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Glutathione homeostasis in response to exercise training and nutritional supplements

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Abstract

Glutathione plays a central role in the maintenance of tissue antioxidant defenses and in the regulation of redox sensitive signal transduction. In muscle cells, the level and redox status of GSH regulates activity of the redox sensitive transcription factor NF-κB. Physical exercise may cause oxidation of GSH in tissues such as the blood, skeletal muscle and liver. Endurance training strengthened GSH dependent tissue antioxidant defenses in most studies. Although studies investigating the effect of sprint training are few, current results show that sprint training may also have a beneficial effect on tissue GSH homeostasis. Skeletal muscle GSH level appears to be tightly regulated by the state of physical activity. Regular exercise enhances and chronic inactivity decreases the level of GSH in this tissue. N-acetyl-L-cysteine (NAC) and α-lipoic acid (LA) are two antioxidant dietary supplements that are able to enhance cellular GSH levels. Because LA can be recycled to its potent dithiol form, dihydrolipoate, by enzymes present in the human cell it has a clear advantage over NAC. Recently an improved form of LA, a positively charged analogue (LA-Plus), has been discovered. LA-Plus has more potent immuno-modulatory activity compared to LA. Both LA and NAC have been shown to have beneficial effects in protecting tissue GSH homeostasis against exercise induced oxidative stress. (Mol Cell Biochem **196**: 31–42, 1999)

Key words: thiols, antioxidant, redox, adaptation, oxidative stress, skeletal muscle, dietary supplement

Introduction

Physical exercise increases energy demand, particularly that of the active tissues such as the skeletal muscle. Depending on the type of activity we get this extra energy from aerobic or anaerobic sources. For example in a 100 m sprint that lasts for about 10 sec or weight lifting events, the required energy supply rate is so high that there is no time for aerobic processes to contribute. All of the energy is spent from energy-rich pyrophosphate bond reserves in the tissue. In longer activities such as the 1500 m run that lasts for around 4 min, however, energy is supplied by both anaerobic (35%) and aerobic (65%) metabolic processes. For endurance events such as the 26 mile marathon run, almost all of the energy is supplied by aerobic metabolism. The metabolic cost of and oxygen consumption by skeletal muscles during exercise also depends on the nature of muscle contraction that is predominantly involved. For example eccentric contraction (muscle lengthens) as during downhill running is associated with much less metabolic cost and oxygen consumption than other types of muscle contractions where the muscle group shortens (concentric) or remains unchanged in length (isometric). Standard exercise forms such as running or bicycling usually involve some specific muscle groups contracting concentrically, and some other muscles eccentrically.

Under resting conditions, oxygen content in arterial and venous blood of the skeletal muscle tissue is 20 and 15 ml per 100 ml blood, respectively. Physical exercise may increase skeletal muscle arterio-venous oxygen difference by 3-fold and blood flow through the tissue by 30-fold. As a result we may have up to 100-fold increase in oxygen flux through the active skeletal muscles during exercise. In 1978 Dillard *et al*. [1] tested whether physical exercise associated

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increased oxygen consumption may cause oxidative tissue damage. They reported that in humans moderate intensity physical exercise increased the content of pentane, a lipid peroxidation by-product, in expired air. Electron paramagnetic resonance spectroscopy allows a direct detection of the short-living free radical species. Using this technique Davies *et al*. [2] showed for the first time in rats that exhaustive treadmill exercise may increase skeletal muscle and liver free radical concentration by 2–3 fold. Recent studies show that repeated exercise augments oxidative stress and that exercise-induced oxidative stress may cause damage to the genetic material [3]. The different mechanisms that may contribute to exercise induced oxidative stress have been recently discussed in a international Olympic committee publication [4].

Exercise training results in a large number of beneficial adaptive changes in various tissues, especially in the skeletal muscle and heart [5–13]. Several studies show that exercise training also enhances the ability of tissues to counteract oxidative damage [14]. In organs such as skeletal muscle, heart and liver, antioxidant defenses appear to be upregulated by physical training. In 1973, Caldarera *et al*. [15] were the first to show that acute exercise increases catalase activity in rat liver, heart and skeletal muscle. Since then a relatively large number of studies have tested the effect of a variety of endurance exercise training regimes on antioxidant defenses [16]. Jenkins *et al*. [17] studied the antioxidant enzymes of the muscle. Needle biopsy samples were collected from the vastus lateralis muscle of healthy men. The subjects were split into high-fit (VO_{2max} > 60 ml/Kg min) and low-fit (VO_{2max} < 60 ml/Kg min) groups. The high aerobic capacity group had significantly greater activities of catalase and superoxide dismutase in their muscle. A strong positive correlation (r = 0.72, $p < 0.01$) between the subject's maximum oxygen uptake and muscle catalase was noted. A similar correlation was also observed between the subject's maximum oxygen uptake and muscle superoxide dismutase (r = 0.60, $p < 0.05$). The study also found that there was a rank order relationship in both the tissue oxygen consumption and antioxidant enzyme activity [17].

Glutathione has emerged to be one of the most fascinating molecules virtually present in all animal cells often in quite high (millimolar) concentrations. It is known to have multifaceted physiological functions including antioxidant defense, detoxification of electrophilic xenobiotics, modulation of redox regulated signal transduction, storage and transport of cysteine, regulation of cell proliferation, synthesis of deoxyribonucleotide synthesis, regulation of immune response, and regulation of leukotriene and prostaglandin metabolism. The effect of exercise training and the role of nutritional supplements in regulating tissue glutathione homeostasis has been briefly reviewed in this work.

Physical exercise and tissue glutathione response

Human blood plasma contains low amounts of reduced glutathione (GSH). Almost all of blood glutathione represents blood cell content, mainly that of the erythrocyte. Oxidation of GSH to glutathione disulfide (GSSG) is a sensitive marker of oxidative stress. In 1988, Gohil *et al*. [18] were the first to report that even submaximal exercise induces blood GSH oxidation. A 100% increase in blood GSSG level was reported within the first 15 min of exercising at 65% VO_{2peak} [18]. Ji *et al*. [19] exercised 8 healthy male cyclists at 70% VO_{2max}. In contrast to the finding of Gohil *et al*. [18], a bout of exercise that lasted for more than 2 h did not elevate blood GSSG. Exercise induced blood glutathione oxidation has been shown in a number of later studies [20–22] and is consistent with previous human experimental studies showing that exhaustive exercising of rats remarkably increases GSSG level in the plasma [23].

The association between exercise intensity and related oxidative stress in healthy young men who exercised for 30 min at their aerobic and anaerobic thresholds one week apart has been studied in our laboratory [21]. Blood samples were drawn before, immediately after and 24 h after tests. In line with the observation of Gohil *et al*., all four exercise bouts (at aerobic and anaerobic thresholds for 30 min each, and two maximal tests [mean duration ~ 14 min]) remarkably increased the level of blood GSSG. Exercise induced perturbations in the blood glutathione redox status and plasma lipid peroxide level were no more observed in the 24 h post-exercise recovery samples. Viguie *et al*. [24] also observed that there was no evidence of persistent or cumulative effects of repeated leg cycling exercise (at 65% VO_{2peak}, 90 min, for 3 consecutive days) on blood glutathione redox status. In moderately trained men, a 50% decline in the blood GSH level was observed during the first 15 min of exercise. This effect was accompanied by an increase in the level of blood GSSG. Total glutathione level in the blood did not change significantly during the exercise. Blood GSH level returned to baseline after 15 min of post-exercise recovery. Although high speed running for brief time intervals (~ 20 sec) to exhaustion did not influence blood GSH oxidation, Sastre *et al*. [25] observed that in trained men blood GSSG levels were 72% higher immediately after exercise than at rest. In young men, intermittent exercise bouts to exhaustion increased blood GSSG by 35% [26]. More recently, Laaksonen *et al*. [22] also observed exercise-induced blood GSH oxidation in young insulin-dependent diabetic men and their corresponding healthy controls. Results obtained so far show that physical exercise may enhance the utilization of blood GSH resulting in decreased GSH/GSSG ratio.

Physical exercise influences GSH metabolism in skeletal muscles and liver of rats [16]. Lew *et al*. [23] reported that exhaustive exercise decreases both liver and muscle glutathione consistently. We investigated the influence of exhaustive treadmill run on the tissue GSH status of rats. Exhaustive exercise decreased total glutathione reserves of the liver and active skeletal muscles red gastrocnemius and mixed vastus lateralis, and that this effect was not observed in the less active longissimus dorsi muscle [27]. Exercise-induced decrease of total glutathione pool in the liver, red gastrocnemius muscle, mixed vastus lateralis muscle and heart of rats was also seen in another independent study carried out in our laboratory. This effect was, however, not seen in the lung [28]. Duarte *et al*. [29] confirmed that a single bout of exercise results in glutathione loss from skeletal muscle. Exercising resulted in a 50% decrease in left soleus muscle total glutathione content, an effect that was interpreted as an index of oxidative stress. Recovery of muscle glutathione level was slow in the post-exercise recovery period. This recovery was remarkably faster in mice supplemented with allopurinol, an inhibitor of the superoxide producing enzyme xanthine oxidase. It was suggested that exercise-induced increase in xanthine oxidase dependent superoxides causes oxidative stress to muscle tissues located in close proximity, and that this stress is manifested as a loss of tissue glutathione.

Role of tissue glutathione in exercise

Although the importance of glutathione in protecting against oxidative stress is well recognized, the role of physiological levels of glutathione and other endogenous antioxidants in protecting against exercise induced oxidative stress is less clear. We evaluated the role of glutathione and selected antioxidant enzymes as determinants of lipid peroxidation at rest and in response to exercise in men (n = 13–14) aged 20–30 years, who cycled for 40 min at 60% of their maximal oxygen consumption (VO_{2max}) [30]. Blood GSSG increased by about 50% in response to exercise. Mean blood reduced GSH decreased by 13% with exercise. Of the measured red blood cell antioxidant enzyme activities, only selenium dependent GSH peroxidase activity increased following exercise. In univariate regression analysis, plasma lipid peroxidation by-product levels at rest predicted exercise induced change in blood total glutathione. Blood GSSG levels at rest were a strong determinant of post-exercise levels. Subjects with a favorable blood glutathione redox status at rest maintained a more favorable redox status in response to exercise induced oxidative stress. Changes in blood GSH, GSSG and total glutathione in response to exercise were closely associated with both resting and exercise induced plasma lipid peroxidation. Results of this study underscores the critical role of glutathione homeostasis in modulating exercise induced oxidative stress, and conversely, the effect of oxidative stress at rest on exercise induced changes in glutathione redox status [30].

A critical role of tissue glutathione in protecting against exercise-induced oxidative stress has been evident particularly from studies involving GSH deficiency. Tissue GSH synthesis is dependent on dietary amino acid supply [31]. Thus, food-deprivation decreases tissue GSH content and refeeding corrects such effect. Exhaustive treadmill exercise tends to further lower hepatic GSH level of food-deprived rats. This effect was more prominent in rats that were refed and had higher levels of baseline hepatic GSH level [32]. Glutathione deficient rats have been prepared by the intraperitoneal injection of L-buthionine-[S,R]-sulfoximine [28]. The L-buthionine-[S,R]-sulfoximine treatment approach selectively inhibits the first enzyme of GSH synthesis, γ-glutamylcysteine synthetase, and turns off intracellular GSH synthesis. L-buthionine-[S,R]-sulfoximine treatment resulted in (i) ~ 50% decrease in the total glutathione pools of the liver, lung, blood and plasma, and (ii) 80–90% decrease in the total glutathione pools of the skeletal muscle and heart. Compared to the placebo treated controls, endurance to exhaustion of glutathione deficient exercising rats was reduced to half. Results of this experiment indicated a crucial role of endogenous GSH in the circumvention of exercise induced oxidative stress and as a determinant of exercise performance [28]. In another recent study, diethyl maleate induced depletion of glutathione also significantly decreased swim-performance of rats [33].

The effect of a more long term GSH deficiency has been studied in mice that were swim-exercised [34]. Global GSH-deficiency in mice was induced by the intraperitoneal injection of L-buthionine-[S,R]-sulfoximine combined with supplementation of 20 mM L-buthionine-[S,R]-sulfoximine in drinking water for 12 days. Using such a protocol, GSH contents in the plasma, liver, kidney heart and skeletal muscles were decreased by 65, 77, 85, 90 and 93% of the control values, respectively. A more enduring exercise bout that lasted for 4–6 h was tested, and no effect of GSH-deficiency on swim-endurance was observed in these mice. Marked decrease in hepatic GSH level was observed during exercise. Consistent with our observation [28], exercise-induced oxidative lipid damage was more in GSH-deficient mice, particularly in skeletal muscle. Increased tissue lipid peroxidation in GSH-deficient state is consistent with the hypothesis that GSH plays a central role in the antioxidant-network, and that impaired GSH defense weakens the efficacy of lipid phase antioxidants as well. Leeuwenburgh and Ji [34] showed that GSH deficiency also influences the activity of antioxidant enzymes. For example, GSH-deficiency was associated with decreased GSH peroxidase activity in the liver, whereas activities of GSSG reductase

and GSH S-transferase were elevated. GSH deficiency may also influence oxidative metabolism in tissues [34]. Results from the heart of GSH deficient mice subjected to prolonged exercise show that GSH is actively used in the myocardium during prolonged exercise at moderate intensity and that GSH deficiency is relatively well tolerated by the heart, possibly compensated for by an increased GSH uptake from the plasma [35].

Response to exercise training

A properly selected exercise training regime improves cardiovascular health and the gross functional capacity of the human body. Does exercise training improve the ability of tissues to defend against oxidative stress? Yes, indeed this has been observed to be the case in several independent studies [14, 36, 37] with a very few exceptions [38]. A recent human study measured resting muscle and blood antioxidant status in untrained and jump-trained humans. Activities of GSH redox cycle enzymes, GSH peroxidase and GSSG reductase, were significantly higher in jump-trained compared with untrained subjects [39]. Endurance training dependent increase in tissue antioxidant defense has been also linked with improved physical performance [40]. We observed that in diabetic patients, a bout of exercise induces oxidative stress and that physical fitness may have a protective effect against such oxidative stress [22, 41]. The protective effect of exercise training against ethanol-induced oxidative injury in specific regions of the brain has been also observed [42]. Adaptation of tissue antioxidant defense systems in response to exercise training appears to be age-sensitive. In an interesting study by Leeuwenburgh *et al*. it was observed that although exercise training selectively increased the activity of antioxidant enzymes in tissues of young rats, there was no such protection against oxidative stress in the senescent muscle.

Kihlström [43] showed that endurance swim training provides enhanced protection to the heart against oxidative stress. This added capacity to detoxify reactive oxygen species was mainly because of elevated glutathione level and a more efficient NADPH supplying system in the trained heart. The training program decreased the activity of GSSG reductase in the myocardium, and increased the activity of thioredoxin reductase. These results concerning the swim training induced decrease of antioxidant enzyme activities in the heart were also observed by this author in a previous investigation [44]. GSSG reductase requires NADPH to maintain a favourable redox status of glutathione. Training increased the activity of the NADPH supplying enzyme glucose 6-phosphate dehydrogenase in the right ventricle. Also, the level of GSH was higher in the trained heart, especially in the left subepimyocardium.

In response to similar sub-maximal exercise, endurance trained rats are able to maintain tissue glutathione redox status better as reflected by the GSSG/total glutathione ratio compared to their untrained counterparts. In this case, the endurance training program significantly increased the activities of GSH peroxidase, GSSG reductase and glucose-6-phosphate dehydrogenase in the skeletal muscle and heart tissues [45].

The effects of aging and exercise training on rat skeletal muscle antioxidant enzyme activities have been tested [46]. Superficial glycolytic and deep oxidative vastus lateralis muscles were collected from rats aging from 2.5 months (young) to 27.5 months (senescent). Old rats had significantly lower GSH peroxidase activity in the deep vastus lateralis muscle. After progressive treadmill training, activity of the hydroperoxide-metabolising enzyme in deep vastus lateralis muscle significantly increased to a level higher than that observed in sedentary young rats. Thus, although aging adversely affects the antioxidant enzyme capacity in skeletal muscle, regular exercise can preserve such protective function. In a different model where Kanter *et al*. [47] tested the effect of swim-training, consistent results were obtained showing that training enhanced GSH peroxidase activity in the blood and liver. In contrast to some of the other studies described above, Tiidus and Houston [48] observed that in female rats, six week treadmill training does not influence the GSH peroxidase activity in skeletal muscle, heart and liver. In a human study, however, endurance training did increase erythrocyte GSH peroxidase activity [26].

We observed that treadmill training of rats increased skeletal muscle citrate synthase activity indicating enhanced oxidative capacity [27]. Hepatic total glutathione content was elevated in the trained rats. However, such an effect was not observed in any of the skeletal muscles studied (i.e. red gastrocnemius, mixed vastus lateralis, and longissimus dorsi). Leg muscle GSH peroxidase activity was higher in trained rats. Treadmill training decreased GSSG reductase activity in red gastrocnemius muscle. This effect may be related to the high intensity of training that may have increased flavoprotein turnover and breakdown in the muscle. Endurance training also increased the activity of γ-glutamyl transpeptidase in both leg muscles, the effect being more pronounced in red gastrocnemius. In the trained leg muscles, activated γ-glutamyltranspeptidase may facilitate the import of substrates required for GSH generation. Decreased γ-glutamyl transpeptidase activity was observed in the control leg muscles after exercise [27]. This effect, however, was not observed in the trained leg muscles indicating that during exercise the trained muscles have a more active substrate import system for GSH generation compared to the untrained controls. γ-Glutamyl transpeptidase activity of the trained liver decreased (~ 50%) after the exercise bout. This response might ensure that

fewer γ-glutamyl compounds are re-trapped in the liver when the needs of the active peripheral tissues are acute. The contention that exercise training strengthens GSH dependent tissue antioxidant defense was further supported by another study where swim-training of rats was associated with a marked increase in the activities of GSH peroxidase and GSSG reductase in the skeletal muscle, heart and liver [49].

GSH dependent antioxidant protection in the skeletal muscle appears to be tightly regulated by the state of physical activity; endurance training enhances and chronic activity-restriction diminishes such protection [27]. Dogs are more naturally endowed to be aerobic runners as compared to the rats that have been the experimental animal in other studies concerning the response of glutathione redox cycle to endurance training and exercise. Beagle dogs, commonly used as a laboratory animal, possess a well developed musculoskeletal system apparently suited for running. Thus, we studied the influence of treadmill training on beagle dogs. Treadmill training (5.5–6.8 km/h, 40 km/day, 5 days/week, 15% uphill grade, for 40 weeks) increased the oxidative capacity of red gastrocnemius, extensor carpi radialis, and triceps muscles of the leg. Training induced changes in the components of GSH metabolism was most pronounced in the red gastrocnemius muscle that is predominantly oxidative by composition. Hepatic and red gastrocnemius total glutathione levels were elevated in response to training. In all three leg muscles mentioned above, training elevated GSH peroxidase activity. This effect was also most pronounced in the red gastrocnemius muscle. GSSG reductase activities in extensor carpi radialis and triceps muscles were higher in the trained dogs. Trained animals with higher hepatic total glutathione reserves also had higher GSH S-transferase activity indicating that the liver of the trained animals had a higher detoxicant status. Training effects were not observed in the splenius muscle of the neck and trunk region which were least active during the training process. In a separate dog experiment [27], the effect of chronic activity-restriction on red gastrocnemius muscle of beagles was studied. The knee and ankle joint of right pelvic limb of each dog was immobilized for 11 weeks in a light fibre-glass cast. The left leg was used as the paired control. Chronic physical inactivity did not influence the activity of GSH dependent enzymes, however, the total glutathione level of the red gastrocnemius muscle was remarkably decreased in the immobilized leg [27]. Decreased total glutathione level and increased GSSG have been also observed to be associated with skeletal muscle atrophy [50].

A 55 week endurance training study with beagle dogs showed that physical training may enhance hepatic GSH S-transferase activity [27]. GSH S-transferases are a family of GSH dependent enzyme that play a central role in drug detoxification and xenobiotic metabolism. In addition, GSH S-transferases may also contribute to hydroperoxide metabolism because they have non-selenium GSH peroxidase activity. Later it was confirmed in rats that swim-training also increases hepatic GSH S-transferase activity when compared to non-trained controls. Electrophoretic and Western blot analyses revealed that a Ya-sized subunit of the transferase is specifically induced by exercise training. Analyses of affinity-purified GSH S-transferases further revealed that a Ya_1 subunit of Ya was most sensitive to exercise training. Non-trained control rats had Ya-subunits predominantly made up of Ya_2, whereas the trained animals had 4.3-fold increased in Ya_1 [51]. GSH S-transferases of exercise trained animals had increased peroxidase activity, an effect that was consistent with the changes in subunit composition. Studies on the regulation of Ya gene expression have revealed that the gene contains a regulatory sequence known as the anti-oxidant response element or ARE in the 5′-flanking region. Transcription of Ya is activated by oxidants such as hydrogen peroxide by a mechanism acting through the ARE [52]. Ya_1 is known to be induced in hydroperoxide overload situations such as selenium deficiency [53]. Thus, exercise-induced regulation of Ya_1 is expected to be oxidant mediated.

Endurance training can upregulate certain antioxidant enzyme activities in rat diaphragm muscle, indicating the potential for improvement of the resistance to intracellular reactive oxygen species [6, 36]. Acute exercise may cause oxidative damage in rat diaphragm through activation of inflammatory pathways and endurance training minimizes oxidative stress caused by acute exercise [54]. The effect of intensity and duration of exercise on training induced tissue antioxidant enzyme responses has been studied [55]. Rats were exercised at low, moderate or high intensity at one of three exercise durations (30, 60 or 90 min/day). The coastal and crural diaphragm, plantaris muscle and parasternal intercostal muscles were investigated. Training effects were highly tissue specific. All training programs markedly increased GSH peroxidase activity in the costal diaphragm, but not in crural diaphragm. Exercise-intensity or duration did not have any major influence on training-induced elevation of GSH peroxidase activity in the coastal diaphragm. In crural diaphragm, however, moderate and high intensity exercise training decreased tissue GSH peroxidase activity when the daily exercise duration was as long as 90 min. None of the training programs influenced GSH peroxidase activity of the parasternal intercostal muscle, although remarkable effects were observed in the plantaris muscle. In the plantaris, daily exercise duration had a marked effect on GSH peroxidase activity response. Longer daily exercise duration triggered a more marked response. Results of another similar study further support that training effects are indeed highly tissue specific [56]. Although exercise training increased GSH peroxidase activity in the red gastrocnemius muscle of rats, such effects were not consistently seen in soleus or even white gastrocnemius muscles. Similar to the

previous results obtained from plantaris muscle, daily exercise duration had a marked effect on GSH peroxidase activity response. Recently it has been shown that porcine skeletal muscle adapts to endurance exercise training in a manner similar to muscle of humans and other experimental animals [57]. Criswell *et al*. [58] observed that high intensity interval exercise is superior to moderate intensity continuous exercise in the elevation of GSH peroxidase activity in rat soleus muscle. Training dependent enhancement of human tissue antioxidant defenses, in certain cases, is thought to be so remarkable that it may completely offset oxidative stress expected to be caused by a severe bout of triathlon race [59].

Both strenuous long duration exercise and exhaustive sprint training may cause oxidative stress [60]. Yet, studies investigating the influence of physical training on tissue antioxidant status have mostly tested the effect of endurance training, which enhances tissue oxidative capacity. Information on the effect of sprint training, which relies primarily on non-oxidative metabolism, on tissue antioxidant defenses is scanty. We examined the effect of a sprint training regimen on rat skeletal muscle and heart GSH system [61]. Soleus muscle, predominantly made up of slow-oxidative fibers, was studied as representative of slow-twitch muscle. Plantaris and extensor digitorum longus muscles, consisting mainly of glycolytic fibers and the superficial white portion of the quadriceps femoris muscle, mainly consisting of fast-oxidative-glycolytic fibers were studied as representative of fast-twitch muscles. Mixed gastrocnemius muscle was examined as an antagonist of extensor digitorum longus muscle. Lactate dehydrogenase and citrate synthase enzyme activities were measured in muscle to test the effects of training on glycolytic and oxidative metabolism, respectively. The efficacy and specificity of the 6 week sprint training protocol was attested by markedly increased anaerobic but not aerobic metabolic capacity in mixed and fast twitch fiber muscles. Endurance training consistently upregulates GSH dependent defenses and other antioxidant enzymes, with effects most marked in highly oxidative muscle. In contrast, sprint training enhanced antioxidant defenses primarily in fast glycolytic muscle. Compared with the control group, GSH peroxidase activities in gastrocnemius, extensor digitorum longus muscles and heart increased following sprint training. The training program also increased GSSG reductase activity in the extensor digitorum longus muscle and heart. Sprint training did not influence glutathione levels or GSH related enzymes in the oxidative soleus muscle. The effect of intermittent sprint cycle training on the level of muscle antioxidant enzyme protection has been also investigated in humans [62]. Resting muscle biopsies, obtained before and after 6 weeks of training and 3, 24, and 72 h after the final session of an additional 1 week of more frequent training, were analyzed for activities of the antioxidant enzymes GSH peroxidase, GSSG reductase, and superoxide dismutase. Intermittent sprint cycle training that induces an enhanced capacity for anaerobic energy generation also improved the level of antioxidant protection in the muscle.

Regulation of NF-κB in skeletal muscle derived cells

Oxidation-reduction (redox) based regulation of signal transduction and gene expression is emerging as a fundamental regulatory mechanism in cell biology [63, 64]. Electron flow through side chain functional CH_2-SH groups of conserved cysteinyl residues in proteins account for their redox-sensing properties. At least two redox sensitive transcription factors, nuclear factor κB (NF-κB) and activator protein-1 (AP-1) have been well defined [63, 64]. Intracellular thiol redox status appears to be a critical determinant of NF-κB activation. At low levels of cytosolic GSSG, T-cells fail to activate NF-κB in response to appropriate stimuli, whereas high GSSG concentration inhibits the binding of activated NF-κB to its cognate DNA site. Thus, it appears that an intermediate optimal level of intracellular GSSG is required for effective NF-κB activation. Droge *et al*. have found that GSH deficiency of T-cells is associated with a suppression of NF-κB function. This effect was suggested to be related to very low levels of GSSG, thought to be necessary for NF-κB activation in some models, in GSH deficient cells [65].

Tumor necrosis factor α (TNFα), a cytokine product of monocytes and macrophages [66] is a rapid and potent activator of NF-κB (Figs 1 and 2). TNFα is suggested to be implicated in muscle wasting of cachexia [67–70]. In support of this it has been observed that sustained increase in serum levels of TNFα contributed by tumor cells [71–74], or in TNFα transgenic mice [75] can induce muscle wasting. Muscle wasting in cachexia is a common phenomenon observed in a large population of individuals suffering from chronic diseases such AIDS, cancer, some inflammatory disorders, sepsis and trauma [76]. Muscle wasting has been recognized as the single most common cause of death among cancer patients [77]. Other muscle pathologies such as eosinophilia myalgia syndrome [78] have been also observed to be associated with increased levels of TNFα. Recently it has been shown that exhaustive exercise of athletes results in increased TNFα levels in the serum [79] suggesting that TNFα may be also implicated in exhaustive exercise induced muscle damage. We investigated the role of endogenous glutathione status in TNFα induced NF-κB activation in skeletal muscle-derived cells [80]. TNFα proved to be a potent inducer of transient NF-κB activation in L6 myoblasts. In buthioninesulfoximine treated GSH

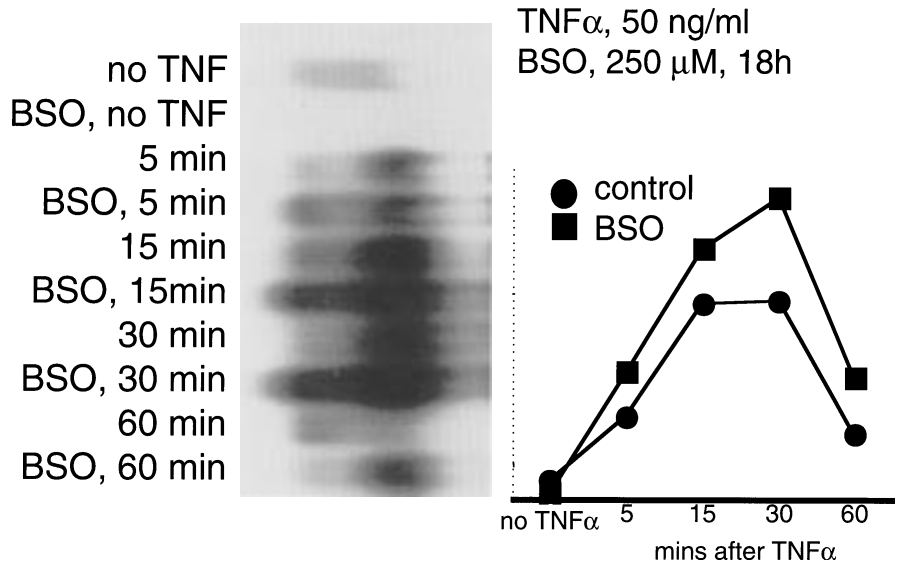

Fig. 1. GSH depletion potentiates TNFα induced NF-κB activation in skeletal muscle derived L6 myoblasts. GSH depletion was caused by buthionine sulfoximine (BSO) treatment (250 μM, 18 h). Lines represent densitometry values of the NF-κB autoradiograph as a function of TNFα treatment time. Circles, BSO non-treated controls; squares, BSO treated GSH depleted cells. Cells were either pre-treated or not with BSO as indicated in the respective lanes, and treated or not with TNFα for the time intervals indicated. The specificity of NF-κB band was verified by cold κB consensus probe competition (not shown) [80].

deficient cells, TNFα induced NF-κB activation was potentiated (Fig. 1) suggesting that such activation is sensitive to cellular GSH, but may have been independent of high levels of intracellular GSSG as previously proposed [65]. Enhancement of cell GSH reserves by treatment of myoblasts with pyrrolidinedithiocarbamate inhibited TNFα induced NF-κB activation (Fig. 2). Bischloroethylnitrosourea treatment is known to inhibit GSSG reductase activity and thus impair NADPH dependent recycling of GSSG to GSH leading to elevated cell GSSG/GSH. In 1,3-bis(chloroethyl)-1-nitrosourea treated cells, TNFα induced NF-κB activation was markedly potentiated suggesting that GSSG may participate in TNFα induced NF-κB activation. The inhibitory effect of pyrrolidinedithiocarbamate on induced NF-κB activation correlated with its effect on intercellular adhesion molecule - 1, the expression of which is known to be NF-κB regulated, expression suggesting changes in cell GSH status not only influences NF-κB activation but also regulates κB dependent transcription [80].

Nutritional supplements

Administered GSH *per se* is not effectively transported into cells [81] except in the small intestine [82–85], it is mostly degraded in the extracellular compartment. The degradation products, i.e. the constituent amino acids, may be used as substrates for GSH neosynthesis inside the cell. Two brief

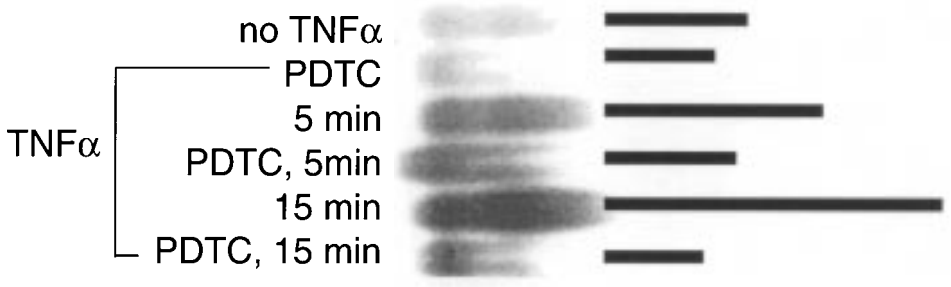

Fig. 2. Inhibition of TNFα induced NF-κB activation in L6 derived skeletal myoblasts with elevated GSH level. Cells were treated (200 μM, 4 h) or not with pyrrolidinedithiocarbamate (PDTC) before TNFα treatment. PDTC treatment increased cell GSH level [80]. Cells were treated or not with TNFα for the time intervals indicated. Bars represent densitometry values of the NF-κB autoradiograph. The specificity of NF-κB band was verified by cold κB consensus probe competition (not shown) [80].

rodent studies claimed that exogenous GSH may remarkably increase endurance to physically exercise [86, 87]. Compared to placebo treated controls 0.5, 0.75 and 1 g/kg intraperitoneal doses of GSH increased endurance to swimming by a marked 102.4, 120 and 140.7%, respectively [87]. At a dose 0.25 g/kg, GSH did not affect endurance when injected once but such a dose could significantly increase endurance when injected once a day for seven consecutive days. In another study, oral GSH at dosages 0.25–1 g/ kg caused a dose-dependent significant improvement in swim endurance [86]. Both above-mentioned studies employed brief bursts of swimming as the exercise challenge and did not report any biochemical data related to either glutathione metabolism or other indices of oxidative stress. We attempted to clarify the possible mechanism of such beneficial effect of GSH supplementation. An extensive biochemical investigation was necessary before any hypothesis regarding the role of exogenous GSH in endurance enhancement could be formulated. Almost all the evidence supporting the contention that a single bout of exercise may induce oxidative stress have been obtained from studies using exercise types that were long in duration, and mostly running or cycling in nature. Because we aimed to test the efficacy of exogenous GSH in controlling exercise induced oxidative stress, an enduring (~ 2 h) treadmill run protocol was used. Intraperitoneal injection of GSH solution (1 g/kg body weight) resulted in a rapid appearance of GSH in the plasma and was followed by a rapid clearance of the thiol. Following the injection, excess plasma GSH was rapidly oxidized. GSH injection did not influence GSH status of other tissues studied. Following repeated administration of GSH, blood and kidney total glutathione levels were increased. Plasma total glutathione of GSH supplemented animals was rapidly cleared during exhaustive exercise. The GSH administration protocol, as used in this study, did not influence the endurance to exhaustive physical exercise of rats. In another report, we showed that treadmill run to exhaustion is associated with a remarkable increase in immunoreactive Mn-SOD (manganese superoxide dismutase, a mitochondrial protein) in the plasma. Glutathione supplementation (500 mg/kg body weight) marginally suppressed such release of the mitochondrial protein to the plasma [88]. The inability of exogenous GSH to provide added antioxidant protection to tissues may be largely attributed to the poor availability of exogenous GSH to the tissues. In another part of this GSH supplementation study conducted in our laboratory we tested the effect of GSH supplementation on exercise-induced leukocyte margination and neutrophil oxidative burst activity [89]. Exercise-associated leukocyte margination was prevented by GSH supplementation. Peripheral blood neutrophil counts were significantly higher in GSH-supplemented groups compared to the placebo control groups. Also, exercise-induced increase in peripheral blood neutrophil oxidative burst activity as measured by luminol-enhanced chemiluminescence per volume of blood tended to be higher in the GSH-supplemented group, and lower in the GSH-deficient rats suggesting that high plasma GSH may have augmented exercise dependent neutrophil priming. In these experiments, for the first time it was shown that GSH supplementation can induce neutrophil mobilization and decrease exercise-induced leukocyte margination, and that exogenous and endogenous GSH can regulate exercise-induced priming of neutrophil for oxidative burst response [89].

N-acetyl-L-cysteine (NAC) and α-lipoic acid (LA) are two nutritional supplements that have remarkable ability to increase cell GSH (Fig. 3) [31]. Both of these agents have been found to be safe for human use. After free NAC enters a cell, it is rapidly hydrolyzed to release cysteine, the rate limiting substrate for intracellular GSH synthesis [31]. α-Lipoic acid is also known as thioctic acid, 1,2-dithiolane-3-pentanoic acid, 1,2-dithiolane-3-valeric acid or 6,8-thioctic acid [31, 64, 90–93]. At physiological pH, α-lipoic acid is anionic and referred to as lipoate. When treated to cells, lipoate is rapidly reduced to dihydrolipoate (DHLA) and released outside the cell. Members of the pyridine nucleotide-disulfide oxidoreductase family of dimeric flavoenzymes e.g. lipoamide dehydrogenase, thioredoxin reductase, and glutathione reductase reduce intracellular lipoate to dihydrolipoate in the presence of cellular reducing equivalents NADH or NADPH (Fig. 3). Thus, a unique advantage of lipoate is that it is able to utilize cellular reducing equivalents, and thus harnesses the metabolic power of the cell, to continuously regenerate its reductive vicinal dithiol form. Because of such recycling mechanism, the lipoate-dihydrolipoate couple can be continuously maintained in a favorable redox state at the expense of the cell's metabolic power [31, 93]. Low concentrations of lipoate has been shown to increase cellular GSH levels by improving the availability of cysteine inside the cell [91, 94]. Because NADH is rapidly consumed to reduce lipoate to dihydrolipoate, treatment of cells with lipoate decreases the $NADH/NAD^+$ ratio. This effect on intracellular reducing equivalent homeostasis is thought to be beneficial in situations such as diabetes and ischemic injury [95]. Because LA can be recycled from its oxidized form to the potent reduced DHLA form by enzymes of the human cell and NAC or its metabolites cannot be regenerated by such mechanism, LA has often been found to be more potent than NAC on a concentration basis [90, 96].

Dihydrolipoate, the reduced form of lipoate, is a potent biologically safe reducing agent and antioxidant [93]. After being enzymatically generated inside the cell, dihydrolipoate rapidly escapes from the cell to the extracellular culture medium [97]. To improve retention in cells, recently we modified the α-lipoic acid (LA) molecule to confer a

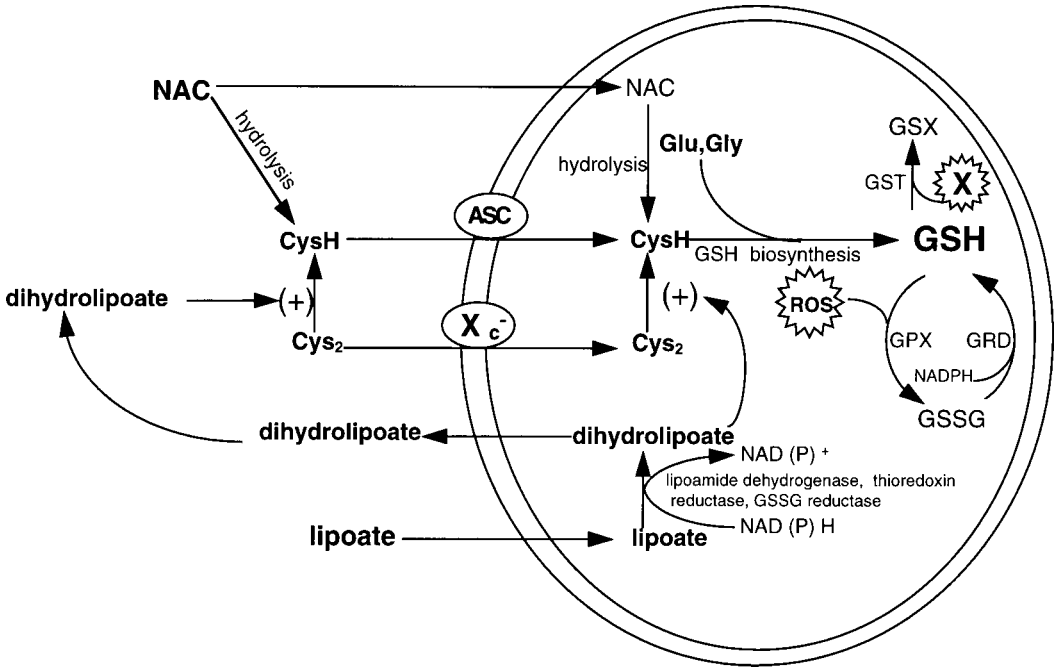

Fig. 3. Improved cysteine supply inside the cell represents an important mechanism by which intracellular GSH may be increased. Hydrolysis of N-acetyl-L-cysteine (NAC) generates cysteine (CysH). Following lipoate supplementation, extracellular dihydrolipoate reduces cystine (Cys_2) outside the cell to cysteine. The cellular uptake mechanism for cysteine by the ASC system is approximately 10 times faster than that for cystine by the x_c^- system. Thus, dihydrolipoate markedly improves cysteine availability within the cell resulting in accelerated GSH synthesis [31]. Glu – glutamine; Gly – glycine; X – electrophilic xenobiotic; GST – GSH S-transferase; GPX – GSH peroxidase; GRD – GSSG reductase; ROS – reactive oxygen species. For more information [31].

positive charge at physiological pH [98]. The protonated form of the new molecule is referred to as LA-Plus (Fig. 4). The uptake of LA-Plus by human T cells was higher compared to that of LA. Several-fold higher amounts of DHLA-Plus, the corresponding reduced form of LA-Plus, was detected in LA-Plus treated cells compared to the amount of DHLA found in cells treated with LA. On a concentration basis, LA-Plus was found to be more biologically potent than LA [98].

The effect of oral N-acetylcysteine supplementation on exercise-associated rapid blood GSH oxidation in the subjects who performed two identical maximal bicycle ergometer exercises three weeks apart has been tested in our laboratory [21]. Before the second maximal exercise test, the men took N-acetylcysteine tablets (200 mg × 4 /day) for two days, and an additional 800 mg in the test morning. In all experiments, blood samples were drawn before, immediately after and 24 h after tests. N-acetylcysteine supplementation increased free radical scavenging capacity of human plasma. Maximal bicycle ergometer test associated rapid blood GSH oxidation was markedly attenuated by N-acetylcysteine supplementation indicating that the treatment spared exercise-associated blood thiol redox status perturbation. In a separate study, trained athletes were orally supplemented with a combination of 1 g GSH and 2 g vitamin C daily for 7 days to test the possible effect of this treatment on exercise-induced blood GSH oxidation [25]. In all five men studied, linearly progressive-intensity treadmill exercise induced blood GSH oxidation. The magnitude of this effect ranged from 34–320% increase in blood GSSG compared to pre-exercise levels. The antioxidant-supplementation protocol was effective in completely protecting against blood GSH oxidation induced by exercise [25].

Lipoic acid is widely used as a food supplement. Almost all of the evidence showing the beneficial effect of lipoic acid on cell GSH has been obtained from *in vitro* studies, however. There is only scanty information regarding whether orally supplemented lipoic acid influences the level of intact lipoate in tissues such as the skeletal muscle and liver. In a recent study we sought to assess the effect of oral supplementation (150 mg/kg, 8 weeks) of lipoic acid on (1) tissue lipoate concentration, GSH levels, and the activities of GSH related enzymes; and (2) exercise induced changes in tissue GSH homeostasis and lipid peroxidation [99]. Lipoic acid supplementation increased the level of lipoate in the red gastrocnemius muscle, and increased total glutathione levels in the liver and blood. Exercise-induced decrease in heart glutathione S-transferase activity was prevented by lipoic acid supplementation. Exhaustive exercise

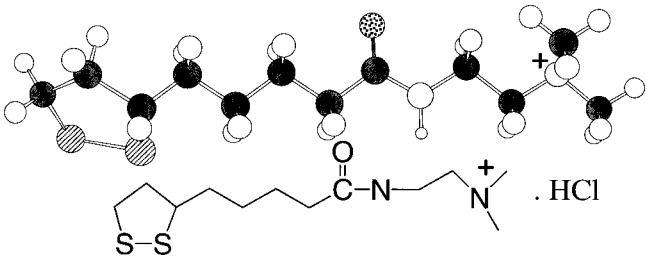

Fig. 4. LA-Plus: A novel analogue of α-lipoic acid with increased biological activity. Chemical name: N,N-dimethyl,N′-2-amidoethyl-lipoate. LA-Plus is positively charged, as shown, at physiological pH. For more information see ref [98].

significantly increased lipid peroxidation metabolite levels in the liver and red gastrocnemius muscle. Lipoic acid supplementation protected against oxidative lipid damage in the heart, liver as well as red gastrocnemius muscle. This study showed that orally supplemented lipoic acid is indeed able to favorably influence tissue antioxidant defenses and counteract oxidative damage at rest and in response to exercise [99].

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