Vol. 46, No. 2, October 1998 BIOCHEMISTRY *and* MOLECULAR BIOLOGY INTERNATIONAL
Pages 297–306

Skeletal muscle and liver lipoyllysine content in response to exercise, training and dietary α-lipoic acid supplementation

Savita Khanna,[1,2] Mustafa Atalay,[1] John K. Lodge,[2] David E. Laaksonen,[1]
Sashwati Roy,[1,2] Osmo Hanninen,[1] Lester Packer[2,3] and Chandan K. Sen[1-4]

[1]*Department of Physiology, University of Kuopio, 70211 Kuopio, Finland;*
[2]*Department of Molecular & Cell Biology, University of California at Berkeley; and*
[3]*Biological Technologies, EETD, Lawrence Berkeley National Laboratory, CA 94720*

Received June 19 1998

Summary - In human cells, α-lipoic acid (LA) is present in a bound lipoyllysine form in mitochondrial proteins that play a central role in oxidative metabolism. The possible effects of oral LA supplementation, a single bout of strenuous exercise and endurance exercise training on the lipoyllysine content in skeletal muscle and liver tissues of rat were examined. Incorporation of lipoyl moiety to tissue protein was not increased by enhanced abundance of LA in the diet. Endurance exercise training markedly increased lipoyllysine content in the liver at rest. A bout of exhaustive exercise also increased hepatic lipoyllysine content. A significant interaction of exhaustive exercise and training to increase tissue lipoyllysine content was evident. In vastus lateralis skeletal muscle, training did not influence tissue lipoyllysine content. A single bout of exhaustive exercise, however, clearly increased the level of lipoyllysine in the muscle. Comparison of tissue lipoyllysine data with that of free or loosely-bound LA results showed a clear lack of association between the two apparently related parameters. Tightly protein-bound lipoyllysine pool in tissues appeared to be independent of the loosely-bound or free LA status in the tissue.

Key word: thioctic acid, antioxidant, mitochondria, thiols, redox, nutrition

INTRODUCTION

Classically, α-lipoic acid (LA) is known for its central role as an essential cofactor in oxidative metabolism (1-3). More recent studies show that exogenously supplemented α-lipoic acid may have several protective function mostly by virtue of its antioxidant and cell redox regulatory functions (4-7). At concentrations as low as 10 - 100 μM, LA has been shown to increase GSH content in human T cells (8). LA and N-acetyl-L-cysteine (NAC; 2-mercapto-propionyl glycine) represent two clinically relevant pro-GSH agents that have been most extensively studied so far (6). Unlike NAC, LA can be recycled to its potent reduced dithiol form, dihydrolipoate (DHLA) (6, 9). DHLA plays a central role in increasing cell GSH (10). Because LA can be recycled to DHLA by enzymes present in human cells it has a clear advantage over NAC on a concentration:effect basis (11, 12).

In vertebrates, endogenous lipoate is found in five mitochondrial proteins: acyltransferase components of the pyruvate, α-ketoglutarate, and branched chain α-ketoacid dehydrogenase complexes (E2p, E2k, and E2b, respectively), protein X of the pyruvate dehydrogenase complex,

[4] Corresponding author. Fax: 510 642 8313. E-mail: cksen@socrates.berkeley.edu or Chandan.Sen@fokka.uku.fi

1039–9712/98/140297–10$05.00/0

and the H-protein of the glycine cleavage system. In these proteins, LA attaches to the ε-amino group of the specific lysine residue of the proteins via an amide linkage. Recently it has been shown that lipoyltransferase I and II lipoylates not only H-protein of the glycine cleavage system but also lipoyl domains of acyltransferases of the pyruvate, α-ketoglutarate, and branched chain α-ketoacid dehydrogenase complexes (13). The lipoyllysine residue functions as a carrier of intermediates of the reactions and reducing equivalents interacting with the active sites of the components of the complexes (3, 14, 15). A defect in the lipoyl-bearing protein X subunit of the pyruvate dehydrogenase complex in humans has been associated with encephalomyelopathy (16).

LA has been detected in the form of lipoyllysine in various natural sources including edible plant and animal products (17). Because of the remarkable therapeutic potential of LA (4, 12, 18), several recent studies have sought to investigate the effect of dietary LA supplementation (19-25). At present there is no information, however, whether LA supplementation may influence the level of lipoyllysine in mitochondrial proteins. Physical exercise is associated with a multi-fold increase in oxygen consumption by tissues, and it has been shown that strenuous exercise may cause oxidative stress (26, 27). Physical exercise is also know to markedly influence skeletal muscle and liver mitochondria function as well as biogenesis (28-36). In this study we sought to examine the possible effect of oral LA supplementation, a single bout of strenuous exercise and endurance exercise training on the lipoyllysine content in skeletal muscle and liver tissues of rat.

MATERIALS AND METHODS

Animals. Animal experiments were approved by the University of Kuopio Animal Research Ethics Committee. Eighty male Wistar rats, 9 weeks old, body weight 275-300 g, were randomly divided into two groups: control (C, n = 40) and exercise trained (T, n = 40). Rats in each of these two groups were either not (P, n = 20) or supplemented (L, n = 20) with LA. These subgroups were further divided into two equal subgroups: rats killed at rest (CPR, CLR, TPR, TLR) and rats killed immediately after exhaustive exercise (CPE, CLE, TPE, TLE). All rats were maintained at 22 ± 2°C with 12:12 h dark : light cycles and fed with standard rat chow, R 36 diet (Lactomin Ab, Stockholm, Sweden).

Supplementation protocol. A solution of racemic mixture of LA (Asta Medica, Frankfurt, Germany) was prepared in phosphate buffered saline (PBS), pH 7.4 and was intragastrically administered daily to rats 150 mg per kg body weight per day for 8 weeks. The corresponding control groups received a matched volume of PBS (P, placebo), pH 7.4.

Exercise protocol. All animals were acquainted with treadmill running (10 % uphill grade , 1.0-1.2 km/h, 15 min/day for 5 days). On the day of killing, CPR, CLR, TPR and TLR rats were killed at rest; CPE, CLE, TPE and TLE rats were killed after running at a final speed of 2.1 km/h at 10% uphill grade until exhaustion. Exhaustion was identified as the loss of righting reflex when rats were laid on their back. Food and supplementation were withheld overnight before killing. Rats in group T were exercise trained using a rodent treadmill set at 10 % uphill grade, 5 days a week for 8 weeks. Training started at 13 weeks of age by familiarizing the rats to the treadmill for 6 days, followed by linear increase in running speed and duration to achieve a final speed and duration of 1.8 km/h and 1.5 h /d as described previously (37).

Sample collection. Rats were matched between groups for the day and time of killing. They were killed by decapitation. Liver and superficial vastus lateralis muscle were quickly dissected out, freed from adipose and connective tissue rinsed in ice-cold saline and blotted, cut into small pieces and submerged in liquid nitrogen and stored in -70°C.

Sample preparation for the detection of lipoyllysine. Tissue samples (250 - 400 mg) were freeze dried overnight. The samples were powdered and suspended in 2 ml of 0.1 M Tris-HCl (pH 7.4) and disrupted using a ultra-sonicator (Branton) for approximately 2.5 mins. The tissue suspension was supplemented with 10 U each of protease VIII (Subtilisin Carlsberg from *Bacillus licheniformis*) and protease XIV (Pronase from *Streptomyces griseus*), and 200 U penicillin "G" and 200 μg streptomycin. The samples were incubated at 37°C for 24 h. After this, samples were freeze dried. Ethanol extraction of the digested and dried samples was performed three times, and finally the supernatant was dried under nitrogen as described previously (17). Lipoyllysine standards were prepared by adding known amounts of lipoyllysine to a solution of bovine serum albumin (2.5 mg/ml). The mixture was protease digested and ethanol extracted as described above. Standards in water, without any other treatment, were also used (17).

Sample preparation for the detection of loosely-bound or free LA. Frozen tissues (500-600 mg) were ground in liquid nitrogen with mortar and pestle. Powdered samples were homogenized with 20 % (w/v) *m*-phosphoric acid (2 ml) on ice 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 brief vortexing and sonication (15 min) in ice bath. Following sonication, samples were shaken for 10 min at +4°C. Samples were centrifuged at 1500 *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 were pooled and evaporated to dryness under nitrogen gas. High performance liquid chromatography (HPLC) mobile phase, as used for LA detection (see a following section for composition), was added to each sample and the samples were left overnight at +4°C. On the following day the samples were centrifuged at 15000 *g* for 5 min. Supernatants were collected and stored at -70°C.

HPLC- amperometric electrochemical detection of lipoyllysine
Samples were separated on a C18 column (5 μM, 4.6 mm i.d. x 150 mm length; Alltech Associates, Deerfield, IL) using the following mobile phase: 65% 0.15 M monochloroacetic acid (pH 2.8), 21% methanol, 14% acetonitrile, and at a flow rate of 1 ml/min. The samples, dried under nitrogen, was dissolved in 0.4 ml of mobile phase and the precipitate was removed by centrifugation (14000 *g* x 15 min). The supernatant was used for injection. Lipoyllysine in the samples was detected using an amperometric detection system (Bioanalytical system, West Lafayette, IN) fitted with a dual gold electrode coated with triple-distilled Hg and operating at 0.05 V potential (17).

HPLC- coulometric electrochemical detection of LA
LA from tissue samples were analyzed using a HPLC-electrochemical (HPLC-EC) detection method as recently described (38). A coulometric detector (ESA, Coulochem II, 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 C18 (250 x 4.6 mm, 5 μm pore size, Alltech, Deerfield, IL) column. The mobile phase, consisting of 50 % (v/v) 50 mM NaH_2PO_4, (pH 2.7), 30 % (v/v) acetonitrile and 20 % (v/v) methanol was delivered using an isocratic solvent delivery module (ESA, Chelmsford, MA) with 1 ml/min flow rate (38).

Statistical analyses. Results are presented as mean ± SD. The overall effects of acute exercise, exercise training and LA supplementation were tested by three-way analysis of variance (ANOVA). Each letter superscripted on the bars represent a class of mean, and non-identical superscripts represent that difference between those two means are significant. For example, **a** is not different from **ab** but different from **c**. The results of statistical analyses are clearly illustrated in Table 1. The interaction of exercise, training and LA supplementation refers to a combined effect of exercise, training and supplementation different from the effect expected from exercise, training or LA supplementation separately. One-way analysis of variance and Duncan's test was done to compare a pair of group means as shown in the respective figures. Significance level was set at $P < 0.05$.

RESULTS

Exercise training markedly increased lipoyllysine content in the liver at rest (Fig. 1). This was true for both LA supplemented and non-supplemented rats. Overall, a bout of exhaustive exercise increased lipoyllysine content in the liver (Table 1). This effect was most markedly visible in trained and LA-supplemented rats. LA supplementation generally decreased tissue lipoyllysine content as an overall effect (Table 1). A significant interaction of exhaustive exercise and training to increase tissue lipoyllysine content was evident. Also, significant interaction of exercise, training and LA supplementation to increase hepatic lipoyllysine was observed when tested by three-way ANOVA (Table 1). In the superficial vastus lateralis skeletal muscle, exercise training did not influence tissue lipoyllysine content. A single bout of exhaustive exercise, however, clearly increased the level of lipoyllysine in the muscle. This was evident both in untrained as well as trained rats (Fig. 2). Overall, LA supplementation tended to ($P = 0.088$) increase tissue lipoyllysine content. A borderline significant ($P = 0.054$) interaction of LA supplementation and exhaustive exercise to increase lipoyllysine content in the muscle was observed. No other two-way or three-way interaction was observed for the variables tested for their effect to influence muscle lipoyllysine content (Table 1).

Neither exercise training nor LA supplementation had any overall influence on LA content in the liver (Table 1). Duncan's test show that LA supplementation increased hepatic LA content in non-trained animals at rest but not in any other treatment group (Fig. 3). A single bout of exhaustive exercise clearly decreased hepatic LA content (Fig. 3). As an overall effect exhaustive exercise also decreased LA content in muscle (Fig. 4, Table 1). In trained rats, LA supplementation increased the level of LA in the muscle as tested by Duncan's test (Fig. 4). However, no overall effect of LA supplementation or treadmill training on LA content in the muscle was observed (Table 1).

DISCUSSION

Lipoyllysine containing proteins play a central role in energy metabolism (3, 13, 15, 39, 40). Activities of critical enzymes such as pyruvate dehydrogenase in glycolysis and α-ketoglutarate dehydrogenase in the citric acid cycle are known to be dependent on lipoyllysine residues bound to these enzymes. It has been suggested that the lipoyllysine residue functions as a carrier of intermediates of the reactions and reducing equivalents interacting with the active sites of the components of the enzyme complexes (3, 15). The process of lipoylation of proteins in the eukaryotic cell is not well understood. A lipoyltransferase enzyme, thought to catalyze the incorporation of lipoyl moiety from lipoyl-AMP to protein, has been purified from bovine liver (41-43). However, this enzyme does not have the ability to activate LA to lipoyl-AMP. Lipoylation of H-protein in bovine liver, therefore, requires the activity of a LA activating enzyme that has been partially purified (44). Thus, at present there is only limited information on the mechanisms regulating the lipoylation of proteins in eukaryotic cells. Results of this study clearly show that LA supplementation does not significantly increase lipoyllysine content in tissues. In contrast, LA supplementation generally decreased hepatic lipoyllysine content as an overall effect. From this it may be concluded that the enhanced abundance of LA in the diet does not improve incorporation of lipoyl moiety to tissue protein.

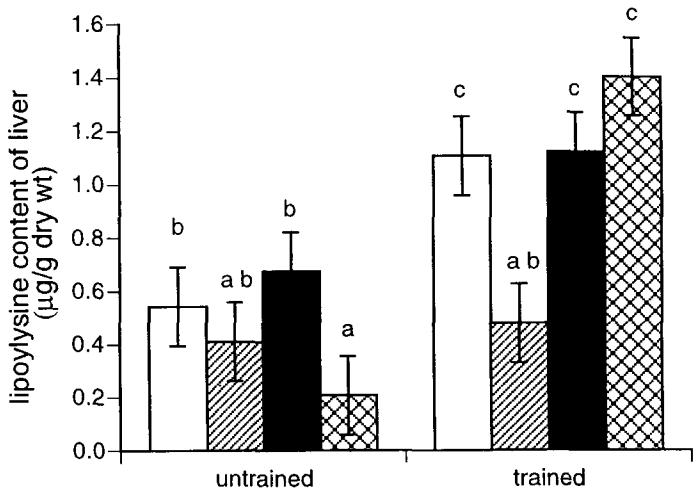

Figure 1. **Lipoyllysine content in the liver.** Open bar, LA non-supplemented at rest; hatched bar, LA supplemented at rest; closed bar, LA non-supplemented post-exercise; cross-hatched bar, LA supplemented post-exercise. LA, lipoate (racemic α-lipoic acid in phosphate buffered saline, pH 7.4). Data are mean ± SD. Means with different superscript are significantly different ($P < 0.05$) as tested by Duncan's test. ANOVA results are shown in Table 1.

Pharmacokinetic studies with LA have shown that this compound is extensively metabolized by β-oxidation in tissues (45). As a result, products such as bisnorlipoic acid, 3-hydroxylipoic acid and 3-ketolipoic acid have been observed to form (46). 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 time in HPLC column compared to that of LA (47). Such metabolic alteration of the LA molecule may provide explanation to the observation that tissue LA content of LA supplemented rats was not high. The antioxidant activity of LA is known to rest in its 1,2-dithiolane ring structure (45). 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 acid as well as tetranorlipoic acid (48-50).

One of the most striking findings of this study is that endurance exercise training increased the content of lipoyllysine specifically in the liver, but not in the skeletal muscle tissue. Training dependent increase in hepatic lipoyllysine content may reflect higher density of mitochondria in the trained tissue. This explanation, however, is less likely to singularly account for the entire effect because no such effect of training was observed in the skeletal muscle although exercise training is known to increase the density of mitochondria in this tissue (33, 34, 51, 52). Exercise training is known to regulate the activity of a large number of enzymes. It is therefore possible that exercise training specifically up-regulates the activity of hepatic enzymes responsible for the incorporation of lipoyl moieties to tissue proteins. This is a very likely mechanism because even a single bout of exhaustive exercise had an overall effect of increasing hepatic lipoyllysine content. The hypothesis

Table 1. Three-way ANOVA analyses[1]

Tissue	Parameter	E	T	L	E x T	E x L	L x T	E x T x L
Liver	Lipoyllysine (Fig 1)	0.012	0.000	0.006	0.002	0.111	0.361	0.000
	Lipoate (Fig 3)	0.007	0.574	0.445	0.733	0.071	0.007	0.631
Muscle	Lipoyllysine (Fig 2)	0.000	0.119	0.088	0.286	0.054	0.080	0.342
	Lipoate (Fig 4)	0.000	0.792	0.334	0.208	0.088	0.164	0.151

[1]Three-way ANOVA [exhaustive exercise (E), training (T) and LA supplementation (L)]. Numbers in the table indicate *P* values (significance).

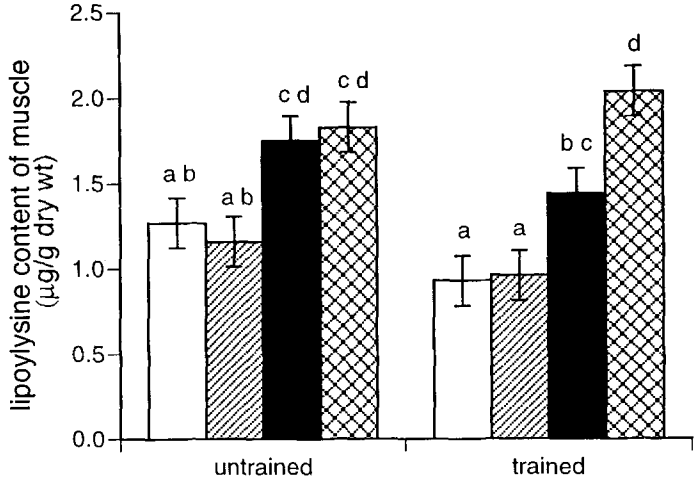

Figure 2. **Lipoyllysine content in the superficial vastus lateralis skeletal muscle.** For other details see legend of Fig. 1.

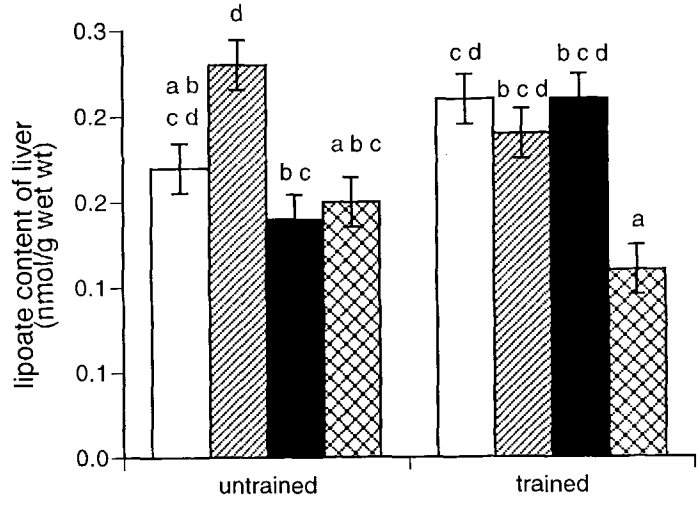

Figure 3. **Lipoate content in the liver.** Lipoate represents the free or loosely-bound tissue lipoate pool that was readily released from the tissue in response to acid extraction. For other details see legend of Fig. 1.

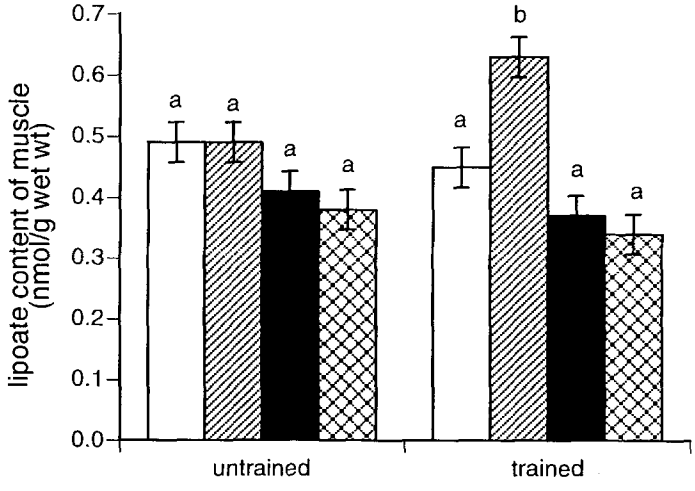

Figure 4. **Lipoate content in the superficial vastus lateralis skeletal muscle.** For other details see legends of Fig. 1 and 3.

that incorporation of lipoyl moieties to proteins is increased in response to exercise was further supported by observations in skeletal muscle. A single bout of exhaustive exercise markedly increased lipoyllysine content in the muscle. This lends further support to the contention that enzymes responsible for the lipoylation of proteins may be sensitive to exercise. This effect appeared to be transient in the muscle but more stable in the liver because exercise training did not influence skeletal muscle lipoyllysine content but did increase its content in the liver.

Comparison of tissue lipoyllysine data with that of free or loosely-bound LA results show a clear lack of association between the two apparently related parameters. This observation is consistent with the finding that LA supplementation, which is expected to increase the content of LA or its metabolites in tissues, does not increase tissue lipoyllysine content. This evidence indicates that the tightly protein-bound lipoyllysine pool in tissues is independent of the loosely-bound or free LA status in the tissue. This is clearly reflected in the observation that although exhaustive exercise increased lipoyllysine content it decreased LA content in the muscle tissue. Also, exercise training increased but did not change liver lipoyllysine and LA content, respectively. Exhaustive exercise is known to increase the activity of enzymes involved in β-oxidation (53). Exercise-induced β-oxidation of LA may account for the exercise-induced loss of LA in the muscle and liver.

In summary, this is the first study that has tested the effect of dietary LA and physical exercise on tissue lipoyllysine content. LA supplementation did not influence lipoyllysine levels in skeletal muscle and liver. Physical exercise, on the other hand, clearly up-regulated lipoyllysine content in both muscle and liver. Endurance training significantly increased hepatic lipoyllysine content. These results indicate that mechanisms involved in the lipoylation of tissue proteins are sensitive to physical activity.

ACKNOWLEDGMENT

Supported by research grants from the Finnish Ministry of Education, Juho Vainio Foundation of Helsinki and National Institutes of Health grant DK 50430 and GM27345. Ph.D. studies of SK, MA and DEL were also supported by a TULES graduate school grant from the Academy of Finland.

REFERENCES

1 Reed, L. J. (1957) *Adv Enzymol* **18**, 319-347
2 Schmidt, U., Grafen, P., Altland, K. and Goedde, H. W. (1969) *Adv Enzymol* **32**, 423-469
3 Reed, L. J. (1998) *Protein Sci* **7**, 220-4
4 Packer, L., Roy, S. and Sen, C. K. (1997) *Adv Pharmacol* **38**, 79-101
5 Sen, C. K. and Packer, L. (1996) *Faseb J* **10**, 709-20
6 Sen, C. K. (1997) *J, Nutr Biochem* **8**, 660-672
7 Sen, C. K. (1998) *Biochem. Pharmacol. in press*
8 Sen, C. K., Roy, S., Han, D. and Packer, L. (1997) *Free Radic Biol Med* **22**, 1241-1257
9 Handelman, G. J., Han, D., Tritschler, H. and Packer, L. (1994) *Biochem Pharmacol* **47**, 1725-30
10 Han, D., Handelman, G., Marcocci, L., Sen, C. K., Roy, S., Kobuchi, H., Flohe, L. and Packer, L. (1997) *Biofactors* **6**, 321-338
11 Merin, J. P., Matsuyama, M., Kira, T., Baba, M. and Okamoto, T. (1996) *FEBS Lett* **394**, 9-13
12 Sen, C. K., Roy, S. and Packer, L. (1997) in *Oxidative Stress Cancer, AIDS and Neurodegenerative Diseases* (Montagnier, L., Olivier, R. and Pasquier, C., eds.), pp. 251-267, Marcel Dekker Inc., New York
13 Fujiwara, K., Okamura-Ikeda, K. and Motokawa, Y. (1996) *J Biol Chem* **271**, 12932-36
14 Reed, L. J., Lawson, J. E., Niu, X. D., Yazdi, M. A. and Fussey, S. P. (1992) *J Nutr Sci Vitaminol (Tokyo)* **Spec No**, 46-51
15 Reed, L. J. and Hackert, M. L. (1990) *J Biol Chem* **265**, 8971-4
16 Marsac, C., Stansbie, D., Bonne, G., Cousin, J., Jehenson, P., Benelli, C., Leroux, J. P. and Lindsay, G. (1993) *J Pediatr* **123**, 915-20
17 Lodge, L., Handelman, G. J., Konishi, T., Matsugo, S., Mathur, V. V. and Packer, L. (1997) *J Appl Nutr* **49**, 3-11
18 Packer, L., Tritschler, H. J. and Wessel, K. (1997) *Free Radic Biol Med* **22**, 359-78
19 Ziegler, D., Hanefeld, M., Ruhnau, K. J., Meissner, H. P., Lobisch, M., Schutte, K. and Gries, F. A. (1995) *Diabetologia* **38**, 1425-33
20 Ziegler, D. and Gries, F. A. (1997) *Diabetes* **46 Suppl 2**, S62-6
21 Ziegler, D., Schatz, H., Conrad, F., Gries, F. A., Ulrich, H. and Reichel, G. (1997) *Diabetes Care* **20**, 369-73
22 Nagamatsu, M., Nickander, K. K., Schmelzer, J. D., Raya, A., Wittrock, D. A., Tritschler, H. and Low, P. A. (1995) *Diabetes Care* **18**, 1160-7
23 Maitra, I., Serbinova, E., Trischler, H. and Packer, L. (1995) *Free Radic Biol Med* **18**, 823-9
24 Khamaisi, M., Potashnik, R., Tirosh, A., Demshchak, E., Rudich, A., Tritschler, H., Wessel, K. and Bashan, N. (1997) *Metabolism* **46**, 763-8
25 Barbiroli, B., Medori, R., Tritschler, H. J., Klopstock, T., Seibel, P., Reichmann, H., Iotti, S., Lodi, R. and Zaniol, P. (1995) *J Neurol* **242**, 472-7
26 Sen, C. K. (1995) *J Appl Physiol* **79**, 675-86
27 Sen, C. K., Packer, L. and Hanninen, O. (1994) *Exercise and oxygen toxicity*, pp. 536, Elsevier Science Publishers B.V, Amsterdam
28 Ardies, C. M., Morris, G. S., Erickson, C. K. and Farrar, R. P. (1987) *Life Sci* **40**, 1053-61
29 Bielecki, J. W., Pawlicka, E. and Gorski, J. (1988) *Acta Physiol Pol* **39**, 421-6

30 Brierley, E. J., Johnson, M. A., James, O. F. and Turnbull, D. M. (1996) *Qjm* **89**, 251-8
31 Chen, J. and Gollnick, P. D. (1994) *Pflugers Arch* **427**, 257-63
32 Dohm, G. L., Huston, R. L., Askew, E. W. and Weiser, P. C. (1972) *Am J Physiol* **223**, 783-7
33 Freyssenet, D., Berthon, P. and Denis, C. (1996) *Arch Physiol Biochem* **104**, 129-41
34 Gollnick, P. D. and King, D. W. (1969) *Am J Physiol* **216**, 1502-9
35 Goodman, C., Henry, G., Dawson, B., Gillam, I., Beilby, J., Ching, S., Fabian, V., Dasig, D., Kakulas, B. and Morling, P. (1997) *Aust J Sci Med Sport* **29**, 95-8
36 Glick, J. L. (1966) *Am J Physiol* **210**, 1215-21
37 Sen, C. K., Marin, E., Kretzschmar, M. and Hanninen, O. (1992) *J Appl Physiol* **73**, 1265-72
38 Sen, C. K., Roy, S., Khanna, S. and Packer, L. (1998) *Meth Enzymol* **299**, in press.
39 Fujiwara, K., Okamura-Ikeda, K. and Motokawa, Y. (1986) *J Biol Chem* **261**, 8836-41
40 Reed, L. J. (1981) *Curr Top Cell Regul* **18**, 95-106
41 Fujiwara, K., Okamura-Ikeda, K. and Motokawa, Y. (1994) *J Biol Chem* **269**, 16605 - 16609
42 Fujiwara, K., Okamura-Ikeda, K. and Motokawa, Y. (1997) *J Biol Chem* **272**, 31974-8
43 Fujiwara, K., Okamura-Ikeda, K. and Motokawa, Y. (1997) *Methods Enzymol* **279**, 184-93
44 Tsunoda, J. N. and Yasunobu, K. T. (1967) *Arch Biochem Biophys* **118**, 395-401
45 Biewenga, G. P., Haenen, G. R. and Bast, A. (1997) *Gen Pharmacol* **29**, 315-331
46 Biewenga, G. (1997) in Ph.D Thesis, pp. 206, Free University of Amsterdam, Amsterdam
47 Teichert, J. and Preiss, R. (1995) *J Chromatogr B Biomed Appl* **672**, 277-81
48 Biewenga, G., de Jong, J. and Bast, A. (1994) *Arch Biochem Biophys* **312**, 114-120
49 Dikalov, S., Khramtsov, V. and Zimmer, G. (1996) *Arch Biochem Biophys* **326**, 207-218
50 Suzuki, Y. J., Tsuchiya, M. and Packer, L. (1993) *Free Radic Res Commun* **18**, 115-122
51 Farrar, R. P., Martin, T. P. and Ardies, C. M. (1981) *J Gerontol* **36**, 642-7
52 Zamora, A. J., Tessier, F., Marconnet, P., Margaritis, I. and Marini, J. F. (1995) *Eur J Appl Physiol* **71**, 505-11
53 Barakat, H. A., Kasperek, G. J., Dohm, G. L., Tapscott, E. B. and Snider, R. D. (1982) *Biochem J* **208**, 419-24