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Glutathione metabolism in skeletal muscle derived cells of the L6 line

C. K. SEN, P. RAHKILA and O. HÄNNINEN
Department of Physiology, University of Kuopio, SF 70211 Kuopio, POB 1627, Finland, and Department of Cell Biology, University of Jyväskylä, SF 40100 Jyväskylä, Finland

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Skeletal muscle derived L6 myoblasts possess a considerably high resting total glutathione (TGSH) pool. Exposure to L-buthionine-[S,R]-sulphoximine resulted in a 90% depletion of the intracellular TGSH pool. All the key enzymes of glutathione metabolism, especially glutathione S-transferase, were observed to be considerably active in the undifferentiated cells. Se-dependent glutathione peroxidase activity appeared to account for most of the total GSH peroxidase activity of the cells. A significant contribution of γ-glutamyl transpeptidase-independent (5 mM acivicin insensitive) mechanism to the extracellular GSH uptake capacity of the muscle cells was evident. Efflux of oxidized glutathione from the cells exposed to *t*-butyl hydroperoxide was rapid and appeared to be energy linked.

Key words: antioxidant, exercise, γ-glutamyl cycle, glutathione, myoblast, oxidative stress, selenium, skeletal muscle.

The γ-glutamyl cycle (Meister 1983) serves a diverse series of critical cellular defensive functions including free radical scavenging, detoxification of electrophiles and the maintenance of thiol-disulphide status. L-γ-glutamyl-L-cysteinyl-glycine is the key compound of the γ-glutamyl cycle (Meister 1983, Deneke & Fanburg 1989, Sen *et al.* 1992, Staal *et al.* 1992). This tripeptide, glutathione (GSH), is the most abundant low molecular weight thiol found in mammalian cells. The liver plays a central role in a complex inter-organ homeostasis of GSH (Deneke & Fanburg 1989).

The primary site for the generation and propagation of oxygen-derived free radical reactions is the lipoidic layers of the cell. α-tocopherol is a lipid soluble molecule that functions as a chain-breaking antioxidant. Peroxyl and alkoxyl radicals formed during lipid peroxidation preferentially combine with an −OH group (containing an easily replaceable hydrogen atom) attached to the hydrophobic structure of the α-tocopherol molecule. In this way, the chain reaction of lipid peroxidation is terminated. However, this antioxidative reaction of α-tocopherol by-produces another weakly reactive radical, tocopherol-O$^{\cdot}$. The water-soluble antioxidants, ascorbic acid and GSH, are suggested to be involved in regenerating α-tocopherol from its radical by-product (Wefers & Sies 1988). GSH, in the aqueous phase, is present in the cytosol as well as in the mitochondrial matrix. The antioxidative function of the thiol is implicated through two general mechanisms of reaction with reactive oxygen species: direct or spontaneous, and glutathione peroxidase catalysed. As a major by-product of such antioxidative reactions glutathione disulphide (GSSG) is produced. Intracellular GSSG thus formed may be reduced back to GSH by glutathione reductase (GRD) activity or released to the extracellular compartment.

Correspondence: Chandan K. Sen, Snellmania 3016, Department of Physiology, University of Kuopio, SF 70211, POB 1627, Kuopio, Finland.

Skeletal muscle glutathione homeostasis appears to be of critical importance in the management of oxidative stress at exercise (Lew *et al.* 1985, 1991, Pyke *et al.* 1986, Ji *et al.* 1988, 1992, Cazzulani *et al.* 1991, Novelli *et al.* 1991, Sen *et al.* 1992). Our previous work has shown that both skeletal muscle and liver glutathione metabolism is responsive to exercise training, a single bout of exhaustive exercise as well as long-term immobilization (Sen *et al.* 1992). The resting TGSH pool and the GSH-dependent antioxidant capacity of oxidative skeletal muscle are significantly increased on exercise training. On the contrary, a lowering of the TGSH pool of the red gastrocnemius muscle has been observed as a result of long-term hind limb immobilization of beagle dogs. All the key enzymes of the γ-glutamyl cycle were observed to be significantly active in skeletal muscle, especially in that of the dog (Sen *et al.* 1992). We and other groups have observed that strenuous exercise leads to a remarkable depletion of both liver and skeletal muscle total glutathione (TGSH, GSH+GSSG) pool (Lew *et al.* 1985, Pyke *et al.* 1986, Sen *et al.* 1992). It has been hypothesized that during exhaustive exercise there occurs a net shuttle of hepatic TGSH to the skeletal muscle to meet the increased antioxidant demand of the active tissue. Recently, it has been reported that exogenous glutathione is potentially capable of enhancing the endurance to muscle effort in mice (Cazzulani *et al.* 1991, Novelli *et al.* 1991). It is believed that the TGSH pool of the skeletal muscle is largely dependent upon hepatic supply of GSH. However, little is known about the contribution of skeletal muscle GSH metabolism to the inter-organ GSH homeostasis.

L6 is an established cell line derived from rat muscles and immortalized by treatment with methylcholantrene. We observed the presence of a considerably high content of TGSH in cultured L6 myoblasts. We were therefore interested in assessing the basic features of glutathione metabolism in this cell line.

METHODS

Materials. L6 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Dulbecco's Modified Eagle Medium (DMEM), foetal calf serum (FCS) and other reagents for the culture medium were obtained from GIBCO Laboratories (Grand Island, NY, USA). NADPH, 1,2-dichloro-4-nitrobenzene (CDNB), acivicin (α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid), L-buthionine-[S,R]-sulphoximine (BSO), sodium selenite and *t*-butyl hydroperoxide (*t*-BOOH) were purchased from Sigma, St Louis, MO, USA. All other chemicals were of the highest analytical grade.

Cell culture. Undifferentiated mononucleated cells (myoblast) were used in this study. The cells were seeded at a concentration of 2×10^5 cells per culture dish (56.7 cm^2, NUNC, Roskilde, Denmark). Cultures were grown in DMEM supplemented with 10% FCS, 5 mM glutamine, 0.3% D-glucose, 50 U ml^{-1} of penicillin, and 50 μg ml^{-1} of streptomycin, in humidified air containing 10% CO_2 at +37 °C. Cell viability was greater than 95% as estimated by trypan blue exclusion. The cells were split once every 2 days. All the experiments and assays were carried out upon cells (6th to 12th passage) in the second day of the last passage.

Determination of GSH uptake and GSSG efflux. Subconfluent monolayers were washed three times in deaerated Ca^{2+}, Mg^{2+} free phosphate buffered saline (PBS), pH 7.4. The cells were then incubated for various time intervals in a 60 μM solution of GSH in PBS at +37 °C. At the end of the desired time interval of incubation, the uptake was stopped by replacing the incubation medium with ice-cold PBS supplemented with 0.5 mM KCN. The culture dish was placed on ice and the monolayer was washed three times. The buffer was then aspirated and the cells were scrapped to obtain a suspension. TGSH and total protein contents were determined. Some experiments were performed following pre-incubation of the monolayer either in 5 mM acivicin (in PBS) for 1 h, or in 1.75 mM BSO (in culture medium) for 20 h under the same conditions in which the cells were grown. For the efflux study, after washing the monolayer three times with PBS, cells were incubated for various time intervals either in PBS, or in 0.5 mM KCN supplemented PBS at +37 °C, containing 150 μM *t*-BOOH. Following the desired time interval of incubation, the cells were washed three times with ice-cold 0.5 mM KCN supplemented PBS and the cell suspension was collected as described above.

Determination of TGSH. 100 μl of cell suspension were added to 10 μl of 5.5 N perchloric acid. The mixture was centrifuged (10000 $g \times 2$ min) to discard precipitated protein. TGSH content in the supernatant was determined by a modification of Tietze's (1969) method as described previously (Sen *et al.* 1992).

Enzyme assays. Subconfluent monolayers were washed in PBS, and then harvested by trypsin treatment. The cell suspension was then centrifuged (2000 $g \times 3$ min) to obtain the cell pellet. The pellet was resuspended (2–3 mg protein ml^{-1}) in the respective enzyme assay buffer containing 0.25% Triton X-100 (v v^{-1}). The suspension was then ultrasonicated

in short bursts on ice for 90 s and stored at −80 °C. In the case of GSHPx assay, prior to being harvested, cells were either grown on the usual growth medium or in the growth medium supplemented with 18 μM of sodium selenite (for 24 or 48 h). Glutathione peroxidase (GSHPx) was assayed (Flohe & Gunzler 1984) either by using cumol hydroperoxide (for total GSHPx or TGSHPx activity) or H_2O_2 (for Se-dependent GSHPx or SE-GSHPx activity) as substrates. Sodium azide was used in the reaction mixture to inhibit catalase activity. The difference in activities of TGSHPx and Se-GSHPx was interpreted as selenium-independent GSHPx (NSe-GSHPx) activity. GRD and γ-glutamyl transpeptidase (GGT) activities were assayed spectrophotometrically (Carlberg & Mannervik 1985, Tate & Meister 1985). Determination of γ-glutamylcysteine synthetase (GCS) and GSH synthetase (GHSH) activities was based on the rate of formation of ADP coupled to the decrease in the absorbance of NADH at 340 nm (Meister 1985, Seelig & Meister 1985). GSH S-transferase (GST) was assayed with CDNB as substrate (Habig *et al.* 1974). Protein contents of homogenous cell suspension were determined by Bradford's microassay (1976).

Data of each observation were obtained as means of triplicates and each experiment was performed four times. Results are expressed as mean ± SEM of the four experiments.

RESULTS

L6 myoblasts were observed to contain considerably high intracellular TGSH (64.5 nmol mg^{-1} protein, Fig. 2). Supplementation of the growth medium of the cultured cells with 1.75 mM BSO resulted in a 90% depletion of intracellular TGSH in 20 h (Fig. 2, Y_2 scale). Table 1 illustrates the activity of most of the key enzymes of the γ-glutamyl cycle in L6 myoblasts. Se-GSHPx appeared to account for most of the TGSHPx activity in the cells (Fig. 1). A significant increase in Se-GSHPx activity of L6 myoblasts was observed following a 24 h exposure of the cells to 18 μM sodium selenite in the culture medium. However, no further increase in the activity of Se-GSHPx was seen when the exposure period was prolonged to 48 h. NSe-GSHPx activity was not affected by the exposure of the cells to sodium selenite (Fig. 1).

GSH uptake kinetics of the myoblast, when incubated in a 60 μM solution of GSH, is represented in Figure 2. GSH uptake rates plateaued after 20 s of incubation time. Pre-incubation of the cells with 5 mM acivicin led to a 50% reduction (as calculated on the basis of the rise of intracellular TGSH in the first 10 s of the uptake experiment in both cases, Fig. 2) in the ability of the myoblasts to uptake GSH from the incubation medium. Release of GSSG, measured as the decrease of intracellular TGSH, following exposure of the myoblasts to 150 μM *t*-

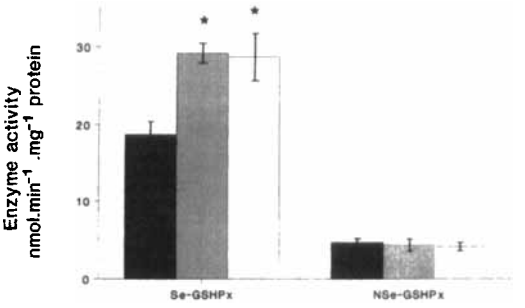

Fig. 1. Selenium-dependent glutathione peroxidase (Se-GSHPx) and non-selenium-dependent glutathione peroxidase (NSe-GSHPx) activities in L6 myoblasts either without (■) or with the exposure to sodium selenite (18 μM in culture medium) for different time intervals (24 and 48 h). Following 24 h of such an exposure (▨), Se-GSHPx activity increased significantly (*, $P < 0.001$, Students *t*-test). No effect was seen in the subsequent 24-h exposure (▩). The 24-h exposure dependent elevation in activity was maintained without any further change. NSe-GSHPx activity accounted for about 25% of the TGSHPx activity and remained unaffected by the exposure. Results are mean ± SEM of four experiments.

Table 1. Activity of the key enzymes of glutathione metabolism in L6 myoblasts

Enzymes	Activity nmol min^{-1} mg $protein^{-1}$
γ-glutamylcysteine synthetase (GCS, EC 6.3.2.2)	13.15 ± 1.18
Glutathione synthetase (GSHS, EC 6.3.2.3)	9.68 ± 0.92
Se-dependent glutathione peroxidase (Se-GSHPx, EC 1.11.1.9)	18.71 ± 1.49
Glutathione reductase (GRD, EC 1.6.2.4)	20.04 ± 1.74
Glutathione S-transferase (GST, EC 2.5.1.18)	87.67 ± 5.48
γ-glutamyl transpeptidase (GGT, EC 2.3.2.2)	16.17 ± 1.30

All values are mean ± SEM.

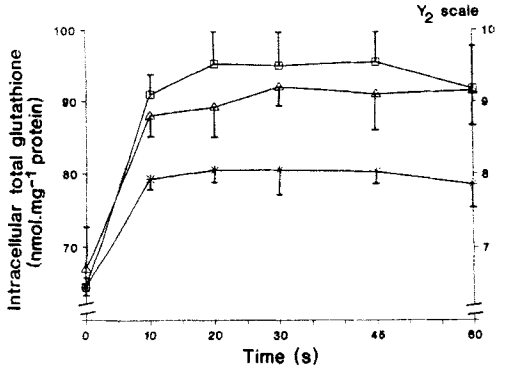

Fig. 2. Change in intracellular total glutathione content following incubation of L6 myoblasts in a 60 μM solution (in PBS) of GSH at +37 °C. —□— represents result from cells without any preincubation (in any inhibitor). —*— represents result from cells pre-incubated in 5 mM acivicin (in PBS) for 1 h, and —△— (Y_2 scale) represents result from cells pre-incubated in 1.75 mM BSO (in culture medium) for 20 h. Values are mean ± SEM of four experiments.

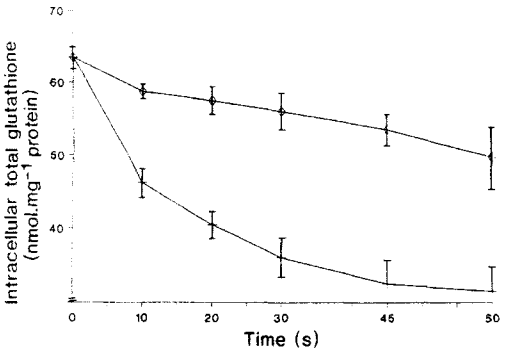

Fig. 3. GSSG release, measured as a decrease in intracellular total glutathione content, from L6 myoblasts following exposure to 150 μM *t*-BOOH (in PBS, +37 °) either with (—◇—) or without (—|—) 0.5 mM KCN. Values are mean ± SEM of four experiments.

BOOH is represented in Figure 3. Within the first minute of exposure to the chemical oxidant, intracellular TGSH content reduced to one-half of the resting value. Supplementation of the incubation medium with 0.5 mM KCN resulted in a considerable inhibition of the above-mentioned rate of GSSG release (Fig. 3).

DISCUSSION

All the key enzymes of GSH metabolism were considerably active in the L6 myoblasts (Table 1). Such an observation is similar to our previous results from animal experiments (Sen *et al.* 1992). However, compared to data from the rat muscle, TGSHPx activity was observed to be considerably lower in the myoblasts. The observed low TGSHPx activity appears to be because of the fact that the culture medium was devoid of Se. Low TGSHPx activity in the muscle of rats fed with Se-deficient diet was also observed by Lang *et al.* (1987). A sharp rise in TGSHPx activity was observed on supplementing the culture medium with sodium selenite (Fig. 1). High activity of GST in the L6 myoblasts suggests that the intracellular GSH pool caters to antioxidant as well as detoxicant needs of the cell. In our previous study with rats (Sen *et al.* 1992), we had observed that activities of the enzymes of glutathione synthesis in the skeletal muscle were very low. GCS, the rate-limiting enzyme in GSH synthesis, was observed to be considerably active in the L6 myoblasts. Activity of GSHS was also detected (Table 1) and it appeared that the intracellular GSH synthesizing apparatus was crucially important in the maintenance of a considerably high intracellular TGSH pool. Supplementation of the growth medium of the cultured cells with 1.75 mM BSO, the most specific inhibitor of GCS (Griffith & Meister 1979, Deneke & Fanburg 1989), resulted in a 90% depletion of intracellular TGSH in 20 h (Fig. 2). Unlike that during *in vitro* culture, a continuous supply of exogenous (plasma-borne) GSH to the developing muscle tissue of the intact animal may be effective in limiting the activities of GCS and GSHS in the organ.

It has been hypothesized that physical exercise is accompanied by a net shuttle of hepatic TGSH to the skeletal muscles (Sen *et al.* 1992). Exercise-associated depletion of hepatic TGSH and elevation of plasma TGSH have been reported (Lew *et al.* 1985, Pyke *et al.* 1986, Sen *et al.* 1992). We observed that specific inhibition of GGT activity of the myoblasts by acivicin resulted in only about 50% blockade of GSH uptake from a 60 μM GSH-supplemented incubation medium to the intracellular compartment with a resting GSH pool in the mM range. A significant contribution of GGT-independent mechanism to the GSH uptake capacity of the muscle cells is thus evident (Fig. 2). Since, compared to other organs, muscle GGT activity is quite low (Sen *et al.* 1992), such mechanism of GSH uptake in the muscles may be considered

to be crucial in determining the availability of elevated levels of circulatory GSH to the muscles during exercise or following administration of exogenous GSH (Hagen *et al.* 1990). A significant alteration of the GSH concentration gradient across the cell membrane, as caused by exposing the cells to BSO, did not affect the uptake pattern (Fig. 2). Such an observation suggests that GSH uptake by muscle cells may not be dependent upon transmembrane concentration gradient of the tripeptide.

Exposure of the L6 myoblasts to *t*-BOOH, a chemical oxidant, resulted in a rapid decline of intracellular TGSH pool (Fig. 3). The intracellular TGSH concentration was reduced to half in the first minute of exposure. Inhibition of cellular mitochondrial respiration by supplementing the incubation medium with 0.5 mM KCN resulted in a remarkable decrease in the rate of GSSG release measured as the fall in intracellular TGSH following exposure of the cells to *t*-BOOH. The mechanism of the GSSG release thus appears to be energy-dependent, similar to that originally observed in erythrocytes (Srivastava & Beutler 1969, Kondo *et al.* 1980) and also evident in the myocardium (Ishikawa *et al.* 1986). Since the initial rate of GSSG release was observed to be rather rapid, extracellular GSSG concentration may be justified to be a suitable indicator of oxidative challenge in the skeletal muscle cell. A multifold rise in plasma GSSG concentration was observed following treadmill running of untrained rats (Lew & Quintanilha 1991).

Skeletal muscle GSH metabolism appears to be a significant component of the complex inter-organ GSH homeostasis.

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