FIG. 2. Effect of BSO on glutathione levels. At the indicated time after plating (arrow) BSO was added to parallel sets of plates and glutathione levels were analyzed.

cation of our results thereby is that the glutaredoxin system, at least in fibroblasts, is not the sole/major hydrogen donor system for ribonucleotide reductase.

Colocalization studies of thioredoxin, ribonucleotide reductase and glutaredoxin show that although most cells contain ribonucleotide reductase, thioredoxin and glutaredoxin, some cell types contain ribonucleotide reductase and thioredoxin but not glutaredoxin (11), ribonucleotide reductase and glutaredoxin but not thioredoxin (12) or neither glutaredoxin or thioredoxin (12). Trx has a broad spectrum of cellular functions (13). Appart of its involvement in ribonucleotide reduction, it modulates the activity of transcription factors such as NF-kB (14) and AP-1 (15), and steroid receptors (16,17). More recently thioredoxin was reported to be identical to cytokine-like factors such as Adult T cell leukemia derived factor (18), T-hybridoma (MP-6) derived B cell stimulatory factor (19), 3B6-IL-1 (20) and early pregnancy factor (21). Also, glutaredoxin is involved in diverse cellular activities (22) e.g. reduction of dehydroascorbate (23), however, the role of glutaredoxin in growth regulation is unknown. The existence of a group of amino acids absolutely conserved among mammalian glutaredoxins and the transforming growth factor-*b* (TGF-*b*) (24) suggests functions of unknown significance.

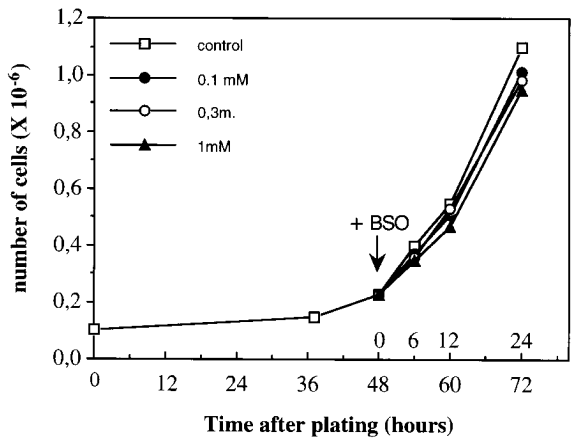

FIG. 3. Effect of BSO on the growth of 3T6 cells. In parallel sets of cultures BSO was added (arrow) and growth was monitored during another 24 hours.

TABLE 1
Incorporation of Isotope from [^{3}H]Thymidine into DNA after Incubation with BSO

	Radioactivity incorporated into DNA (cpm/min)		
	6 h	12 h	24 h
BSO (mM)			
0	44,100	39,700	32,100
0.1	43,200	40,600	29,600
0.3	40,400	38,400	27,500
1	40,300	38,600	26,500

Cultures at different time points after BSO addition were labelled with thymidine for 30 and 60 min. Incorporation into DNA was measured as described in Materials and Methods. The results are expressed as cpm/min and are corrected per 10^6 cells.

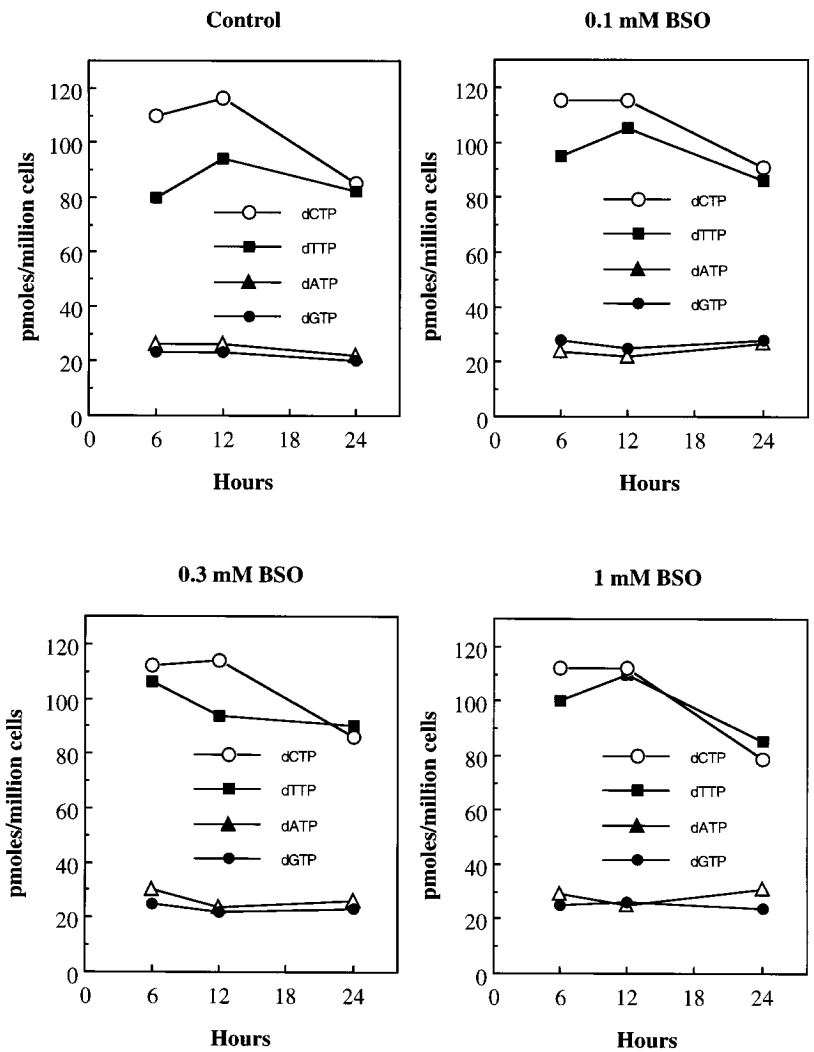

FIG. 4. Effect of BSO on the size of dNTP pools. Various amounts of BSO were added to growing 3T6 cells. The size of the four dNTP pools was determined at different time points after BSO addition.

Thus, considering the growing list of other functions for glutaredoxin and thioredoxin we can assume that the participation in ribonucleotide reduction is not their sole function. Since ribonucleotide reductase is essential for cell survival, the cell to ensure its proper supply of dNTPs may have developed more than one hydrogen donor system. This systems may act interchangeably depending on the cell type and the metabolic status of the cell. The fact that in testis exist cells which contain ribonucleotide reductase but no thioredoxin or glutaredoxin suggests the existence of even a third hydrogen donor for mammalian ribonucleotide reductase. In *E. coli* a third hydrogen donor system for ribonucleotide reductase in a double mutant lacking thioredoxin and glutaredoxin has been identified (25,26). A third hydrogen donor for mammalian ribonucleotide reductase might be just another glutaredoxin or thioredoxin variant or rather, an absolutely different reducing system.

ACKNOWLEDGMENTS

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