α-Lipoic acid supplementation: tissue glutathione homeostasis at rest and after exercise

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[1]*Department of Physiology, Faculty of Medicine, University of Kuopio, 70211 Kuopio, Finland; and* [2]*Department of Molecular and Cell Biology and* [3]*Biological Technologies Section, Lawrence Berkeley National Laboratory, University of California, Berkeley, California 94720–3200*

Khanna, Savita, Mustafa Atalay, David E. Laaksonen, Mustafa Gul, Sashwati Roy, and Chandan K. Sen. α-Lipoic acid supplementation: tissue glutathione homeostasis at rest and after exercise. *J. Appl. Physiol.* 86(4): 1191–1196, 1999.—Antioxidant nutrients have demonstrated potential in protecting against exercise-induced oxidative stress. α-Lipoic acid (LA) is a proglutathione dietary supplement that is known to strengthen the antioxidant network. We studied the effect of intragastric LA supplementation (150 mg/kg, 8 wk) on tissue LA levels, glutathione metabolism, and lipid peroxidation in rats at rest and after exhaustive treadmill exercise. LA supplementation increased the level of free LA in the red gastrocnemius muscle and increased total glutathione levels in the liver and blood. The exercise-induced decrease in heart glutathione *S*-transferase activity was prevented by LA supplementation. Exhaustive exercise significantly increased thiobarbituric acid-reactive substance levels in the liver and red gastrocnemius muscle. LA supplementation protected against oxidative lipid damage in the heart, liver, and red gastrocnemius muscle. This study reports that orally supplemented LA is able to favorably influence tissue antioxidant defenses and counteract lipid peroxidation at rest and in response to exercise.

thioctic acid; thiol; nutrition; lipid peroxidation; antioxidant

SEVERAL STUDIES HAVE consistently shown in both humans as well as experimental animals that physical exercise may induce oxidative stress (20, 28, 33). Considerable attention has been directed toward the study of antioxidant supplementation with physical exercise to improve the health benefits of physical exercise by decreasing unwanted side effects such as oxidative stress (20, 28). α-Lipoic acid (LA) or thioctic acid (1,2-dithiolane-3-pentanoic acid) is an antioxidant (22, 27, 29, 32) that is commonly used as a dietary supplement, particularly in the United States and Germany. At physiological pH, LA is anionic and is commonly referred to as lipoate. Trace amounts of LA naturally occurring in human tissues are present in α-keto acid dehydrogenase and pyruvate dehydrogenase complexes in which it is covalently bound to a protein-lysyl residue (23). Exogenously supplied LA is readily taken up by a variety of cells and tissues in which it is rapidly reduced by NADH- or NADPH-dependent enzymes to dihydrolipoate [6,8-dithiooctanoic acid; dihydrolipoic acid (DHLA); Ref. 23]. DHLA is a strong reductant with a standard reduction potential of −0.32 V (21). DHLA is known to regenerate major physiological antioxidants of lipid and aqueous phases, such as vitamin E, ascorbate, and GSH (23). The ability of LA to enhance intracellular content of the crucial endogenous antioxidant GSH has been reported (18, 34).

GSH homeostasis plays a central role in defending tissues against oxidative stress (27, 28). Thus it is important to understand how tissue GSH levels may be affected by LA. It was previously shown that supplementation of GSH per se was not effective in increasing GSH levels in tissues, such as liver and muscle, that are known to be highly active in synthesizing GSH (10). Availability of cysteine, a precursor for glutathione synthesis, inside the cell is a critical determinant of cellular glutathione level (27). Intracellular levels of GSH in most cells are determined by the x_c^- system, which mediates cellular import of cystine, and the ASC system, a neutral amino acid transporter that mediates the cellular import of cysteine (2, 3, 15, 27). Because of rapid autooxidation of cysteine to cystine, almost all of the cyst(e)ine present in biological systems is oxidized cystine. For example, the plasma cysteine-to-cystine ratio was estimated to be 1:10 in favor of the oxidized form (12). Several compounds have been tested for their efficacy to serve as cellular cysteine delivery agents. Two clinically relevant pro-GSH agents that have been extensively studied to date are *N*-acetyl-L-cysteine (NAC; 2-mercaptopropionyl glycine) and LA (27). LA has a clear advantage over NAC because, unlike NAC, LA can be reduced in human tissues to its potent dithiol form, DHLA, by enzymes such as lipoamide dehydrogenase, thioredoxin reductase, and glutathione reductase at the expense of cellular-reducing equivalents (26, 36). Once the cysteine originating from NAC is oxidized to cystine, there is no apparent mechanism by which NAC or the cysteine originating from it could be recycled back to the corresponding reduced form. Thus NAC serves as a one-time cysteine donor. In contrast, LA has the ability to serve as a continuous supplier of cysteine. DHLA, generated inside the cells by the enzymatic reduction of LA, provides cysteine by reducing cystine, which is abundant in the extracellular compartment (16). While reducing cystine to cysteine, DHLA oxidizes to LA. LA, generated as a result, is taken up by the cell, in which it is again enzymatically reduced to DHLA. Enzyme-catalyzed reduction of LA to DHLA requires NADH or NADPH that are supplied by the metabolism of glucose. Thus LA utilizes the metabolic power of the cell to continuously regenerate its potent DHLA form.

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Thus LA is able to maintain a continuous supply of cysteine as long as it gets reduced to DHLA by intracellular enzymes (16, 27).

LA is used as a dietary supplement. However, almost all of the evidence showing the beneficial effect of LA on cell GSH has been obtained from in vitro studies. Also, there is a lack of information regarding whether orally supplemented LA influences the level of intact LA in tissues such as the skeletal muscle and liver. Two types of muscle tissue, oxidative red gastrocnemius and glycolytic superficial white vastus lateralis (1), were studied. Our aim was *1*) to assess the effect of oral supplementation of LA on tissue LA concentration, GSH levels, and the activities of GSH-related enzymes, and *2*) to test the effect of LA supplementation on exercise-induced changes in tissue GSH homeostasis and lipid peroxidation.

MATERIALS AND METHODS

Animals. Animal experiments were approved by the University of Kuopio Animal Research Ethics Committee. Forty male Wistar rats (9 wk old, body weight 275–300 g) were randomly divided into two groups: control (C, $n = 20$) and LA supplemented ($n = 20$). These groups were further divided into two equal subgroups: rats killed at rest (CR, LAR) and rats killed immediately after exhaustive exercise (CEx, LAEx). All rats were housed in a room maintained at 22 ± 2°C with 12:12-h dark-light cycles and fed with standard rat chow, R 36 diet (Lactomin, Stockholm, Sweden).

Supplementation protocol. A solution of racemic mixture of LA (Asta Medica, Frankfurt, Germany) was prepared in PBS, pH 7.4, and was administered intragastrically to rats daily (150 mg·kg body $wt^{-1}\cdot day^{-1}$) for 8 wk. The corresponding C groups received a matched volume of PBS, pH 7.4. The supplementation dose was determined on the basis of a previous study in which 1.65 g of LA/kg diet was shown to prevent signs of vitamin E deficiency in mice (25). Because LA intake by each rat may be more accurately controlled when it is supplemented intragastrically compared with when rats are fed a diet enriched with LA, we selected the former mode of administration. In human studies, up to 1,200 mg of intravenous infusion of LA have been used (40). Our intent was to study the effect of chronic LA supplementation. In a previous study in which the effect of LA supplementation in preventing symptoms of vitamin E deficiency was tested in mice, a 5-wk supplementation protocol was used. In this study in rats, we decided to extend the supplementation period to 8 wk.

Exercise protocol. All animals were acquainted with motorized treadmill running (10% uphill grade, 1.0–1.2 km/h, 15–30 min/day for 3 days). On the day of killing, CR and LAR rats were killed at rest, and CEx and LAEx rats were killed after running at a final speed of 1.8 km/h at 10% uphill grade until exhaustion. A standard mild electrical shock deterrent was used intermittently when necessary to coerce the rats to run. Exhaustion was identified as the loss of righting reflex when rats were laid on their back. Food and supplementation were withheld overnight before the rats were killed. Endurance time to exhaustion was not significantly different between nonsupplemented and LA-supplemented animals (not shown).

Sample collection. Rats were matched among groups for the day and time of killing. They were killed by decapitation. Liver, heart, kidney, and superficial vastus lateralis and red gastrocnemius muscles were quickly dissected out, freed from adipose and connective tissue, rinsed in ice-cold saline, blotted, cut into small pieces, submerged in liquid nitrogen, and stored at −70°C. Mixed blood was collected from decapitated rats in 0.5 volumes of ice-cold 10% (wt/vol) 5-sulfosalicylic acid contained in microcentrifuge tubes. The resultant mixture was immediately vortexed and centrifuged at 10,000 *g* at 4°C. Immediately after the centrifugation, the clear supernatant obtained from 10% (wt/vol) 5-sulfosalicylic acid-treated deproteinized blood was stored at −70°C for the determination of total glutathione (TGSH).

Tissue preparation and biochemical analyses. For the determination of TGSH, tissues were homogenized on ice in brief bursts by an Ultra-Turax homogenizer (Janke and Kunkel) in a 1:10 (wt/vol) dilution of ice-cold 0.5 N perchloric acid. Resultant homogenates were centrifuged at 10,000 *g* for 15 min (4°C), and the supernatant was stored at −70°C. On the day of measurement, the supernatant was diluted with distilled water, and TGSH was measured spectrophotometrically as described earlier (31). For the assays of glutathione peroxidase (GPX), glutathione disulfide reductase (GRD), and glutathione *S*-transferase (GST), frozen tissues were crushed in liquid nitrogen and homogenized in extraction buffer (50 mM Tris, 0.25 M sucrose, 1 mM EDTA, pH 7.4). The homogenate was centrifuged at 10,000 *g* at 4°C for 15 min. The supernatant was centrifuged again at 105,000 *g* at 4°C for 60 min, and the postmicrosomal supernatant was stored at −70°C. Activities of tissue GPX, GRD, and GST were determined spectrophotometrically from the postmicrosomal supernatant, as described previously (31). Protein content of the supernatant was determined according to Bradford (7). Tissues were homogenized and analyzed for the presence of thiobarbituric acid reactive substances (TBARS) as described before (30).

Determination of free LA in tissues. Frozen tissues (500–600 mg) were ground with mortar and pestle in liquid nitrogen. Powdered samples were homogenized with 20% (wt/vol) *m*-phosphoric acid (2 ml) on ice by using a Teflon homogenizer. After vigorous vortexing and brief bursts of ultrasonication, homogenates were transferred to glass tubes. The homogenates were extracted with hexane (3 ml) and isopropanol (250 μl) by a brief vortexing and sonication (15 min) in an ice bath. After sonication, samples were shaken for 10 min at 4°C. Samples were centrifuged at 1,500 *g*, and the hexane layer was collected in a glass tube. The above-mentioned hexane extraction was repeated twice from the same sample. Hexane collected from all three washes was pooled and evaporated to dryness under nitrogen gas. HPLC mobile phase, as used for LA detection (see *HPLC electrochemical detection of LA* for composition), was added to each sample, and the samples were left covered overnight at 4°C. On the following day, the samples were centrifuged at 15,000 *g* for 5 min. Supernatants were collected and stored at −70°C.

HPLC electrochemical detection of LA. LA from tissue samples was analyzed by using a HPLC electrochemical detection method, as recently described (35). A coulometric detector (Coulochem II; ESA Laboratories, Chelmsford, MA) was used for the analysis. The electrodes of the coulometric detector were set as follows: *electrode 1*, +0.45 V; *electrode 2*, +0.85 V; and guard cell, +0.90 V. LA was separated on a Altima C18 (250 × 4.6 mm, 5-μm pore size; Alltech, Deerfield, IL) column. The mobile phase, consisting of 50% (vol/vol) 50 mM NaH_2PO_4, (pH 2.7), 30% (vol/vol) acetonitrile, and 20% (vol/vol) methanol was delivered by using an isocratic solvent delivery module (ESA) with 1 ml/min flow rate (35).

Statistical analyses. Results are presented as means ± SE. The overall effects of LA supplementation as well as exhaustive exercise were tested by two-way ANOVA. Interaction of

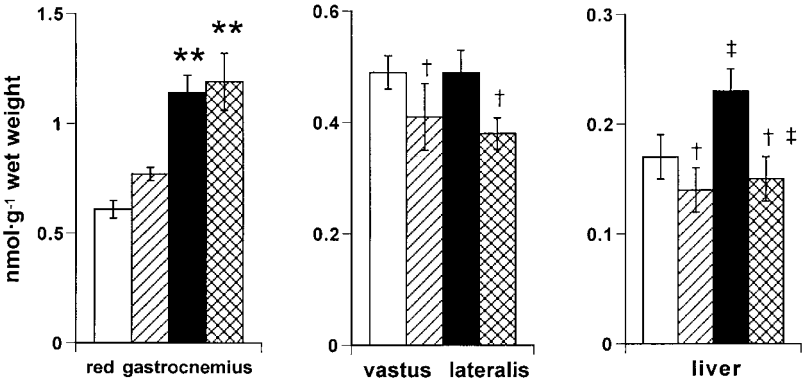

Fig. 1. Lipoic acid content in red gastrocnemius muscle, vastus lateralis muscle, and liver of nonsupplemented (at rest, open bars; after exercise, hatched bars) and lipoic acid-supplemented (at rest, solid bars; after exercise, crosshatched bars) rats. Values are means ± SE; $n = 10$ rats/group. Difference due to lipoic acid supplementation: $^{**}P < 0.001$, $\ddagger P = 0.092$ (represents nonsignificant trend). Difference due to exhaustive exercise: $\dagger P < 0.05$.

LA supplementation and exercise refers to a combined effect of supplementation and exercise different from the effect of LA supplementation and exercise separately. In certain cases (see Table 1 legend), one-way ANOVA and Duncan's tests were done to compare a pair of group means. The level of statistical significance was set at $P < 0.05$.

RESULTS

Tissue lipoate levels in response to oral supplementation of lipoate. Using a highly sensitive method for the determination of LA from biological samples, we were able to detect LA from acidified skeletal muscle and liver extracts (Fig. 1). LA supplementation increased the level of LA in the red gastrocnemius muscle ($P < 0.001$) and tended to increase the level in liver ($P = 0.092$) but did not reach statistical significance. However, LA supplementation did not influence the level of LA in the superficial vastus lateralis muscle (Fig. 1). Exhaustive exercise did not influence LA content of red gastrocnemius muscle but decreased tissue LA contents in both vastus lateralis muscle ($P < 0.05$) and liver ($P < 0.05$) (Fig. 1).

Tissue TGSH level and activity of glutathione-related enzymes in response to oral supplementation of LA. LA supplementation significantly increased total TGSH levels in the liver ($P < 0.001$) and blood ($P < 0.05$) (Fig. 2). No significant change in TGSH levels in kidney, heart, red gastrocnemius muscle, and vastus lateralis muscle was observed in response to LA supplementation. Exercise-induced loss of hepatic TGSH ($P < 0.001$) was observed in both LA-nonsupplemented as well as LA-supplemented rats. Although LA supplementation did not increase the TGSH level in the heart, exercise-induced loss of GSH from the heart tended to be less in supplemented rats compared with nonsupplemented rats (23.1% loss in nonsupplemented vs. 3.6% loss in supplemented), but this did not reach significance (Fig. 2).

LA supplementation did not influence the activity of GPX in any of the tissues studied (Table 1). GPX activity tended to be higher in the heart of LA-supplemented and LAEx rats compared with the corresponding nonsupplemented group ($P = 0.089$ for the interaction between LA and exercise). The activity of GRD was not affected by LA supplementation or exercise in the tissues studied. GST activity in the heart of nonsupplemented animals decreased ($P < 0.05$) in response to exercise. Such exercise-dependent loss of GST activity was prevented by LA supplementation (interaction of LA supplementation and exercise, $P < 0.05$). The activity of GST in other tissues was not affected by the interventions (Table 1).

Resting and exercise-induced tissue lipid peroxidation in response to oral supplementation of lipoate. In LA-supplemented rats, lower levels of TBARS were detected in the liver ($P < 0.05$), red gastrocnemius muscle ($P < 0.05$), and heart ($P < 0.001$) compared with the corresponding nonsupplemented groups (Fig. 3). LA supplementation did not influence the TBARS level of other tissues such as superficial vastus lateralis muscle (Fig. 3) or kidney (not shown).

Exhaustive exercise significantly increased TBARS levels in the liver ($P < 0.001$) and red gastrocnemius

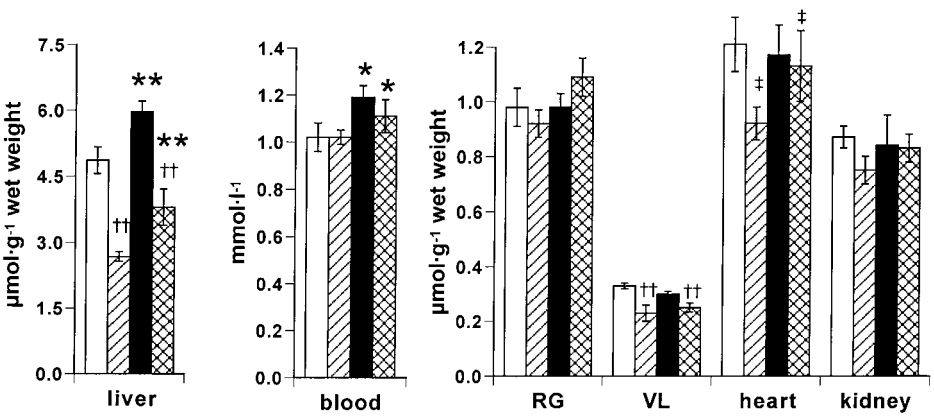

Fig. 2. Effect of lipoic acid supplementation and exhaustive exercise on total glutathione content in liver, blood, red gastrocnemius muscle (RG), vastus lateralis muscle (VL), heart, and kidney of nonsupplemented (at rest, open bars; after exercise, hatched bars) and lipoic acid-supplemented (at rest, solid bars; after exercise, crosshatched bars) rats. Values are means ± SE; $n = 10$ rats/group. Difference due to lipoic acid supplementation: $^{**}P < 0.001$, $^{*}P < 0.05$. Difference due to exhaustive exercise: $\dagger\dagger P < 0.001$, $\ddagger P = 0.095$ (represents nonsignificant trend).

Table 1. *Effect of lipoate supplementation and exercise*

	Liver	RG	VL	Heart	Kidney
			Glutathione peroxidase		
CR	795 ± 39.6	126 ± 11.2	17.1 ± 0.7	316 ± 9.8	446 ± 26.5
CEx	786 ± 40.8	120 ± 15.7	17.9 ± 0.9	303 ± 10.8	484 ± 53.7
LAR	765 ± 25.7	109 ± 9.9	18.3 ± 1.5	313 ± 9.7	471 ± 29.0
LAEx	806 ± 39.4	138 ± 14.2	18.1 ± 0.9	336 ± 10.6‡	448 ± 16.7
			Glutathione disulfide reductase		
CR	134 ± 5.0	26.3 ± 1.9	18.9 ± 0.7	31.8 ± 1.1	384 ± 10.7
CEx	130 ± 3.1	22.4 ± 2.9	18.5 ± 0.7	33.0 ± 1.5	424 ± 30.1
LAR	142 ± 5.2	25.3 ± 2.2	20.6 ± 0.6	35.4 ± 1.8	389 ± 8.6
LAEx	137 ± 5.9	23.0 ± 2.1	16.9 ± 0.9	32.7 ± 0.9	376 ± 14.2
			Glutathione S-transferase		
CR	1,908 ± 128	134 ± 3.1	91.1 ± 3.8	152 ± 4.8	286 ± 13.5
CEx	1,735 ± 66	129 ± 6.1	90.0 ± 4.4	136 ± 4.6*	310 ± 18.5
LAR	1,928 ± 53	128 ± 5.9	92.0 ± 5.8	144 ± 4.3	295 ± 18.0
LAEx	1,942 ± 56	125 ± 3.0	81.8 ± 2.8	149 ± 4.0†	315 ± 9.7

Values are means ± SE in nmol·min^{-1}·mg protein^{-1}. RG, red gastrocnemius muscle; VL, superficial vastus lateralis muscle; CR, control rats at rest; CEx, control rats after exhaustive exercise; LAR, lipoate-supplemented rats at rest; LAEx, lipoate-supplemented rats after exhaustive exercise. Difference due to exhaustive exercise: $*P <$ 0.05 (1-way ANOVA and Duncan's test). Interaction between lipoate-supplemented and exercise: $\dagger P < 0.05$, $\ddagger P = 0.089$ (represents nonsignificant trend).

muscle ($P < 0.001$) and tended to increase TBARS in the vastus lateralis muscle (Fig. 3). Cardiac (Fig. 3) and renal (not shown) TBARS levels were not affected by the exhaustive exercise protocol used in this study.

DISCUSSION

Despite a rapidly growing interest in LA and its therapeutic potential as a proglutathione agent, information regarding the effect of orally supplemented LA on the level of GSH in tissues is limited (8). This is the first study investigating the effect of LA feeding on the activity of GSH-related enzymes in tissues. Furthermore, using a recently developed assay system (35), we have been able to estimate tissue LA levels more sensitively and consistently than those reported before by the same laboratory (17). The new method was sensitive enough to detect endogenous LA present in tissues.

Previously, it has been reported that oral administration of ^{14}C-labeled LA to rats results in 66% absorption of the ^{14}C label (24). The observation that LA feeding increased the intact LA level in the red gastrocnemius muscle (87.39 ± 22.79%) and liver (35.19 ± 14.63%) but not in the superficial vastus lateralis muscle may be explained by the fact that LA is subject to metabolic modification in tissues. Also, exercise-induced loss of LA from tissues may result from metabolic processing of intact LA in the tissue. Pharmacokinetic studies with LA have shown that this compound is extensively metabolized by β-oxidation in tissues (6). After such catabolism, LA is transformed to products such as bisnorlipoic acid, 3-hydroxylipoic acid, and 3-ketolipoic acid (4). 3-Hydroxylipoic acid is a precursor of 3-ketolipoic acid in the β-oxidation sequence. Other investigators have also detected metabolic products of LA that have different retention times in the HPLC column compared with that of LA (39).

Recent studies have uncovered the mechanism by which LA increases cellular GSH (18, 27, 34). DHLA markedly improves cysteine availability within the cell, resulting in accelerated GSH synthesis (27). In view of this mechanism of action of LA, it may be expected that the effect of LA on tissue GSH would be most marked in organs that have a high activity of GSH-synthesizing enzymes. We observed that LA supplementation increased the GSH level of blood and liver. Such observations may be explained by the fact that both of these tissues, i.e., erythrocytes in blood and the liver, have remarkably high activity of GSH-synthesizing enzymes (10, 37).

Despite the fact that neither an increased amount of intact LA nor enhanced GSH levels were observed in the heart of LA-supplemented rats, LA supplementation tended to protect against exercise-induced loss of heart TGSH. Also, as an overall effect, LA supplementation protected against exercise-induced hepatic TGSH loss. The antioxidant activity of LA is known to rest in its 1,2-dithiolane ring structure (6). Thus β-oxidation products of LA, which would have a smaller fatty acid-like carbon chain but an unaffected dithiolane ring, should retain the antioxidant properties of LA. Indeed this has been shown to be true for bisnorlipoic

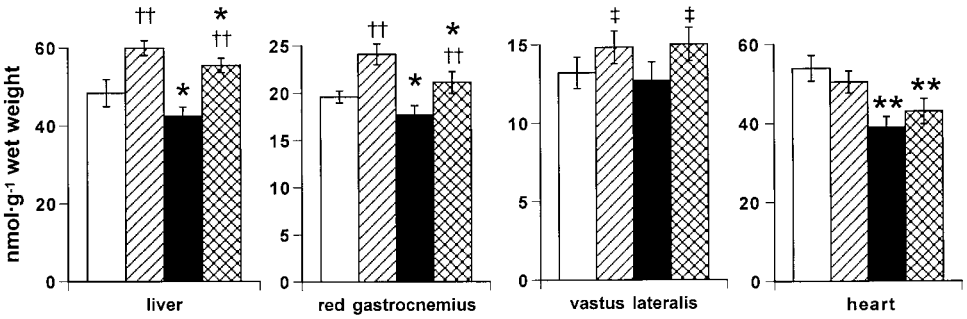

Fig. 3. Effect of lipoic acid supplementation and exhaustive exercise on lipid peroxidation levels in liver, red gastrocnemius muscle, vastus lateralis muscle, and heart of nonsupplemented (at rest, open bars; after exercise, hatched bars) and lipoic acid-supplemented (at rest, solid bars; after exercise, crosshatched bars) rats. Lipid peroxide level in tissues was determined by presence of thiobarbituric acid-reactive substances. Values are means ± SE; $n = 10$ rats/group. Difference due to lipoic acid supplementation: $**P < 0.001$, $*P < 0.05$. Difference due to exhaustive exercise: $\dagger\dagger P < 0.001$, $\ddagger P = 0.076$ (represents nonsignificant trend).

acid as well as tetranorlipoic acid (5, 11, 38). In view of the present findings, it seems likely that such metabolites of LA may have contributed to heart antioxidant defenses. In LA-supplemented rats, exercising was associated with a tendency to increase tissue GPX activity. This would result in improved antioxidant defense in that tissue as well.

Besides being a powerful antioxidant, GSH is a major cellular electrophile conjugator as well. GST catalyzes the reaction between the SH group of GSH and potential alkylating agents, thereby neutralizing their electrophilic sites and rendering them more water soluble. GSTs represent a major group of phase II detoxification enzymes (19). We observed that exhaustive exercise decreased the activity of GST in the heart. Supplementation of rats with LA abolished the exercise-induced decrease of GST activity in the heart. Previously, it has been shown that oxidative stress decreases the steady-state mRNA levels encoding constitutively expressed GST isozymes (Ya1, Ya2, Yb1, Yb2, and Yc1) as well as the activity of these isozymes (14). Thus the antioxidant properties of LA may be involved in preserving GST activity under conditions of exercise-induced oxidative stress.

Oxidative damage is a continuously ongoing process, and low levels of reactive oxygen species and markers of oxidative stress are detectable in tissues of animals even at rest (9, 13). Several studies have shown that strenuous exercise may cause oxidative damage (28, 33). Consistently, we observed that exercise increased lipid peroxidation in the liver and skeletal muscles. An overall protective effect of LA supplementation against oxidative lipid damage was evident in the heart and liver as well as in red gastrocnemius muscle. Such protection against exercise-induced oxidative stress is likely mediated in part by LA itself and the metabolites of LA having potent antioxidant properties. Because LA and its metabolites are mostly hydrophilic antioxidants, strengthening the antioxidant defense network and potentiating the effects of vitamin E may be a more important mechanism by which LA may protect against lipid peroxidation, as reported previously (22, 23).

In summary, this study shows that oral supplementation of LA increases the content of this antioxidant in the oxidative red gastrocnemius muscle and liver. In agreement with in vitro studies, LA treatment was able to increase the TGSH level of tissues, e.g., blood and liver, having high GSH-synthesizing activity. Although LA supplementation did not increase the level of free LA in the heart, the heart also appeared to benefit from LA supplementation, particularly against oxidative lipid damage induced by exhaustive running exercise. LA supplementation also protected in a tissue-specific manner against inactivation of GST, depletion of TGSH, and induction of lipid peroxidation in response to exhaustive running exercise. This study shows that orally supplemented LA is able to favorably influence tissue antioxidant defenses.

This study was supported by research grants from the Finnish Ministry of Education and Juho Vainio Foundation of Helsinki (to C. K. Sen). The PhD studies of S. Khanna, M. Atalay, M. Gul, and D. E. Laaksonen were also supported by a graduate school grant from the Academy of Finland.

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Received 4 May 1998; accepted in final form 3 December 1998.

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