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The role of superoxide radical in TNF-α induced NF-κB activation*

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ABSTRACT

Electron spin resonance (ESR) spin trapping with 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide (DEPMPO) was utilized to investigate the generation of oxygen free radicals from macrophages stimulated by tumor necrosis factor-α (TNF-α). TNF-α stimulated macrophages generated hydroxyl (·OH) and superoxide anion ($O_2^{\cdot -}$) radicals. Incubation of TNF-α with macrophages resulted in an activation of DNA binding activity of the nuclear transcription factor NF-κB. Superoxide dismutase (SOD), but not catalase or sodium formate, inhibited this NF-κB activation, suggesting that $O_2^{\cdot -}$ rather than H_2O_2 or ·OH, radicals play the most critical role in this induction. β-Nicotinamide adenine dinucleotide phosphate (NADPH) did not affect the NF-κB activation, while allopurinol, an inhibitor of xanthine oxidase, repressed it, suggesting that xanthine/xanthine oxidase, and not NADPH dependent oxidase, may be a source of $O_2^{\cdot -}$ radicals which induce NF-κB activation. $O_2^{\cdot -}$ is generated via reduction of molecular oxygen by xanthine and xanthine oxidase, as demonstrated by the oxygen consumption assay. The results indicate that TNF-α induces oxygen radical generation from macrophages. $O_2^{\cdot -}$ seems to play a key role in TNF-α-induced NF-κB activation in macrophages. Xanthine and xanthine oxidase appears to be a source of $O_2^{\cdot -}$ radicals responsible for TNF-α-induced NF-κB activation.

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Introduction

Tumor necrosis factor-α (TNF-α) is a 17kD protein produced primarily by macrophages.

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This cytokine inhibits the growth of a wide variety of tumor cells, partly by inducing apoptosis. TNF-α can initiate a cascade of events which contribute to cell recruitment, including the secretion of chemotactic cytokines by immune and non-immune cells and the expression of adhesion molecules by endothelial cells.[1–8] In recent years, certain transcription factors have been reported to regulate the production of TNF-α. Nuclear factor κB (NF-κB) is one of these.[9]

TNF-α promoter contains a number of motifs, including binding sites for NF-κB. This inducible transcription factor is ubiquitous in many eukaryotic cell types. NF-κB regulates the transcription of a great variety of genes, especially those involved in inflammatory and immune responses.[10–12] TNF-α has been reported to be able to cause NF-κB activation.[13–15] Reactive oxygen species (ROS) have been suggested to play a role in this activation.[16–19] These species include, but are not limited to, superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH). However, to date there has been no direct evidence to demonstrate the generation of ROS from TNF-α stimulated cells. Most evidence available is indirect (eg, the effects of antioxidants).[19] This may be attributed partly to the fact that the detection of oxygen free radicals in intact cells is very difficult. Electron spin resonance (ESR) spin trapping is currently a method of choice, but the spin adducts formed are not stable and can be metabolized by the cells quite rapidly. A new spin trap, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide (DEPMPO), was developed recently.[20] This spin trap, upon reacting with oxygen free radicals, generates relatively stable adducts and makes otherwise undetectable free radicals detectable.

In this paper, the following questions will be addressed: (a) Does TNF-α induce free radical generation by macrophages? If so, what kinds of free radicals are generated? (b) Do free radicals play a key role in TNF-α induced NF-κB activation? If they do, what radicals play the most important role? (c) What is the source of the radical generation?

Materials and Methods

Materials

Recombinant mouse TNF-α was purchased from Endogen (Woburn, MA). Catalase and superoxide dismutase (SOD) were obtained from Boehringer Mannheim (Indianapolis, IN). β-Nicotinamide adenine dinucleotide phosphate (NADPH), sodium formate and allopurinol were bought from Sigma (St. Louis, MO). 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide (DEPMPO) was purchased from Oxis International, Inc. (Portland, OR).

Free Radical Measurements

ESR spin trapping was used to examine free radical generation. Spin trapping is necessary because of the reactive nature of the free radicals to be studied. This technique involves an addition-type reaction of a short-lived radical with a diamagnetic compound (spin trap) to form a relatively long-lived free radical product, the so-called spin adduct, which can be studied by conventional ESR. The intensity of the spin adduct signal corresponds to the amount of short-lived radicals trapped, and the hyperfine splittings of the spin adduct are generally characteristic of the original, short-lived, trapped radical.

All measurements were conducted with a Varian E9 ESR spectrometer and a flat cell assembly. Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) as reference standards. An EPRDAP 2.0 program (U.S. EPR, Inc., Clarksville, MD) was employed for data acquisition and analysis. Reactants were mixed in test tubes to a total final volume of 1 ml. The reaction mixture was then transferred to a flat cell for ESR measurement.

Oxygen consumption measurements

Oxygen consumption measurements were carried out with a Gilson oxygraph, Model 516

(Gilson Medical Electronics, Middleton, WI). The cell concentration was 1.0×10^6/ml, and measurements were made over a period of 10 minutes.

Oligonucleotide

Oligonucleotides were synthesized by the phosphoramidite method on a DNA/RNA Synthesizer (Applied Biosystems, Model 392, Foster City, CA). A NF-κB binding sequence (5'GAAATTCCAAAGAGTCATCAGA3') from the promotor region of the human IL-2 receptor α chain gene was used to synthesize a NF-κB binding oligonucleotide. The synthesized single-stranded oligonucleotides were deprotected at 50°C overnight, dried in a speed vacuum and then dissolved in the Tris-EDTA buffer. Complimentary strands were denatured at 80°C for 5 minutes and annealed at room temperature. The double-stranded probe was labeled with ^{32}P-dCTP (Amersham, Arlington Heights, IL) using a Klenow fragment (Bethesda Research Laboratories, Gaithersburg, MD).

Cell culture

A mouse monocyte-macrophage cell line, RAW 264.7 cells, was obtained from American Type Culture Collection (ATCC) (Rockville, MD). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Mediatech, Washington, DC) supplemented with 10 percent fetal bovine serum, 2 mM glutamine and 1000 units/ml penicillin-streptomycin.

Nuclear extraction

Nuclear extracts were prepared by a modified method of Sun, et al.[21] RAW 264.7 cells suspended in DMEM plus 10 percent fetal bovine serum were cultured in 35 mm cell culture plates at 5×10^6 cells/plate for 24 hours. The medium was replaced with DMEM, with no fetal bovine serum to starve the cells, for 2 hours. The cells were then returned to a complete medium and treated with 50 ng/ml of TNF-α plus or minus other agents for 3 hours (figure 4). At the end of the culture period, cells were harvested and treated with 500 μl lysis buffer (50 mM potassium chloride (KCl), 0.5 percent NP-40, 25 mM N-[2-Hydroxyethyl] piperazine-N^1-[2-ethanesulfonic] acid (HEPES) pH 7.8, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 20 μg/ml aprotinin and 100 μM dithiothreitol (DTT) on ice for 4 minutes. After a 1-minute centrifugation at 14,000 rpm, the supernate was saved as a cytoplasmic extract. The nuclei were washed once with the same buffer without NP-40. The washed nuclei were suspended in a 100 μl volume of extraction buffer (500 mM KCl, 10 percent glycerol with the same concentrations of HEPES, PMSF, leupeptin, aprotinin and DTT as the lysis buffer), and pipetted three times for proper mixing. After centrifugation at 14,000 rpm for 5 minutes, the supernate was harvested, and this nuclear protein extract was stored at −70°C. The protein concentration was determined using a BCA protein assay reagent (Pierce, Rockford, IL).

Electrophoretic mobility shift assay (EMSA)

The DNA-protein binding reaction was conducted in a 24 μl reaction mixture including 1 μg Poly dI.dC, (Sigma, St. Louis, MO), 3 μg nuclear protein extract, 3 μg BSA, 4×10^4 cpm of ^{32}P-labeled oligonucleotide probe (1 μg), 4 μl distilled water and 12 μl of 2 × anneal buffer (24 percent glycerol, 24 mM HEPES pH 7.9, 8 mM Tris-HCl pH 7.9, 2 mM ethylenediaminetetra-acetic acid (EDTA), 2 mM DTT). This mixture was incubated on ice for 10 minutes in the absence of the radiolabelled probe, then incubated for 20 minutes at room temperature in the presence of radiolabelled probe. After incubation, the DNA-protein complexes were resolved on a 5 percent acrylamide gel (National Diagnostics, Atlanta, GA) that had been pre-run at 210 V for 30 minutes with 0.5 × Tris-boric acid-EDTA buffer. The loaded gel was run at 210 V for 90 minutes, then dried and placed on Kodak X-OMAT film

(Eastman Kodak, Rochester, NY) for autoradiography. The film was developed after overnight exposure at -70°C.

The concentrations given in the figure legends are final concentrations. All experiments were performed at room temperature and under ambient air except those specifically indicated.

Results

1. Detection of free radical generation

ESR spin trapping was used to detect oxygen radical generation from TNF-α-stimulated macrophages. This method is specific and sensitive and is considered to be the best technique for detection and identification of free radical generation. Figure 1a shows a typical spectrum recorded from a suspension containing TNF-α, RAW 264.7 macrophages and DEPMPO (a spin trapping reagent). This spectrum can be simulated (figure 2) as a combination of two radical adducts, DEPMPO/$O_2^{\cdot -}$ and DEPMPO/·OH. The hyperfine splittings for these adducts are listed in table I. These hyperfine splittings are essentially the same as those reported in the literature for these adducts.[20] Thus, the spectrum in figure 1a was assigned to a combination of DEPMPO/$O_2^{\cdot -}$ and DEPMPO/·OH. SOD was used to verify $O_2^{\cdot -}$ generation by TNF-α-stimulated macrophage. As shown in figure 1b, SOD inhibited the DEPMPO/$O_2^{\cdot -}$ spin adduct signal, further demonstrating that $O_2^{\cdot -}$ radicals were generated. Figure 3 shows the time course of DEPMPO/$O_2^{\cdot -}$ and DEPMPO/·OH generation. The TNF-α-stimulated formation of these adducts reached a saturation level in about 1 hour. Sodium formate, an ·OH radical scavenger, decreased the DEPMPO/·OH signal (figure 1c), indicating the generation of ·OH radicals by TNF-α stimulated macrophages.

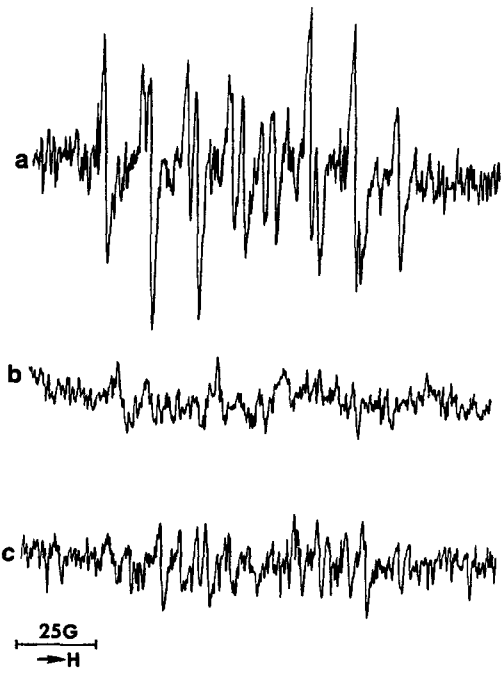

FIGURE 1. (a) ESR spectrum recorded 45 minutes after the addition of 1 μg/ml TNF-α to 1.0×10^6 cells/ml RAW 264.7 macrophages and 100 mM DEPMPO in a phosphate buffered solution (pH 7.4). (b) Same as (a) but with 500 units/ml SOD added. (c) Same as (a) but with 50 mM sodium formate added. The spectrometer settings were as follows: receiver gain, 1.5×10^5; time constants 0.3 second; modulation amplitude, 1.0 G; scan time, 4 minutes; magnetic field, 3470 ± 100 G.

2. Role of $O_2^{\cdot -}$ in TNF-α induced NF-κB activation

RAW 264.7 cells were used to study TNF-α-induced NF-κB activation and the role of free radicals in this process. The cells were incubated with TNF-α in the presence of various reagents (figure 4) for 3 hours and then were harvested for extraction of the nuclear proteins. The proteins were analyzed by electrophoretic mobility shift assay (EMSA) for the DNA binding activity. The results are shown in figure 4a. For clarity, the gel density photometer readings are provided in figure 4b. As shown in figure 4a, the untreated RAW cells (lane 1) exhibited a basal level of NF-κB activation. Macrophages treated with TNF-α showed enhanced DNA binding activity (lane 2). Catalase, a scavenger of H_2O_2, did not inhibit TNF-α-induced NF-κB activation (lane 3). SOD, an inhibitor of $O_2^{\cdot -}$ radicals, suppressed NF-κB activation (lane 4), while sodium formate, an ·OH radical scavenger, did

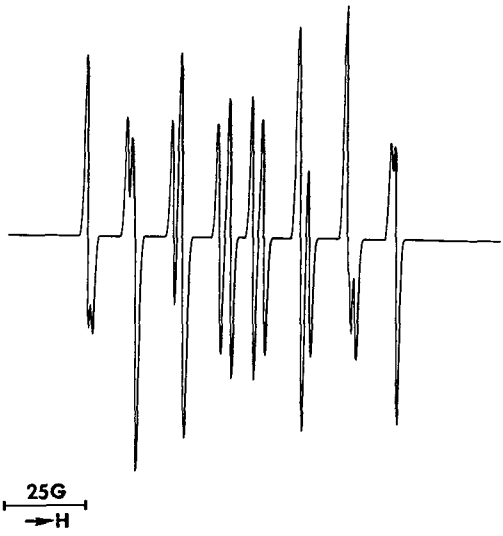

FIGURE 2. Computer-simulated spectrum of a composition of DEPMPO/·OH and DEPMPO/$O_2^{\cdot -}$. The spectra were simulated using the hyperfine splitting constants listed in Table 1.

not exhibit any significant effect (lane 5). The results suggest that $O_2^{\cdot -}$ radicals, rather than H_2O_2 or ·OH radicals, are responsible for TNF-α-induced NF-κB activation.

3. THE MAJOR SOURCE OF $O_2^{\cdot -}$ RADICALS RESPONSIBLE FOR TNF-α INDUCED NF-κB ACTIVATION

The major enzymatic sources for $O_2^{\cdot -}$ radical generation in macrophages are NADPH oxidase and xanthine oxidase.[22] NADPH oxidase is a multicomponent enzyme complex found in the plasma membrane. This oxidase functions to catalyze the one electron transfer from cytosolic NADPH to molecular oxygen, resulting in $O_2^{\cdot -}$ generation. Addition of NADPH to TNF-α-stimulated macrophages resulted in a two-fold increase in oxygen consumption (data not shown). If this enzyme were the major source of $O_2^{\cdot -}$ radicals responsible for TNF-α-induced NF-κB activation, NADPH would in turn increase the NF-κB binding activity. As shown in figure 4 (lane 6), addition of NADPH did not alter TNF-α-induced NF-κB activation. This result suggests that NADPH oxidase is not a major source of $O_2^{\cdot -}$ radicals responsible for NF-κB activation.

Xanthine oxidase is a cytosolic enzyme. It uses molecular oxygen as its electron acceptor and generates $O_2^{\cdot -}$ radicals. Allopurinol curbs $O_2^{\cdot -}$ radical generation by inhibiting the xanthine oxidase.[23] As shown in figure 4, lane 7, allopurinol deterred TNF-α-induced NF-κB binding activity. The results suggest that the cytoplasmic xanthine oxidase may be an important site of $O_2^{\cdot -}$ radical generation responsible for TNF-α-induced NF-κB activation.

4. OXYGEN CONSUMPTION

As shown in figure 5, cells stimulated by TNF-α consumed nearly twice as much molecular oxygen as untreated cells. SOD decreased the amount of the oxygen consumed by regeneration of molecular oxygen via dismutation of $O_2^{\cdot -}$. Allopurinol, an inhibitor of xanthine oxidase, curtailed oxygen consumption, indicating that xanthine and xanthine oxidase is a source of $O_2^{\cdot -}$ generation via one electron reduction of molecular oxygen.

Discussion

While it is generally believed that TNF-α-stimulated macrophages generate ROS, no direct evidence has yet been reported. Using ESR spin trapping with DEPMPO as a spin trap, the present study suggests that TNF-α indeed stimulates macrophages to generate $O_2^{\cdot -}$ and ·OH radicals. As stated in the Introduction, the detection of oxygen radical formation is difficult because of the instability of spin adducts in intact cells. However, DEPMPO adducts of $O_2^{\cdot -}$ and ·OH are relatively stable and offer the method of choice for detection of $O_2^{\cdot -}$ and ·OH generation from cellular systems.

It is well known that ROS are involved in a variety of pathological processes. They can cause DNA damage, lipid peroxidation, protein modification and activation of certain nuclear transcription factors, such as activator

TABLE I

Electron Spin Resonance by Hyperfine Splitting Constants of DEPMPO/$O_2^{\cdot-}$ and DEPMPO/·OH

Spin Adduct	A_N(G)	$A_H\beta$(G)	A_H^γ(G)	A_P(G)
DEPMPO/·OH	14.1	13.0	0.27 (3H)	47.5
DEPMPO/$O_2^{\cdot-}$	13.4	12.0	0.8 (1H)	52.5
			0.44 (6H)	

protein-1 (AP-1) and NF-κB.[17–18,26–30]). It is possible that ROS may be a common mediator for the activation of NF-κB induced by a variety of agents, such as cytokines, UV light, ionizing radiation and pathogen-related stimuli.[13–18,24–28] It has been frequently proposed that TNF-α is able to induce NF-κB activation via free radical reactions. The results obtained in the present investigation indicate that $O_2^{\cdot-}$ radicals are the major species responsible for TNF-α-induced NF-κB activation. The following experimental observations support this conclusion: (a) ESR spin trapping measurements show that TNF-α-stimulated cells generated $O_2^{\cdot-}$ radicals, (b) SOD inhibited TNF-α-induced NF-κB activation, while catalase or sodium formate did not, indicating that $O_2^{\cdot-}$, but not H_2O_2 or ·OH plays a significant role and (c) allopurinol, which can restrain $O_2^{\cdot-}$ generation by inhibiting xanthine oxidase, depressed the TNF-α-induced NF-κB activation.

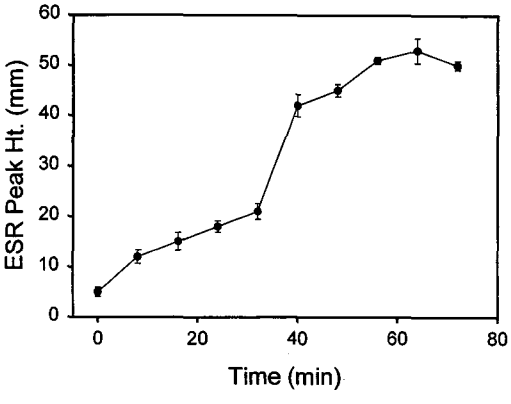

FIGURE 3. Time course of DEPMPO/$O_2^{\cdot-}$ and DEPMPO/·OH generation from TNF-α stimulated RAW 264.7 macrophages. The experimental conditions were the same as those in Figure 1a. Results are means ± SE (n = 3).

The $O_2^{\cdot-}$ radicals can be generated by macrophages by two major enzymes, NADPH oxidase and xanthine oxidase. They can also be generated by the mitochondrial electron transport chain, cytochrome p-450, nitric oxide synthase and arachidonic acid metabolism.[22,31–33] The present study shows that addition of NADPH to TNF-α-stimulated cells resulted in a two-fold increase in oxygen radical generation and oxygen consumption (data not shown). However, NADPH did not affect TNF-α-induced NF-κB activation. It appears that the specific location of the radical generation is very important. The concentration of SOD varies in different parts of the cells. In addition, the oxygen radicals are generally reactive. They cannot travel long distances without reacting with their targets. Allopurinol inhibited both TNF-α-induced NF-κB activation and oxygen consumption, suggesting that xanthine and xanthine oxidase may be a source of $O_2^{\cdot-}$ radicals responsible for TNF-α-induced NF-κB activation. Molecular oxygen is likely reduced to $O_2^{\cdot-}$radicals, as demonstrated by oxygen consumption assay and inhibitory effect by allopurinol. However, the critical source of $O_2^{\cdot-}$ may be cell-dependent. For example, in L929 cells, the mitochondrial electron transport chain is reportedly the major source of $O_2^{\cdot-}$ radicals responsible for TNF-α-induced NF-κB activation.[19]

The following conclusions may be drawn from the results of the present study: (a) Stimulation of RAW 264.7 cells with TNF-α

leads to generation of both $O_2^{\cdot -}$ and ·OH radicals; (b) $O_2^{\cdot -}$ radicals are the likely species responsible for TNF-α-induced NF-κB activation; and (c) Xanthine and xanthine oxidase appears to be a major source of $O_2^{\cdot -}$ radicals responsible for TNF-α-induced NF-κB activation.

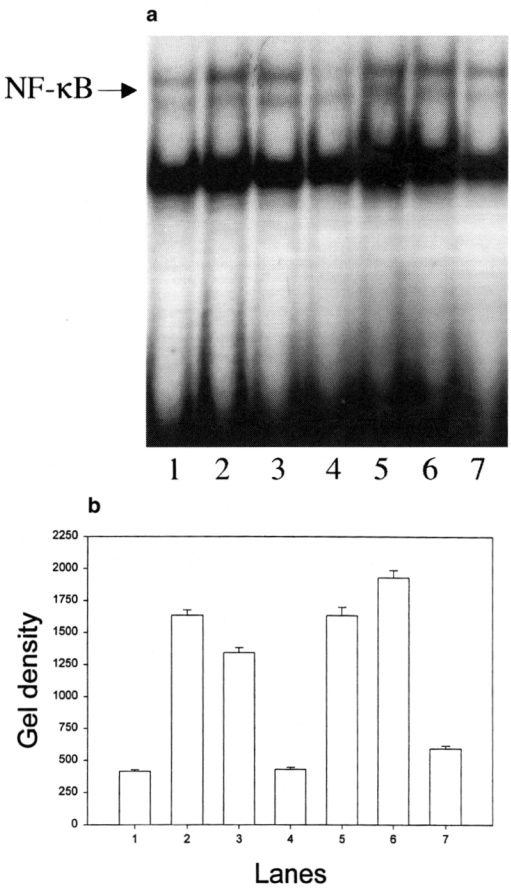

FIGURE 4. (a) Role of $O_2^{\cdot -}$ radicals in TNF-α-induced NF-κB activation in RAW 264.7 macrophages. The cells were adjusted to a density of 5×10^6/ml and treated for 3 hours with different stimuli, then subjected to extraction of the nuclear proteins as stated in the Materials and Methods. DNA binding activity of the NF-κB protein was detected with a probe of ^{32}P labeled double-stranded NF-κB binding oligonucleotide by an EMSA assay. Lane 1, untreated cells; lane 2, cells + 50 ng/ml TNF-α; lane 3, cells + 50 ng/ml TNF-α + 10,000 units/ml catalase; lane 4, cells + 50 ng/ml TNF-α + 500 units/ml SOD; lane 5, cells + 50 ng/ml TNF-α + 1 mM sodium formate; lane 6, cells + 50 ng/ml TNF-α + 1 mM NADPH; lane 7, cells + 50 ng/ml TNF-α + 25 μM allopurinol. (b) Gel density photometer readings of Figure 4a. Results are means ± SE (n = 3).

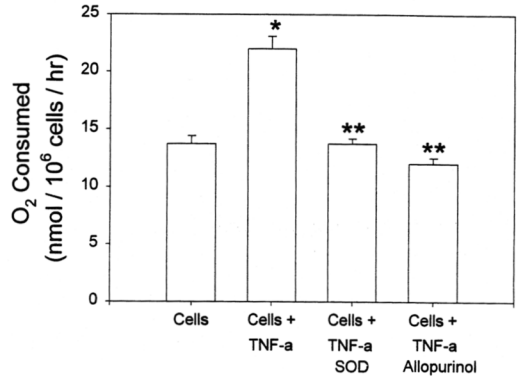

FIGURE 5. Oxygen consumption by TNF-α stimulated RAW 264.7 macrophages. Bars represent: (1) untreated cells (1.0×10^6 cells/ml), (2) same as (1) but with 1 μg/ml TNF-α added, (3) same as (2) but with 500 units/ml SOD added, (4) same as (2) but with 25 μM allopurinol added. The asterisk indicates a significant increase in oxygen consumption from control (n = 3, $P < 0.02$). The double asterisks denote a significant decrease in oxygen consumption from cells treated by TNF-α.

References

1. Tartaglia LA, and Goeddel DV. Two TNF receptors. Immunol Today 1992;13:151–153.
2. Feingold K, Soued M, and Grunfeld C. Tumor necrosis factor stimulates DNA synthesis in the liver of intact rats. Biochem Biophys Res Commun 1988;153: 576–582.
3. Fiers W. Tumor necrosis factor. Characterization at the molecular, cellular and in vivo level. FEBS Lett 1991;285:199–212.
4. Emgeimann H, Holtmann H, Brakebusch C, Avni YS, Sarov I, Nophar Y, Hadas E, Leitmer O, and Wallach D. Antibodies to a soluble form of a tumor necrosis factor (TNF) receptor have TNF-like activity. J Biol Chem 1990;265:14497–14504.
5. Perkins RC, Scheule RK, Hamilton R, Gomes G, Friedman G, and Holian A. Human alveolar macrophages asbestos exposure. Exp Lung Res 1993;19:55–65.
6. Ljungman AG, Lindahl M, and Tagesson C. Asbestos fibers and man made mineral fibers: Induction and release of tumor necrosis factor-α from rat alveolar macrophages. Occup Environ Med 1994;51:777–783.
7. Li XY, Lamb D, and Donaldson K. The production of TNF-α and IL-1 like activity by bronchoalveolar leukocytes after intratracheal instillation of crocidolite asbestos. Int J Exp Pathol 1993;74:403–410.
8. Craighead JE and Mossman BT. The pathogenesis of asbestos-associated disease. N Engl J Med 1982;306: 1446–1455.
9. Osborn L, Kunkel S, and Nabel GJ. Tumor necrosis factor alpha and interleukin 1 stimulate the human

immunodeficiency virus enhancer by activation of the nuclear factor kappa B. Proc Natl Acad Sci USA 1989; 86:2336–2340.
10. Baeuerle PA. The inducible transcription activator NF-kappa B: *regulation by distinct protein subunits.* Biochim Biophys Acta 1991;1072:63–80.
11. Baeuerle PA and Henkel T. Function and activation of NF-κB in the immune system. Annu Rev Immunol 1994;12:141–179.
12. Grilli M, Jason J-S, and Lenardo MJ. NF-kappa B and Rel: participants in a multiform transcriptional regulatory system. Int Rev Cytol 1993;143:1–62.
13. Schmidt KN, Amstad P, Cerutti P, and Baeuerle PA. The role of hydrogen peroxide and superoxide as messengers in the activation of transcription factor NF-κB. Chem & Bio 1995;2:13–22.
14. Li J-J, Westergaard C, Ghosh P, and Colburn NH. Inhibitors of both nuclear factor-κB and activator protein-1 activation by block the neoplastic transformation response. Cancer Res 1997;57:3569–3576.
15. Schreck R, Albermann K, and Baeuerle PA. Nuclear factor κB: An oxidative stress-responsive transcription factor of eukaryotic cells (a review). Free Rad Res Commun 1992;17:221–237.
16. Staal FJT, Roederer M, Herzenberg LA, and Herzenberg LA. Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus. Proc Natl Acad Sci USA 1990;87:9943–9947.
17. Schreck R, Rieber P, and Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. EMBO J 1991;10:2247–2258.
18. Schreck R, Meier B, Mannel D, Droge W, and Baeuerle PA. Dithiocarbamates as potent inhibitors of *nuclear factor kappa B activation in intact cells.* J Exp Med 1992;175:1181–1194.
19. Schulze-Osthoff K, Beyaert R, Vandevoorde V, Haegeman G, and Fiers W. Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF. EMBO J 1993;12: 3095–3104.
20. Frejaville C, Karoui H, Tuccio B, Moigne FL, Culcasi M, Pietri S, Lauricella R, and Tordo P. 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide: a new efficient phosphorylated nitrone for the in vitro and in vivo spin trapping of oxygen-centered radicals. J Med Chem 1995;38:258–265.
21. Sun SC, Elwood J, Beraud C, and Greene WC. Human T-cell leukemia virus type I Tax activation of NF-kappa B/Rel involves phosphorylation and degradation of I kappa B alpha and RelA (p65)-mediated induction of the c-rel gene. Mol Cell Biol 1994;14: 7377–7384.
22. *Forman JH and Cadenas E. Oxidative Stress and Signal Transduction.* International Thomson Publishing. 1997.
23. Watts RW, Watts JE, and Seegmiller JE. Xanthine oxidase activity in human tissues and its inhibition by allopurinol (4-hydroxypyrazolo[3,4-d] pyrimidine). J Lab Clin Med 1965;66:688–697.
24. Sun Y and Oberley LW. Redox regulation of transcriptional activators. Free Rad Biol Med 1996;21:335–348.
25. Siebenlist U, Franzoso G, and Brown K. Structure, regulation and function of NF-κB. Annu Rev Cell Biol 1994;10:405–455.
26. Remacle J, Raes M, Toussaint O, Renard P, and Rao G. Low levels of reactive oxygen species as modulators of cell function (a review). Mutat Res 1995;316:103–122.
27. Meyer M, Schreck R, and Baeuerle PA. H_2O_2 and antioxidants have opposite effects on activation of NF-κB and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. EMBO J 1993;12:2005–2015.
28. Meyer M, Pahl HL, and Baeuerle PA. Regulation of the transcription factors NF-κB and AP-1 by redox changes. Chem Biol Interact 1994;91:91–100.
29. Shi X, Dong Z, Huang C, Ma W, Liu K, Ye J, Chen F, Leonard SS, Ding M, Castranova V, and Vallyathan V. The role of hydroxyl radicals as a messenger in the activation of nuclear transcription factor NF-κB. Mol Cell Bio 1998: in press.
30. Chen F, Lu Y, Demers LM, Rojanasakul Y, Shi X, Vallyathan V, and Castranova V. Role of hydroxyl radical in silica-induced NF-kappa B activation in macrophages. Ann Clin Lab Sci 1998;28:1–13.
31. Boveris A and Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem J 1973;134:707–716.
32. Turrens JF and Boveris A. Generation of superoxide *anion by the NADH dehydrogenase of bovine heart* mitochondria. Biochem J 1980;191:421–427.
33. Turrens JF, Alexandre A, and Lehninger AL. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. Arch Biochem Biophys 1985;237:408–414.