BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **247,** 223–228 (1998)
ARTICLE NO. RC988764

A Positively Charged α-Lipoic Acid Analogue with Increased Cellular Uptake and More Potent Immunomodulatory Activity

Chandan K. Sen,*[,1] Oren Tirosh, Sashwati Roy,* Michael S. Kobayashi, and Lester Packer
Department of Molecular & Cell Biology, University of California at Berkeley, Berkeley, California 94720-3200; and
**Department of Physiology, University of Kuopio, 70211 Kuopio, Finland*

Received April 28, 1998

α-Lipoic acid (LA) is taken up by cells and reduced to its potent dithiol form, dihydrolipoate(DHLA), much of which is rapidly effluxed out from cells. To improve retention in cells, the LA molecule was modified to confer a positive charge at physiological pH. N,N-dimethyl,N′-2-amidoethyl-lipoate was synthesized. The protonated form of the new molecule is referred to as LA-Plus. The uptake of LA-Plus by human Wurzburg T cells was higher compared to that of LA. Several-fold higher amounts of DHLA-Plus, the corresponding reduced form of LA-Plus, were detected in LA-Plus treated cells compared to the amount of DHLA found in cells treated with LA. At 100 μM, LA did not but LA-Plus inhibited H_2O_2 induced NF-κB activation and NF-κB directed IL-2 receptor expression. Both LA and LA-Plus synergised with selenium in inhibiting H_2O_2 induced NF-κB activation. At 150 μM LA-Plus, but not LA, inhibited TNFα induced NF-κB activation. At 5 μM LA-Plus, but not LA, protected against both spontaneous and etoposide induced apoptosis in rat thymocytes. LA-Plus is thus an improved form of LA with increased therapeutic potential.

The redox state of biological thiols regulate a number of key signal transduction processes of the immune system (1-3). Several immune disorders have been associated with lowered thiol status in certain cells such as lymphocytes (2, 4). Correction of such perturbations in cellular thiol homeostasis have resulted in beneficial clinical measures of outcome (5). Several chemical agents that are known to be able to improve intracellular thiol redox status have been used as tools to study redox sensitive signal transduction (3, 6). Most such agents are useful tools to study redox sensitive signal transduction experimentally and do not have clinical safety records. Among the clinically relevant agents tested so far for their ability to favorably regulate redox sensitive molecular responses N-acetyl-L-cysteine (NAC) and α-lipoic acid (LA) hold most promise (6, 7). Although in many respects the effects of NAC are quite similar to that of LA, much higher concentration (mM) of NAC is required to produce comparable effects (6, 8). This is primarily because, in contrast to NAC, LA is able to utilize the activity of enzymes present in the human cell to continuously regenerate its potent dithiol form, dihydrolipoate (DHLA). In the concentration range 50-100 μM LA has been shown to markedly improve reduced thiol status of human lymphocytes (9).

LA has been detected in the form of lipoyllysine in various natural sources including edible plant and animal products (10). It has been shown that following enzymatic reduction of LA to DHLA in cells, most of the DHLA is rapidly effluxed from the cell to the culture medium (11). We hypothesized that improved retention of DHLA in the cell would enhance the biological efficacy of LA treatment on a concentration:effect basis. Chemical manipulations of the LA molecule were done to confer a positive charge on the molecule at physiological pH. Cellular uptake, and the ability of this novel analogue to *1)* regulate inducible NF-κB activation in T cells, and *2)* inhibit apoptosis in rat thymocytes were tested.

MATERIALS AND METHODS

Synthesis of N,N-dimethyl,N′-2-amidoethyl-lipoate · HCl [*N,N-dimethyl,N′-2-amidoethyl, 6,8-dimercapto octanoic acid, according to IUPAC nomenclature*]. One equivalent of the R enantiomer of LA was dissolved in 100 ml of anhydrous dichloromethane. Three equivalents of N,N-dimethylethylenediamine was added to this solution. The reaction mixture was stirred for 5 min after which 1.2 equivalents of N-hydroxy-succinimide was added. This was followed by the

[1] To whom correspondence should be addressed. Fax: 510 642 8313. E-mail: cksen@socrates.berkeley.edu or Chandan.Sen@fokka.uku.fi.

0006-291X/98 $25.00

addition of 1.2 equivalents of dicyclohexylcarbodiimide. The mixture was stirred at room temperature over night. Next day, the reaction mixture was transferred to a separatory funnel. The desired compound was extracted into the aqueous phase by adding concentrated HCl. After this, organic extraction of the compound was carried out by addition of 100 ml of chloroform and 1 N NaOH solution. The organic layer was separated from the aqueous layer, dried with anhydrous magnesium sulfate, filtered and evaporated to dryness. The obtained compound was re-dissolved in dichloromethane. HCl gas was bubbled through the organic solvent up to saturation. The dichloromethane solvent was evaporated and the HCl salt of N,N-dimethyl,N′-2-amidoethyl-lipoate, referred to as LA-Plus in this work, was precipitated by using anhydrous ether.One equivalent of the R enantiomer of LA was dissolved in 100 ml of anhydrous dichloromethane. Three equivalents of N,N-dimethylethylenediamine was added to this solution. The reaction mixture was stirred for 5 min after which 1.2 equivalents of N-hydroxy-succinimide was added. This was followed by the addition of 1.2 equivalents of dicyclohexylcarbodiimide. The mixture was stirred at room temperature over night. Next day, the reaction mixture was transferred to a separatory funnel. The desired compound was extracted into the aqueous phase by adding concentrated HCl. After this, organic extraction of the compound was carried out by addition of 100 ml of chloroform and 1 N NaOH solution. The organic layer was separated from the aqueous layer, dried with anhydrous magnesium sulfate, filtered and evaporated to dryness. The obtained compound was re-dissolved in dichloromethane. HCl gas was bubbled through the organic solvent up to saturation. The dichloromethane solvent was evaporated and the HCl salt of N,N-dimethyl,N′-2-amidoethyl-lipoate, referred to as LA-Plus in this work, was precipitated by using anhydrous ether.

Cells and culture. Human lymphoma Wurzburg cells is a derivative of Jurkat T-cells (American Type Culture Collection, Bethesda) provided to us by L. A. Herzenberg of Stanford University, CA (12). Cells were grown in phenol red free RPMI 1640 medium supplemented with 10% foetal calf serum (FCS), 1% (w/v) penicillin-streptomycin, 110 mg/L sodium pyruvate and 2 mM L-glutamine (University of California, San Francisco) in humidified air containing 5% CO_2 at 37°C . When indicated, cells were treated with the R-enantiomer of LA or LA-Plus.

Thymocytes were isolated from male Sprague Dawley rats (50-75 g) as described (13) and suspended at a density of 5×10^6 cells/ml in culture medium consisting of RPMI-1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin. The cells were maintained in the culture medium at 37°C in humidified air containing 5% CO_2.

High performance liquid chromatography (HPLC). LA and DHLA from cell extracts were analyzed as described recently (14). A coulometric detector (ESA, Coulochem II, Chelmsford, MA) was used. The mobile phase, consisting of 50 % (v/v) of solution A [50 mM NaH_2PO_4, (pH 2.7)], and 50% (v/v) of solution B [70 % (v/v) acetonitrile and 30 % (v/v) methanol] was delivered using an isocratic

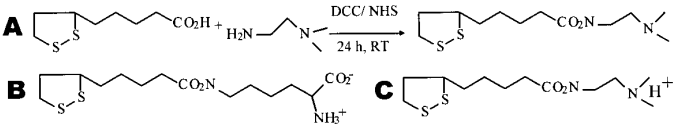

FIG. 1. Chemical structures. (A) Overall reaction for the synthesis of N,N-dimethyl,N′-2-amidoethyl-lipoate (free base form shown) from lipoic acid and N,N-dimethylethylenediamine using a dicyclohexylcarbodiimide (DCC) and N-hydroxy-succinimide (NHS) coupling strategy; reaction time, 24 h; RT, room temperature. (B) Lipoyllysine, the naturally occurring form of lipoic acid. C, LA-Plus (the protonated form of N,N-dimethyl,N′-2-amidoethyl-lipoate, pH 7.4).

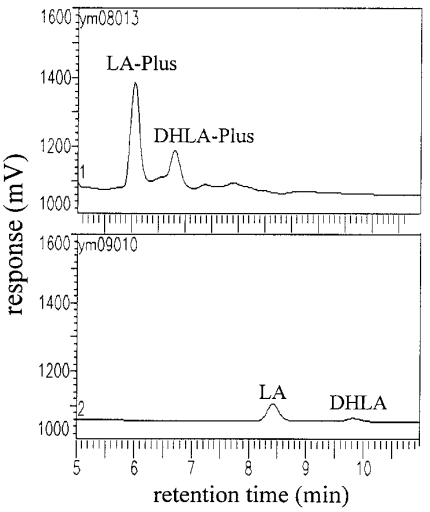

FIG. 2. Detection of oxidized and reduced forms of LA or LA-Plus from cell extracts. HPLC chromatograms obtained from Wurzburg cells treated with 100 μM of LA-Plus (top) or LA (bottom) for 1 h.

solvent delivery module (ESA, Chelmsford, MA) set at 1 ml/min flow rate. For the determination of LA-Plus and its corresponding reduced dithiol form, DHLA-Plus, cells were extracted and analyzed as described for the determination of LA and DHLA except that the mobile phase consisted of 65% (v/v) solution A and 35% (v/v) solution B, and the flow rate was set at 0.8 ml/min.

Nuclear extraction and electrophoretic mobility shift assay (EMSA). Cultured cells were pelleted by brief centrifugation (16000 $g \times 1$ min). Nuclear extracts were prepared from ~2-2.5 $\times$ 10^6 cells as previously described (15). EMSAs were performed essentially as described earlier (15).

Interleukin 2 receptor (IL-2R) expression. Wurzburg cells in culture were washed with Dulbecco's phosphate buffered saline (D-PBS, pH 7.4) containing 10 mM Na_2EDTA and incubated with a solution of CD25 monoclonal antibody (clone: B1.49.9) coupled with phycoerythrin purchased from Immunotech (Cedex, France) for 30 min at 4°C. IL-2R expression was detected using a flow cytometer (EPICS XL Coulter, Miami, FL) .

Determination of DNA fragmentation. Permeabilized cells were used as previously described (16) with minor modifications. Cells were washed with D-PBS and fixed for 20 min on ice by resuspending the pellet in 1% paraformaldehyde in D-PBS. The fixed cells were then centrifuged (600 $g \times 5$ min) and gently resuspended in a hypotonic propidium iodide solution (50 μg/ml in 0.1% w/v sodium citrate and 0.1% v/v Triton X-100). The suspension was incubated at 4°C in dark for 12-14 h before being analyzed by a flow cytometer. A 15 mW powered argon ion laser was used for excitation at 488 nm and emission was measured at 575 nm.

Statistics. Difference between means was tested by Students *t*-test with the level of significance set at $P < 0.01$.

RESULTS

Synthesis, detection and cellular uptake of LA-Plus. The yield of LA-Plus (Fig. 1), calculated on a molar basis of LA used and HCl salt of N,N-dimethyl,N′-2-amidoethyl-lipoate produced, was 80%. The calculated

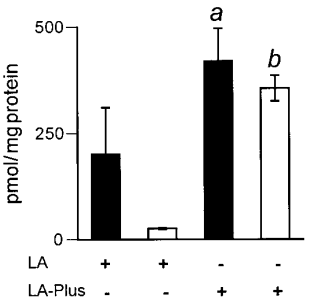

FIG. 3. Uptake and retention of oxidized and reduced forms of LA or LA-Plus in cells. Wurzburg cells in culture were treated with 100 μM of LA-Plus or LA for 1 h. Solid bars represent the respective oxidized forms; open bars represent the respective reduced forms. Data are mean ± SD of at least 3 experiments. *a*, significantly higher than LA content in LA treated cells; *b*, significantly higher than DHLA content in LA treated cells.

molecular weight of LA-Plus was 277. Fast atom bombardment mass spectrometry verified that the molecular weight of LA-Plus was 277 Da. Purity of the compound was over 99% as observed by high performance liquid chromatography (HPLC). A HPLC assay system was set up to detect LA-Plus and DHLA-Plus in the same run (Fig. 2). Figure 3 shows that the uptake of LA-Plus by Wurzburg T cells was significantly higher compared to that of LA. Several-fold higher amounts of DHLA-Plus was detected in LA-Plus treated cells compared to the amount of DHLA found in cells treated with an equimolar amounts of LA (Fig. 3).

Regulation of NF-κB activation and NF-κB directed protein expression. At a concentration of 100 μM, LA marginally potentiated but LA-Plus inhibited H_2O_2 induced nuclear translocation of NF-κB proteins (Fig. 4). Treatment of cells with selenium alone did not affect H_2O_2 induced NF-κB activation, but when treated in combination with LA or LA-Plus potentiated the inhibitory effect of both agents (Fig. 4). H_2O_2 treatment induced the expression of the NF-κB regulated protein IL-2R. Consistent with the effect of both LA and LA-Plus on H_2O_2 induced nuclear translocation of NF-κB proteins, H_2O_2 induced expression of IL-2R tended (not significantly) to be increased in response to LA treatment, but was inhibited in cells treated with LA-Plus (Fig. 5). Compared to LA, the improved ability of LA-Plus to inhibit induced NF-κB activation was also observed in cells that were activated with TNFα (Fig. 6).

Inhibition of spontaneous and etoposide-induced apoptosis in rat thymocytes. Following 6 h of isolation, almost all of the DNA in thymocytes was intact and a very low fraction of fragmented DNA was detected (Fig. 7A). After 48 h of culture, a significant increase in DNA fragmentation was evident. Such spontaneous DNA fragmentation was not affected by 5 or 10 μM of LA, but was markedly inhibited by 5 μM LA-Plus (Fig. 7A). Treatment of cells with etoposide, a topoisomerase II inhibitor, accelerated spontaneous apoptosis. This induced apoptosis was not prevented by 5 or 10 μM LA treatment, but was completely inhibited by 5 μM LA-Plus (Figs. 7B and 8). Figure 8 illustrates flow cytometry histograms showing the pattern of propidium iodide staining of intact and fragmented DNA in different treatment groups.

DISCUSSION

In nature, α-lipoic acid (Fig. 1A) exists in the bound form as lipoyllysine (Fig. 1B) (10). The chemical structure of LA-Plus (Fig. 1C) bears close resemblance with lipoyllysine. At physiological pH, LA-

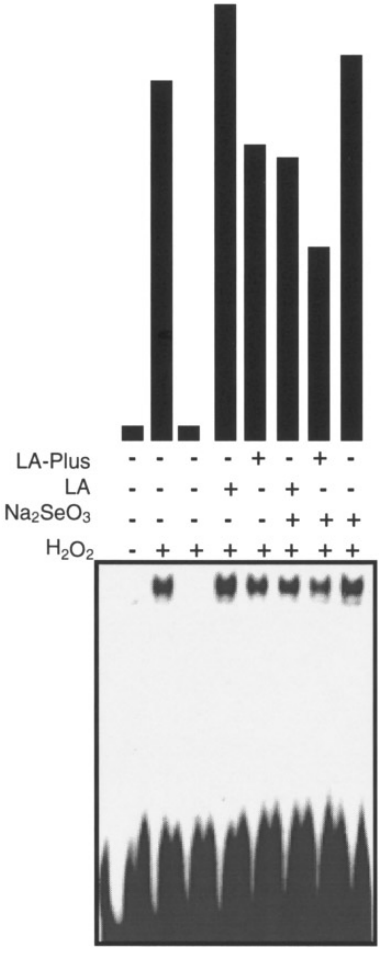

FIG. 4. Regulation of H_2O_2 induced NF-κB activation in human Wurzburg T cells. Bars represent densitometric data of NF-κB bands. Na_2SeO_3, 1 μM, 24 h before H_2O_2 treatment; LA or LA-Plus, 100 μM, 1 h before H_2O_2 treatment; H_2O_2, 250 μM × 2 h. The specificity of the NF-κB band was demonstrated (lane 3 from left) by treating the nuclear protein of positive control extracts (lane 2 from left) with an excess of cold competitor NF-κB consensus probe for 15 mins before the ^{32}P labelled probe was added.

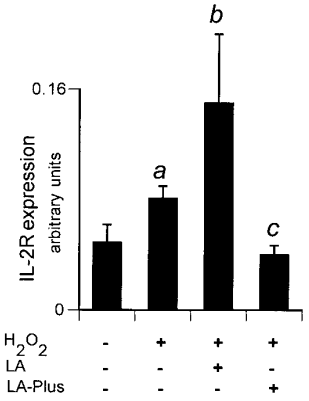

FIG. 5. Regulation of H_2O_2 induced interleukin 2 receptor expression in human Wurzburg T cells. Treatments were done exactly as described in Fig. 4 except that H_2O_2 treatment was for 6 h. Data are mean ± SD of at least 3 experiments. *a*, significantly higher than in H_2O_2 non-treated cells, *b*, trend to be higher (not significant) than in H_2O_2 treated cells. *c*, significantly lower than in H_2O_2 treated cells.

Plus bears a positive charge (Fig. 1C) and may thus be expected to be taken up by cytoplasmic organelles such as mitochondria. Previously it has been shown that LA is taken up by cells and reduced to DHLA which is rapidly effluxed from cells (11). The uptake of LA-Plus by and retention of DHLA-Plus in Wurzburg cells was much better relative to those following LA treatment. Thus, LA-Plus may be expected to be effective at much lower concentration than that required of LA. This contention was validated in our NF-κB studies where 100 and 150 μM of LA-Plus

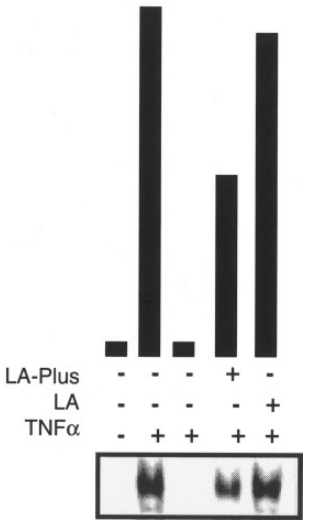

FIG. 6. Regulation of TNFα induced NF-κB activation in human Wurzburg T cells. Bars represent densitometric data of NF-κB bands. LA or LA-Plus, 150 μM, 1 h before TNFα treatment; TNFα, 50 ng/ml, 30 min. The specificity of the NF-κB band was demonstrated (lane 3 from left) as described in Fig. 4.

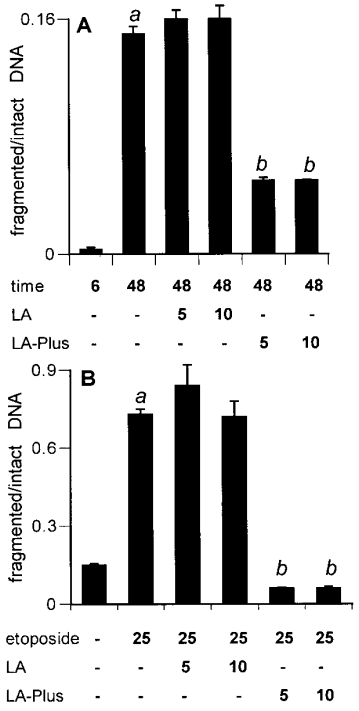

FIG. 7. Protective effect of LA-Plus against DNA fragmentation in rat thymocytes. (A) Effect of LA and LA-Plus on spontaneous DNA fragmentation. time, represents hours after isolation of thymocytes from rats. Thymocytes were either treated or not with LA or LA-Plus (as shown in micromolar concentrations) after 6 h of isolation. Measurements were done after the time shown following isolation. *a*, significantly higher than the 6 h time point. *b*, significantly lower than the 48 h time point with either no or LA treatment. (B) Effect of LA and LA-Plus on DNA fragmentation induced by 25 μM etoposide for 48 h. Thymocytes were either treated or not with LA or LA-Plus (as shown in micromolar concentrations) after 6 h of isolation. The agents were added 5 minute before etoposide treatment. *a*, significantly higher than etoposide non-treated cells. *b*, significantly lower than etoposide treated cells that were either not or treated with LA. Data are mean ± SD of at least 3 experiments.

inhibited NF-κB activation in response to H_2O_2 and TNFα, respectively. NF-$\kappa\beta$ is known to be implicated in human immunodeficiency virus related gene expression. Based on the ability of LA to inhibit inducible NF-κB activity it has been previously proposed that LA may have therapeutic potential in AIDS treatment (17). In that study mM amounts of LA was required for the effect, however (17). At 100 μM, LA tended to potentiate H_2O_2 induced NF-κB activation. In cells treated with such concentration of LA, the amount of DHLA detected was very low. It is likely that the low amount of DHLA was insufficient to inhibit induced NF-κB activation, and that the potentiating effect is mostly due to the relatively higher amounts of the disulfide form in cells that may have synergized with H_2O_2 in activating NF-κB. Selenite treatment alone did not influence H_2O_2 induced NF-

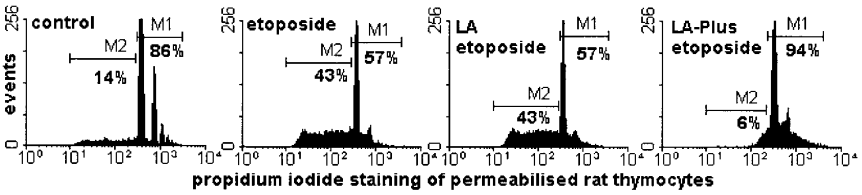

FIG. 8. Flow cytometric histograms showing DNA fragmentation in rat thymocytes. Cells were treated with etoposide, LA (5 μM) and LA-Plus (5 μM) as indicated in the legend of Fig. 7. M1 and M2 represents propidium iodide staining of intact and fragmented DNA, respectively. The percentage of total DNA in each domain is indicated.

κB activation. However, both LA as well as LA-Plus synergized with selenite to inhibit H_2O_2 induced translocation of NF-κB proteins to the nucleus. Activities of antioxidant selenoproteins such as glutathione peroxidase and thioredoxin reductase in cells are known to be upregulated by selenium supplementation (18). Moreover, dithiols such as glutaredoxin and thioredoxin efficiently donate electrons to glutathione peroxidase thus detoxifying hydroperoxides (19). It is therefore likely that intracellular DHLA and DHLA-Plus, which are dithiols, may serve as electron donors to glutathione peroxidase the activity of which is expected to be upregulated in selenite treated cells. Under appropriate conditions of cell activation, IL-2R expression may serve as a marker of NF-$\kappa\beta$ transactivation. Chemical modification of LA to LA-Plus markedly potentiated the NF-κB regulatory effect of LA as observed by nuclear translocation as well as inducible IL-2R expression results.

The thymus is an organ that is larger in embryos and that gradually involutes throughout life. Apoptosis plays a major role in this age-related involution process. Thus, thymocytes are in a continuous state of spontaneous apoptosis. LA-Plus, but not LA, significantly retarded the rate of spontaneous apoptosis in isolated thymocytes at a concentration as low as 5 μM. This may be expected to slow the rate of age-related involution of thymus. The regular process of age dependent regression of the thymus may be complicated by rapid involution when thymocyte apoptosis is accelerated. Because etoposide induced apoptosis of rat thymocytes involves reactive oxygen species (20), antioxidants protect against such apoptosis. Previously it has been shown that 4 mM DHLA or 2 mM lipoamide is necessary to inhibit etoposide induced apoptosis in rat thymocytes, and that LA does not protect even at such high concentrations (21). Consistent with the previous report 10 μM LA failed to afford any protection against etoposide induced apoptosis. In contrast, only 5 μM of LA-Plus was able to completely inhibit etoposide induced apoptosis in isolated rat thymocytes. Thus at a concentration almost three orders of magnitude less than the concentration at which LA has been previously reported to be ineffective, LA-Plus treatment provided complete protection.

In summary, a novel analogue of LA that has structural similarities with lipoyllysine, the naturally occurring form of LA has been synthesized. This new analogue, LA-Plus, is positive charged at physiological pH. The uptake of LA-Plus by and retention of the corresponding reduced form in human Wurzburg T cells was better relative to those following LA treatment. LA-Plus was multi-fold more potent than LA in inhibiting H_2O_2 or TNFα induced NF-κB activation in Wurzburg T cells, and in inhibiting both spontaneous as well as etoposide induced apoptosis in rat thymocytes. LA-Plus is thus an improved form of LA with increased therapeutic potential.

ACKNOWLEDGMENTS

This study was supported by National Institutes of Health Grant DK 50430 and a gift to the Regents of the University of California. C.K.S. and O.T. contributed equally to this work.

REFERENCES

1. Sen, C. K., and Packer, L. (1996) *FASEB J.* **10,** 709–720.
2. Droge, W., Schulze-Osthoff, K., Mihm, S., Galter, D., Schenk, H., Eck, H. P., Roth, S., and Gmunder, H. (1994) *FASEB J.* **8,** 1131–1138.
3. Sen, C. K. (1998) *Biochem. Pharmacol.,* in press.
4. Droge, W., Eck, H. P., and Mihm, S. (1992) *Immunol. Today* **13,** 211–214.
5. Herzenberg, L. A., De Rosa, S. C., Dubs, J. G., Roederer, M., Anderson, M. T., Ela, S. W., Deresinski, S. C., and Herzenberg, L. A. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 1967–1972.
6. Sen, C. K. (1997) *J. Nutr. Biochem.* **8,** 660–672.
7. 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, Dekker, New York.
8. Merin, J. P., Matsuyama, M., Kira, T., Baba, M., and Okamoto, T. (1996) *FEBS Lett.* **394,** 9–13.
9. Sen, C. K., Roy, S., Han, D., and Packer, L. (1997) *Free Radical Biol. Med.* **22,** 1241–1257.
10. Lodge, J. K., Youn, H.-D., Handelman, G. J., Konishi, T., Matsugo, S., Mathur, V., and Packer, L. (1997) *J. Appl. Nutr.* **49,** 3–11.
11. Handelman, G. J., Han, D., Tritschler, H., and Packer, L. (1994) *Biochem. Pharmacol.* **47,** 1725–1730.

12. Sen, C. K., Roy, S., and Packer, L. (1996) *FEBS Lett.* **385,** 58–62.
13. Raffay, M., and Cohen, G. M. (1991) *Arch. Toxicol.* **65,** 135–139.
14. Sen, C. K., Roy, S., Khanna, S., and Packer, L. (1998) *Methods Enzymol.* **299,** in press.
15. Sen, C. K., Khanna, S., Reznick, A. Z., Roy, S., and Packer, L. (1997) *Biochem. Biophys. Res. Commun.* **237,** 645–649.
16. Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. (1991) *J. Immunol. Methods* **139,** 271–279.
17. Suzuki, Y. J., Agarwal, B. B., and Packer, L. (1992) *Biochem. Biophys. Res. Commun.* **189,** 1709–1715.
18. Marcocci, L., Flohe, L., and Packer, L. (1997) *Biofactors* **6,** 351–358.
19. Bjornstedt, M., Kumar, S., Bjorkhem, L., Spyrou, G., and Holmgren, A. (1997) *Biomed. Environ. Sci.* **10,** 271–279.
20. Slater, A. F., Nobel, C. S., and Orrenius, S. (1995) *Biochim. Biophys. Acta* **1271,** 59–62.
21. Bustamante, J., Slater, A. F., and Orrenius, S. (1995) *Free Radical Biol. Med.* **19,** 339–347.