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Glutathione Regulation of Tumor Necrosis Factor-α-Induced NF-κB Activation in Skeletal Muscle-Derived L6 Cells

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TNFα is implicated in several skeletal muscle pathologies including muscle wasting of cachexia. Muscle wasting and other conditions such as physical exercise and immobilization are also associated with disturbances in muscle glutathione status. Hence, it was of interest to investigate the role of endogenous glutathione status in TNFα induced NF-κB activation in skeletal muscle-derived cells. TNFα proved to be a potent inducer of transient NF-κB activation in L6 myoblasts. In buthioninesulfoximine (BSO) treated cells, TNFα induced NF-κB activation was markedly potentiated suggesting that such activation is sensitive to cellular GSH, but may have been independent of high levels of intracellular GSSG. Because this activation was inhibited by the antioxidant pyrrolidinedithiocarbamate (PDTC) the involvement of reactive oxygen species in this activation system seems likely. NF-κB activation in L6 cells was also observed in response to direct H_2O_2 treatment. Results from GSSG reductase inhibited cells suggest that GSSG may participate in, but is not required for, TNFα induced NF-κB activation. The inhibitory effect of PDTC on NF-κB activation correlated with its effect on ICAM-1 expression suggesting that this GSH status modifying agent not only influenced nuclear translocation of NF-κB proteins but also regulated κB dependent transcription.

Tumor necrosis factor α (TNFα), a cytokine product of monocytes and macrophages (1) is a rapid and potent activator of NF-κB. TNFα is suggested to be implicated in muscle wasting of cachexia (2-5). In support of this it has been observed that sustained increase in serum levels of TNFα contributed by tumor cells (6-9), or in TNFα transgenic mice (10) 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 (11). Muscle wasting has been recognized as the single most common cause of death among cancer patients (12). Other muscle pathologies such as eosinophilia myalgia syndrome (13) 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 (14) suggesting that TNFα may be also implicated in exhaustive exercise induced muscle damage.

Glutathione (L-γ-glutamyl-L-cysteinylglycine), ubiquitously distributed and often found in mM concentration in cells, provides a major mechanism for reactive oxygen species detoxification and maintenance of intracellular thiol redox state. Following interaction of reactive oxygen species with reduced glutathione (GSH), oxidized glutathione disulfide (GSSG) is produced. Increased GSSG/GSH ratio is a characteristic biological response to oxidative stress. Elevated skeletal muscle GSSG/GSH ratio have been observed in physiological oxidative stress situations such as that triggered by strenuous exercise (15) or immobilization (16).

In this study L6 myoblasts were used to investigate TNFα-induced activation of NF-κB in relation to the endogenous glutathione status. The expression of intercellular adhesion molecule-1 (ICAM-1), known to be regulated by NF-κB activity (17), was studied as a marker of NF-κB transactivation.

MATERIALS AND METHODS

Materials. L6 cells were obtained from American Type Culture Collection (MD). Dulbecco's Phosphate Buffered Saline (DPBS) and

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Dulbecco's Modified Eagle Medium (DMEM) supplemented with high glucose, L-glutamine, pyridoxine hydrochloride and 110 mg/l sodium pyruvate were obtained from GIBCO BRL (Life Technologies, NY). Fetal calf serum (FCS) and other reagents for the culture medium were obtained from the cell culture facility of the University of California-San Francisco. TNFα stock solution (4° C) was a kind gift from Grace Wong (Genentech Inc., San Francisco, CA). L-Buthionine-[S,R]-sulfoximine (BSO) and the ammonium salt of pyrrolidinedithiocarbamate (PDTC) were purchased from SIGMA (St. Louis, MO). Hydrogen peroxide solution (30%; 10.15 M) was obtained from Fisher Scientific (Fair Lawn, NJ). Molar concentration of H_2O_2 was calculated using the coefficient value 0.0394 $cm^{-1} \cdot mM^{-1}$ at 240 nm. All other chemicals were of the highest analytical grade. BCNU [1,3-bis(chloroethyl)-1-nitrosourea] was a gift from Jill Johnson of Drug Synthesis & Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD).

Cell culture. Undifferentiated mononucleated cells (myoblast) were used in this study. For experiments, cells were seeded at a concentration of 0.2×10^6 cells per well in 6-well flat bottom tissue culture treated polystyrene plate (FALCON, Becton Dickinson Labware, NJ). Cultures were grown in DMEM supplemented with 10% FCS, 5 mM glutamine, 0.3% D-glucose, 50 U/ml of penicillin, and 50 mg/ml of streptomycin, in humidified air containing 5% CO_2 at +37°C. Cell viability was greater than 97% as estimated by trypan blue exclusion or propidium iodide staining (18). The cells were split once every third day. All experiments were carried out on cells (6th to 12th passage) in the third day of the last passage.

Cellular GSH. Cells in monolayer were washed thrice with DPBS and then treated with 0.5 ml of 4% monochloroacetic acid. The extract was mixed by resuspending and transferred to an eppendorf that was snap frozen in liquid nitrogen. Before HPLC analysis, the extract was centrifuged (16000 *g*, 5 min) and the supernatant filtered using 0.45 μM microfilterfuge tubes fitted with nylon membrane (Rainin, Woburn, MA). The sample pellet was dissolved in 1N NaOH for determination of total protein using a BCA protein assay kit (PIERCE, Rockford, IL).

A HPLC system coupled with a gold-mercury electrode electrochemical detector (HPLC-EC) was used. HPLC separation of GSH was done using a Alltech Altima C-18 (150 mm $\times$ 4.6 mm, 5 μM) column as described before (18).

Nuclear extraction and electrophoretic mobility shift assay (EMSA). Cultured cells were harvested by partial trypsinization (1$\times$ Trypsin-EDTA, 1 min) and pelleted by brief centrifugation (16000 *g*, 1 min). Nuclear extracts were prepared from $\sim$1-1.5 $\times$ 10^6 cells as previously described (19). EMSAs were performed essentially as described earlier (19). Binding reaction mixtures (20 μl) containing 5 μg protein ($\leq$10 μl) of nuclear extract, 3 μg poly(dI-dC) (Pharmacia, Sweden), ^{32}P-labeled probe (NF-κB consensus oligonucleotide), 2 mM $MgCl_2$, 1 mM DTT, 10 % (v/v) glycerol and 4 mM Tris-HCl (pH 7.9) were incubated for 30 min at room temperature. Proteins were separated by electrophoresis in a 0.8 mm thick native 6% polyacrylamide gel using a Tris-borate-EDTA running buffer (12.5 mM Tris-borate containing 0.25 mM Na_2EDTA, pH 8.0), followed by autoradiography and densitometry. The specificity of the NF-κB band was demonstrated by treating the nuclear protein of positive control extracts with an excess of cold competitor probe or cold mutant (one base pair altered, Santa Cruz Biotechnology Inc.) probe for 15 mins before the EMSA was performed (not shown).

ICAM-1 expression. L6 myoblasts were detached from monolayer by washing twice with DPBS and then scrapping cells treated with DPBS containing 10 mM Na_2EDTA. Detached cells suspended in DPBS were immunostained with a monoclonal mouse anti-rat ICAM-1 antibody (clone: 1A29) coupled with fluorescein isothiocyanate (MAS735, Harlan Sera-Lab Limited, Loughborough, U.K.). ICAM-1 expression was detected flowcytometrically (EPICS XL Coulter, Miami, FL) using a 488 nm argon ion laser set at 525 nm emission.

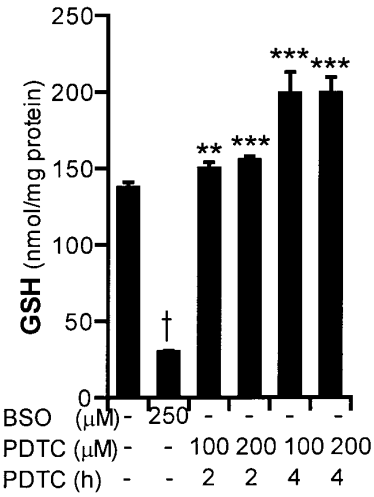

FIG. 1. L6 cell GSH content in response to BSO or PDTC treatment. ** $P < 0.01$, *** $P < 0.001$ for the effect of PDTC to increase cell GSH level compared to PDTC untreated control cells. † $P < 0.001$ for the effect of BSO treatment (18 h) to decrease cell GSH level compared to BSO untreated control cells. Data are mean ± SD.

RESULTS

Cell glutathione. Based on previously reported (20) intracellular water space in L6 cells under isosmotic conditions (1.67 μl/10^6 cells) and cell GSH content observed in the present study (Fig. 1) the intracellular GSH concentration in L6 myoblasts was 9 mM. Intracellular GSH content in these cells was manipulated by treatment of cells with the GSH synthesis inhibitor BSO or the reducing agent PDTC. BSO treatment (250 μM, 18h) resulted in 80% loss of cellular GSH (Fig. 1) but did not influence cell viability (not shown). PDTC treatment for 4h, in contrast, increased L6 myoblast GSH stores by 45% (Fig. 1).

NF-κB activation. TNFα (50 ng/ml) was evidently a potent and rapid inducer of NF-κB activation in L6 myoblasts. The activation response was rapid and transient with a peak response following 15-30 mins of TNFα challenge. GSH-deficiency clearly potentiated TNFα induced NF-κB activation, also the activation was more sustained compared to the transient nature of activation in the corresponding control cells (Fig. 2). TNFα induced NF-κB activation, on the other hand, could be markedly suppressed in PDTC treated cells that had elevated levels of GSH (Fig. 3). To test whether TNFα induced NF-κB activation was sensitive to the redox state of intracellular GSH, cellular GSSG reductase activity was arrested by BCNU treatment (10 μM, 4h). BCNU treatment is known to impair GSSG reductase and NADPH dependent recycling of GSSG to GSH and thus leads to an elevated cell GSSG/GSH (21, 22). In BCNU treated cells, TNFα induced NF-κB activation was markedly potentiated (Fig. 4).

The regulation of TNFα induced NF-κB activation by the endogenous thiol antioxidant GSH suggested the possibility that the activation process may be reactive oxygen species mediated. To verify whether NF-κB activation in L6 cells is indeed sensitive to reactive oxygen species, cells were directly treated with various concentrations of a membrane permeable reactive oxygen species, H_2O_2. H_2O_2 treatment triggered NF-κB activation at concentrations as low as 100-250 μM (Fig. 5).

ICAM-1 expression. Under appropriate conditions of cell activation, ICAM-1 expression may serve as a marker of NF-κB transactivation. Under resting conditions L6 myoblasts express a low baseline level of ICAM-1. TNFα treatment increased ICAM-1 expression such that after 24 h of treatment expression of the protein was doubled (Fig. 6). TNFα induced expression of ICAM-1 was markedly inhibited in cells pretreated with PDTC (Fig. 6).

DISCUSSION

In the present study, it was evident that TNFα induced NF-κB activation was markedly potentiated in GSH-deficient cells suggesting that such activation may have been GSSG independent. Previously, Droge et al. have observed that under certain conditions such as phorbol ester (23) induced activation of T lineage cells, GSH depletion caused by BSO treatment inhibits NF-κB activation. From this it was hypothesized that GSSG may be directly involved in the NF-κB activation cascade (23). Such BSO induced inhibition of NF-κB activation was, however, not observed in experiments with Wurzburg T cells that were activated with TNFα (24).

TNFα is known to induce of oxidative stress (25, 26). Because TNFα induced NF-κB activation was inhibited by the pro-GSH agent PDTC the involvement of reactive oxygen species in this activation system seems likely. This hypothesis is consistent with a direct role of H_2O_2 in the NF-κB activation cascade that has been previously suggested by Baeuerle and associates (27). Experiments with H_2O_2 treatment of L6 cells show that the NF-κB activation cascade in this cell type is indeed sensitive to reactive oxygen species.

TNFα induced NF-κB activation was potentiated in BCNU treated cells. So, under these conditions it is likely that GSSG did participate in the activation system. This is consistent with the hypothesis of Droge et

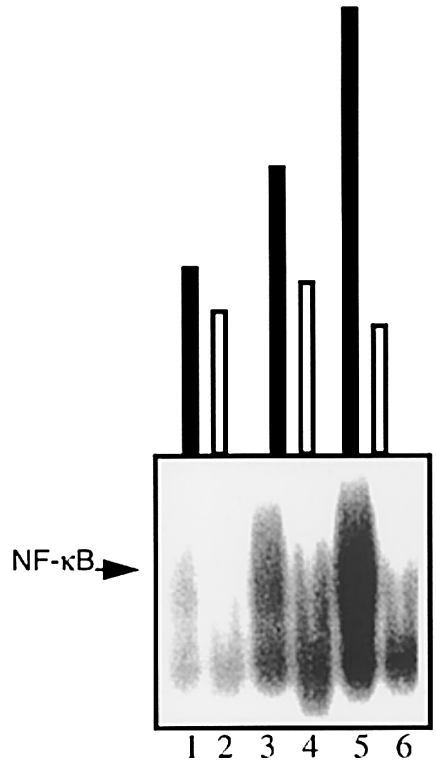

FIG. 3. Inhibition of TNFα induced NF-κB activation by PDTC. Cells were treated (200 μM, 4h; lanes 2,4,6) or not (lanes 1,3,5) with PDTC before TNFα treatment. Lanes 1 & 2, no TNFα treatment. Nuclear protein was extracted at two time points after TNFα treatment: 3-4, 5 min; 5-6, 15 min. Other details are as indicated in Figure 2.

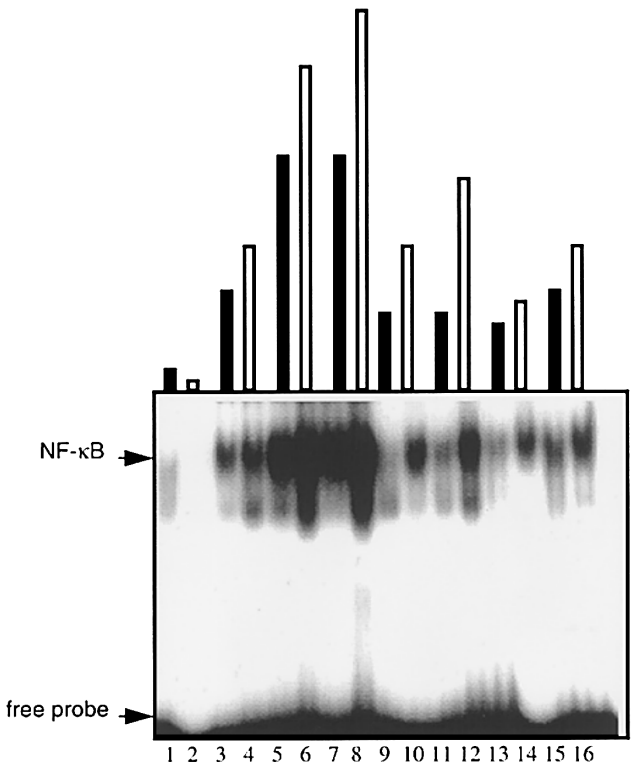

FIG. 2. Kinetics of TNFα induced NF-κB activation and the effect of cellular GSH depletion. GSH depletion was caused by BSO treatment (250 μM, 18h). Bars represent densitometry values of the NF-κB autoradiograph. Filled bars, BSO non-treated controls; open bars, BSO treated GSH depleted cells. 1-2, no TNFα treatment; 3-16 TNFα treated (50 ng/ml). Nuclear protein was extracted at various time points after TNFα treatment: 3-4, 5 min; 5-6, 15 min; 7-8, 30 min; 9-10, 1h; 11-12, 2h; 13-14, 4h; and 15-16, 6h.

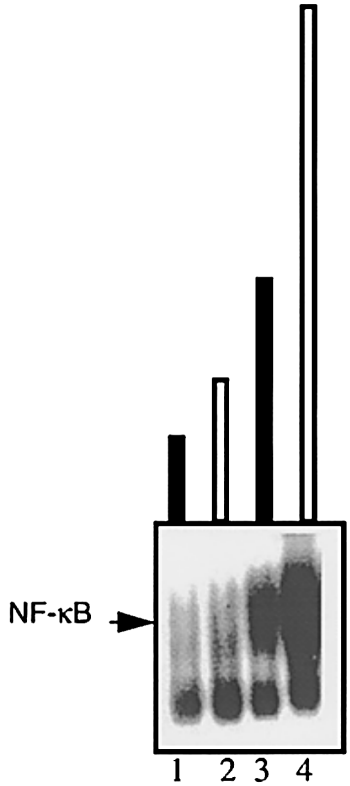

FIG. 4. Potentiation of TNFα induced NF-κB activation in GSSG reductase activity arrested cells. GSSG reductase activity was arrested by treatment of cells with 10 μM BCNU for 4 h. Cells were treated (lanes 2,4) or not (lanes 1,3) with BCNU before TNFα treatment. Lanes 1 & 2, no TNFα treatment; lanes 3 & 4, TNFα treated 15 mins. Other details are as indicated in Figure 2.

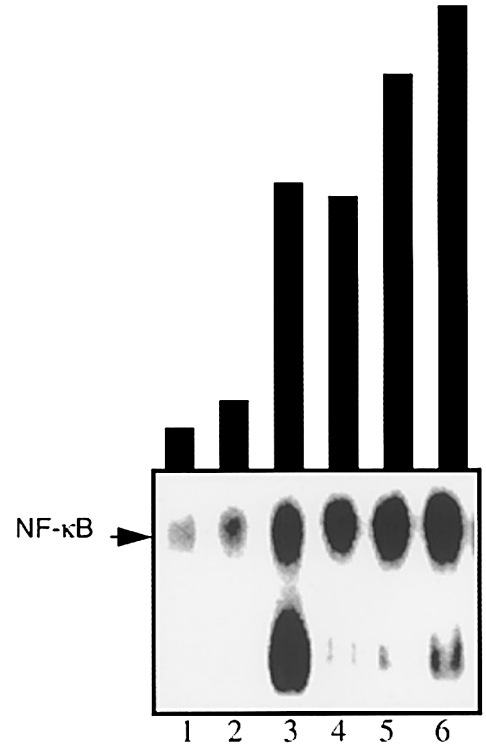

FIG. 5. H_2O_2 induced NF-κB activation. Cells were treated (2 h; lanes 2-6) or not (lane 1) with various concentrations of H_2O_2 as indicated below. Lane **2,** 100 μM; **3,** 250 μM; **4,** 500 μM; **5,** 1 mM; **6,** 2 mM. Other details are as indicated in Figure 2.

al. (23) that under certain conditions GSSG may be involved in the NF-κB activation cascade. Thus, results of this study suggest that GSSG may participate in, but is not required for, TNFα induced NF-κB activation.

The effect of PDTC on NF-κB EMSA was consistent with its effect on ICAM-1 expression suggesting that this pro-GSH agent not only influenced the translocation of NF-κB protein to the nucleus but also regulated NF-κB dependent transcription. For the first time it is reported in this work that TNFα is a strong inducer of ICAM-1 expression in skeletal muscle-derived cells. ICAM-1 is known to be a major component mediating leukocyte adhesion and may account for leukocyte infiltration into TNFα exposed skeletal muscle.

TNFα was a potent inducer of transient NF-κB activation in L6 myoblasts. Previously it has been reported that TNFα is implicated in muscle wasting in cachexia (2-5). TNFα is also suspected to be implicated in other muscular disorders such as eosinophilia myalgia syndrome (13) and immobilization dystrophy (A. Z. Reznick, unpublished observation). Recently it has been shown in a murine model that TNFα causes muscle wasting by inducing oxidative stress and nitric oxide synthase (iNOS) expression in skeletal muscle (28). Induction of iNOS expression, a gene known to contain κB site in its promoter, suggests that the effects of TNFα on muscle wasting may involve NF-κB activation. However, these authors cite unpublished data to

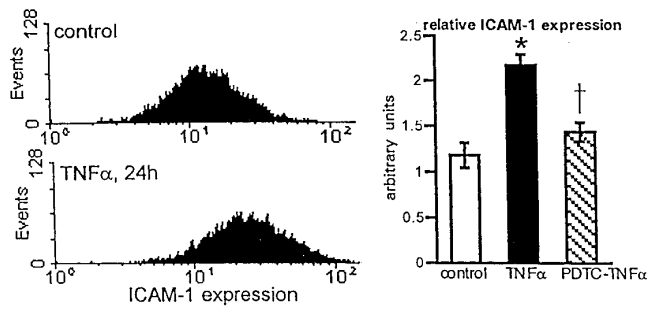

FIG. 6. TNFα induced ICAM-1 expression and its regulation. *Left Panel:* flow cytometric histogram illustrating ICAM-1 expression profile of TNFα untreated (above) or treated (50 ng/ml below) L6 cells. *Right Panel:* open bar, TNFα or PDTC non treated control cells; filled bar, TNFα treated (50 ng/ml, 24h); hatched bar, PDTC (200 μM, 4h) pretreated and TNFα treated (in the presence of PDTC). Data were collected from at least 10,000 gated viable cells. * $P < 0.001$ for the effect of TNFα to induce ICAM-1 expression. † $P < 0.001$ for the effect of PDTC pretreatment to downregulate TNFα induced ICAM-1 expression. Data are mean ± SD.

exclude the involvement of NF-κB activation in the muscle wasting process (28). Because TNFα induced NF-κB in muscle-derived cells is evidently rapid and transient it may be difficult to detect such activation if samples are not collected at appropriate time points.

Several disorders such as advanced malignancy and HIV infection that lead to cachexia are also known to be associated with abnormalities in GSH metabolism. Conditions such as elevated plasma glutamate levels and decreased plasma cysteine levels result in decreased tissue GSH stores (29). Decreased skeletal muscle GSH has been observed in tumor bearing mice (30). Conditions such as lowered cellular GSH level or elevated cellular GSSG level that were observed to potentiate TNFα induced NF-κB activation may be expected to favor the development of cachexic processes in TNFα exposed skeletal muscle tissue. Effective inhibition of TNFα induced NF-κB activation by pro-GSH agents indicates the remarkable potential of thiol based antioxidants (31) in TNFα related muscle pathologies.

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REFERENCES

1. Akira, S., Hirano, T., Taga, T., and Kishimoto, T. (1990) *FASEB J* **4,** 2860–2867.
2. Beutler, B., and Cerami, A. (1986) *Nature* **320,** 584–558.
3. Fong, Y., Moldawer, L. L., Marano, M., Wei, H., Barber, A., Manogue, K., Tracey, K. J., Kuo, G., Fischman, D. A., Cerami, A., *et al.* (1989) *Am. J. Physiol.* **256,** R659–R665.
4. Strassmann, G., Fong, M., Kenney, J. S., and Jacob, C. O. (1992) *J. Clin. Invest.* **89,** 1681–1684.
5. Spiegelman, B. M., and Hotamisligil, G. S. (1993) *Cell* **73,** 625–627.
6. Oliff, A., Defeo-Jones, D., Boyer, M., Martinez, D., Kiefer, D., Vuocolo, G., Wolfe, A., and Socher, S. H. (1987) *Cell* **50,** 555–563.
7. Tracey, K. J., Morgello, S., Koplin, B., Fahey, T. J. d., Fox, J., Aledo, A., Manogue, K. R., and Cerami, A. (1990) *J. Clin. Invest.* **86,** 2014–2024.
8. Costelli, P., Carbo, N., Tessitore, L., Bagby, G. J., Lopez-Soriano, F. J., Argiles, J. M., and Baccino, F. M. (1993) *J. Clin. Invest.* **92,** 2783–2789.
9. Brenner, D. A., Buck, M., Feitelberg, S. P., and Chojkier, M. (1990) *J. Clin. Invest.* **85,** 248–255.
10. Cheng, J., Turksen, K., Yu, Q. C., Schreiber, H., Teng, M., and Fuchs, E. (1992) *Genes Dev.* **6,** 1444–1456.
11. Tracey, K. J., and Cerami, A. (1993) *Annu. Rev. Cell. Biol.* **9,** 317–343.
12. Pisters, P. W., and Pearlstone, D. B. (1993) *Crit. Rev. Clin. Lab. Sci.* **30,** 223–272.
13. Ronen, N., Gross, B., Ben-Shachar, D., and Livne, E. (1996) *Adv. Exp. Med. Biol.* **398,** 177–182.
14. Weinstock, C., Konig, D., Harnischmacher, R., Keul, J., Berg, A., and Northoff, H. (1997) *Med. Sci. Sports Exerc.* **29,** 345–354.
15. Sen, C. K. (1995) *J. Appl. Physiol.* **79,** 675–686.
16. Kondo, H., and Itokawa, Y. (1994) *in* Exercise and Oxygen Toxicity (Sen, C. K., Packer, L., and Hanninen, O., Eds.), pp. 319–342, Elsevier Science, Amsterdam.
17. Collins, T., Read, M. A., Neish, A. S., Whitley, M. Z., Thanos, D., and Maniatis, T. (1995) *FASEB J.* **9,** 899–909.
18. Sen, C. K., Roy, S., Han, D., and Packer, L. (1997) *Free Radic. Biol. Med.* **22,** 1241–1257.
19. Sen, C. K., Roy, S., and Packer, L. (1996) *FEBS Lett.* **385,** 58–62.
20. Sen, C. K., Hanninen, O., and Orlov, S. N. (1995) *J. Appl. Physiol.* **78,** 272–281.
21. Frischer, H., and Ahmad, T. (1977) *J. Lab. Clin. Med.* **89,** 1080–1091.
22. Karplus, P. A., Krauth-Siegel, R. L., Schirmer, R. H., and Schulz, G. E. (1988) *Eur. J. Biochem.* **171,** 193–198.
23. 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.
24. Sen, C. K., and Packer, L. (1996) *FASEB J.* **10,** 709–720.
25. Feng, L., Xia, Y., Garcia, G. E., Hwang, D., and Wilson, C. B. (1995) *J. Clin. Invest.* **95,** 1669–1675.
26. Sakaguchi, S., Furusawa, S., Yokota, K., Sasaki, K., Takayanagi, M., and Takayanagi, Y. (1996) *Pharmacol. Toxicol.* **79,** 259–265.
27. Muller, J. M., Rupec, R. A., and Baeuerle, P. A. (1997) *Methods* **11,** 301–312.
28. Buck, M., and Chojkier, M. (1996) *EMBO J.* **15,** 1753–1765.
29. Droge, W., Gross, A., Hack, V., Kinscherf, R., Schykowski, M., Bockstette, M., Mihm, S., and Galter, D. (1997) *Adv. Pharmacol.* **38,** 581–600.
30. Hack, V., Gross, A., Kinscherf, R., Bockstette, M., Fiers, W., Berke, G., and Droge, W. (1996) *FASEB J.* **10,** 1219–1226.
31. Sen, C. K., and Packer, L. (1997) *Am. J. Clin. Nutr.,* in press.