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Original Contribution

OXYGEN FREE RADICALS MEDIATE THE INDUCTION OF MANGANESE SUPEROXIDE DISMUTASE GENE EXPRESSION BY TNF-α

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Abstract—In this study, the hypothesis that oxygen free radicals act as intracellular messengers is examined. Treatment of human oral carcinoma SCC-25 cells with 200 ng/ml human TNF-α for 6 h greatly increased manganese superoxide dismutase (MnSOD) gene expression as detected by western blotting, RT-PCR, and nuclear run-on experiments. In the presence of the oxygen free radical spin trapping reagent, 5,5-dimethyl pyrroline-*N*-oxide (DMPO), the induction of MnSOD gene expression by TNF-α was significantly reduced. Electron paramagnetic resonance experiments showed that the production of oxygen free radicals was enhanced in TNF-α treated cells. Taken together, these observations suggest that the induction of MnSOD expression by TNF-α is at least partially mediated by intracellular formation of oxygen free radicals, and that superoxide is most likely the initiating species involved in the mediation of MnSOD gene expression by TNF-α. © 2000 Elsevier Science Inc.

Keywords—Manganese-containing superoxide dismutase, DMPO, Gene expression, Nuclear run-on, EPR, Free radicals

INTRODUCTION

Tumor necrosis factor-alpha (TNF-α) has been demonstrated to induce the expression of manganese superoxide dismutase (MnSOD), a mitochondrial enzyme involved in the detoxification of superoxide radicals [1,2]. The induction has been shown to be rapid, and substantial in many cell types. The induction of MnSOD by TNF-α occurs in many normal cells and in some but not all tumor cells; the induction also appears to be a physiological process in whole animals in response to environmental or pathological stresses [3].

TNF-α has been demonstrated to activate cellular production of reactive oxygen species (ROS) in many cell types [4–6]. For example, TNF-α has been shown to induce ROS at both plasma membranes and mitochondrial membranes [5,6]. We hypothesized that oxygen free radicals or ROS may act as signal molecules to modulate gene expression and cellular activity. In particular, we hypothesized that the enhanced production of oxygen free radicals caused by TNF-α induces MnSOD gene expression.

In the present study, we demonstrate for the first time that TNF-α induces MnSOD expression at the transcriptional level in human oral carcinoma SCC-25 cells. However, in the presence of the oxygen free radical spin trapping reagent 5,5-dimethylpyrroline-*N*-oxide (DMPO), the induction of MnSOD expression is greatly reduced. The results seen with TNF-α were similar to those observed with a simple superoxide generating system—xanthine plus xanthine oxidase. The induction of MnSOD by both TNF-α and xanthine-xanthine oxidase (X/XO) and the inhibition of MnSOD induction by DMPO suggest a similar induction mechanism in both systems. Furthermore, electron paramagnetic resonance experiments using spin trapping techniques demonstrated that superoxide and hydroxyl radical production are significantly increased in TNF-α treated SCC-25 cells. These data are consistent with the hypothesis that oxygen free radicals are mediators in the induction of MnSOD expression by TNF-α in human oral carcinoma SCC-25 cells.

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MATERIALS AND METHODS

Reagents

Recombinant human TNF-α, hydrocortisone, xanthine, xanthine oxidase, *E. coli* MnSOD, bovine catalase, and the spin trapping reagent, 5,5-dimethylpyrroline-*N*-

oxide (DMPO), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's cell culture medium (DMEM), Ham's-F12, and other related materials were from the Hybridoma Facility at The University of Iowa. Fetal bovine serum was purchased from Hyclone (Salt Lake City, UT, USA). All chemicals were either cell culture grade or molecular biology grade.

Rabbit polyclonal antibody against human kidney MnSOD was prepared in Dr. L. W. Oberley's laboratory [7]. Secondary antibody from goat against rabbit IgG conjugated with horseradish peroxidase was purchased from Sigma Chemical Co. Human MnSOD cDNA was provided by Dr. G. Bell at the University of Chicago and human GAPDH cDNA was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) [8].

Cell culture

The human oral carcinoma SCC-25 cell line was originally obtained from ATCC [9–11]. Cells were grown in 90% DMEM-Ham's F-12, 0.4 μg/ml of hydrocortisone, and 10% heat-inactivated fetal bovine serum (FBS) medium containing 100 U/ml of penicillin and 100 μg/ml streptomycin. The cultures were kept at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in an incubator, with medium replacement every three days. For routine subculture, cells were treated with 0.25% trypsin–0.1% ethylenediaminetetraacetate (EDTA) solution. Trypsin was removed after 5 min, and the culture dishes or flasks kept at 37°C in the incubator until cells became detached. Fresh medium was then added and the cells were dispensed into new dishes or flasks in a 1:4 or 1:10 ratio. Cell viability was assayed by the trypan blue dye exclusion method.

Cell harvest

For routine Western blot analysis and RNA purification, the 90% confluent cultured cells either with or without treatment at specific times were washed with sterile 1× PBS (phosphate-buffered saline, pH 7.8) twice, and scraped off the dishes or flasks in the same solution. The cells were pelleted using a tabletop centrifuge at 1200 × *g* for 10 min. The cell samples were then resuspended in 50 mM potassium phosphate buffer (PB, pH 7.8) and either immediately assayed or stored in a −70°C freezer for later processing.

Protein concentration measurement

Samples used for western analysis were first analyzed for protein concentration. The Bradford method of Bio-Rad (Hercules, CA, USA) was used according to the manufacturer's instructions [12]. Briefly, the harvested cell samples were sonicated using a Vibra Cell Sonicator (Sonics and Materials, Inc., Danbury, CT, USA) for 20 s on ice twice in 50 mM PB (pH 7.8). The sonicated samples or standard protein were incubated with reagents at room temperature for 20 min, and subjected to spectrophotometric measurement at 595 nm. The protein concentration of individual samples was determined by comparison with the defatted bovine serum albumin standard curve [12].

Western blot analysis

Equal amounts of protein (usually 20 μg per well) from different samples were subjected to 15% SDS PAGE gel electrophoresis, then transferred to a nitrocellulose membrane according to the standard protocol [13]. Antibody against human MnSOD developed in Dr. L. W. Oberley's lab was used to immunoreact with specific antigens on the nitrocellulose membrane for 2 h at room temperature or overnight at 4°C. Secondary antibody of goat-anti-rabbit IgG conjugated with peroxidase in a 1:1000 dilution in TBS (Tris-buffered saline) was used to react with the first antibody against human MnSOD protein under the same conditions. Color development was processed by addition of 1 mg/ml 4-chloro-1-naphthol in 20% methanol/PBS. All experiments in this study were repeated two to three times with similar results.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

TNF-α or X/XO treated human oral carcinoma cells as well as nontreated cells were washed twice with 4 ml of sterile 1× PBS (pH 7.8) and scraped off the culture dishes in 2 ml of the same buffer solution. Total cellular RNA was isolated according to the procedure of Chomczynski and Sacchi using RNAzol B solution (Tel-Test, Inc., Friendwood, TX, USA) [14]. Samples were vortexed and kept on ice for 5 min and 0.5 ml of chloroform was added to the mixture followed by centrifugation at 20,000 × *g* for 20 min. The aqueous phase was transferred to a new sterile tube, and an equal volume of isopropanol was added to precipitate the RNA. After washing with ice cold 70% ethanol twice, the RNA pellet was air-dried and dissolved in 30 μl of autoclaved double distilled water. Total RNA was quantified by spectrophotometry at 260/280 nm.

Reverse transcription of cDNA was carried out in a 50 μl mixture containing 10 μl 5× reverse transcription (RT) buffer, 2 μg of total RNA, 0.5 mM dNTP (dATP, dCTP, dGTP, dTTP), 1.5 μg of random hexamer, and

400 units of Moloney murine leukemia virus (MMLV) reverse transcriptase. The mixture was incubated at 42°C for 1 h. After that, the mixture was heated at 95°C to inactivate the MMLV reverse transcriptase.

The PCR reaction was carried out in 50 μl reaction mixture containing 5 μl of 10× PCR reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 3 mM $MgCl_2$), 2 μl of RT cDNA, 1.0 μl of 10 mM dNTP, 1.0 μl of 10 μM sense and antisense primers, and 2 units of Taq DNA polymerase. After 4 min denaturation at 94°C, the PCR amplification was carried out for 30 cycles with denaturation at 94°C for 45 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min, followed by an extension at 72°C for 5 min. The primers for the MnSOD RT-PCR reaction were GAGTTGCTGGAAGCCATCAAACGT (sense), and GTATCTTTCAGTTACATTCTCCCA (antisense). The expected size of the amplified MnSOD cDNA fragment is 306 base pairs. The primers for the GAPDH RT-PCR reaction were ATTCCATGGCACCGTCAAGGCT (sense), and TCAGGTCCACCACTGACACGTT (antisense). The expected size of the amplified GAPDH cDNA fragment is 570 base pairs. All primers were synthesized by the DNA Core Facility at The University of Iowa.

The PCR amplification reaction mixture (10 μl) was loaded on a 2% agarose gel and separated by electrophoresis. The gel was stained with ethidium bromide and photographed with Polaroid film type 667.

Nuclear run-on assay

For these experiments, human oral carcinoma SCC-25 cells were cultured in T-75 flasks in complete medium. Once cells reached 90% confluence, complete medium was replaced by serum free medium, and cells cultured for 6 h. Then, a group of four flasks was supplemented to 200 ng/ml of TNF-α, another group of four flasks was supplemented with X/XO (50 μM of xanthine and 20 milliunits/ml of xanthine oxidase), and a similar group remained unsupplemented. After a 6-h incubation period, the flasks were washed twice with 4 ml of sterile PBS, and the cells were quickly scraped from each group of flasks with 2 ml of lysis buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 3 mM $CaCl_2$, 1 mM DTT, and 0.5% Nonidet P-40. The cells were homogenized using a Dounce homogenizer with a loose-fitting pestle and then centrifuged at 500 × g for 10 min at 4°C to sediment the nuclei. After washing with another 2 ml of lysis buffer, the pellet was resuspended in 150 μl of 50 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA, and 40% glycerol. The nuclei were either immediately assayed for in vitro transcription or stored in liquid nitrogen for later study.

The in vitro transcription was conducted following a procedure similar to that described by Greenberg and Ziff [15], using digoxigenin (Dig)-labeled UTP. All nuclei (about 3×10^7) collected from four flasks were used in the transcription reaction, which was allowed to proceed for 45 min at 30°C. After treatment with RNase-free DNase and proteinase K, extraction with phenol-chloroform was conducted. Glycogen (10 μg) was added to the supernatant to precipitate RNA by isopropanol. Equal amounts of RNA (2 μg) were used to hybridize to the Nytran membrane (Boehringer-Mannheim, Indianapolis, IN, USA), which contained 5 μg denatured DNA of empty plasmid, or plasmid containing MnSOD or GAPDH cDNA, respectively prepared by a slot-blot apparatus.

Hybridizations were carried out at 65°C for 48 hr in aqueous solution containing 5 × SSC (20 × SSC: 3M sodium chloride, 0.3M sodium citrate, pH 7.0), 5× Denhardt solution (100× Denhardt : 10g Ficoll 400, 10g polyvinylpyrrolidone, 10g BSA in 500 ml double distilled H_2O), and 0.5% SDS. Membranes were washed four times for 15 min at 68°C with 2×SSC with 0.2% SDS. Finally the hybridization was detected by anti-Dig antibody according to manufacturer's instructions (Boehringer-Mannheim).

EPR spin trapping techniques

Five × 10^5 cells were seeded in 60 mm dishes and cultured until 90% confluent. Culture medium was removed and cells were washed with 1× PBS twice. TNF-α (200 ng/ml) in sterile PB buffer (pH 7.8) were cultured with cells for 2 h. Then, 75 mM DMPO was added to the cell culture and subsequently incubated for 1 h before the cells were processed for EPR examination [5,16].

Control as well as TNF-α treated cells were scraped from the flasks gently and transferred into 2 ml sterile tubes. Cell number was determined by counting with a hemocytometer in a separate control dish treated with trypsin/EDTA. Cell density was adjusted accordingly to 5×10^6 cells/ml. EPR spectroscopy was performed on 500 μl samples (2.5×10^6 cells) in a TM flat cell using a Bruker (Karlsruhe, Germany) ESP-300 EPR Spectrometer in the Free Radical Research Institute, The University of Iowa. Instrument settings were as follows: microwave power, 10 mW; klystron frequency, 9.764 GHz; modulation amplitude, 1.056 Gauss; scan rate, 80 G/84 s; and time constant, 0.16 s [17]. Spectra resulted from five signal-averaged scans.

RESULTS

To determine if TNF-α induced MnSOD expression in human oral carcinoma SCC-25 cells, western blotting

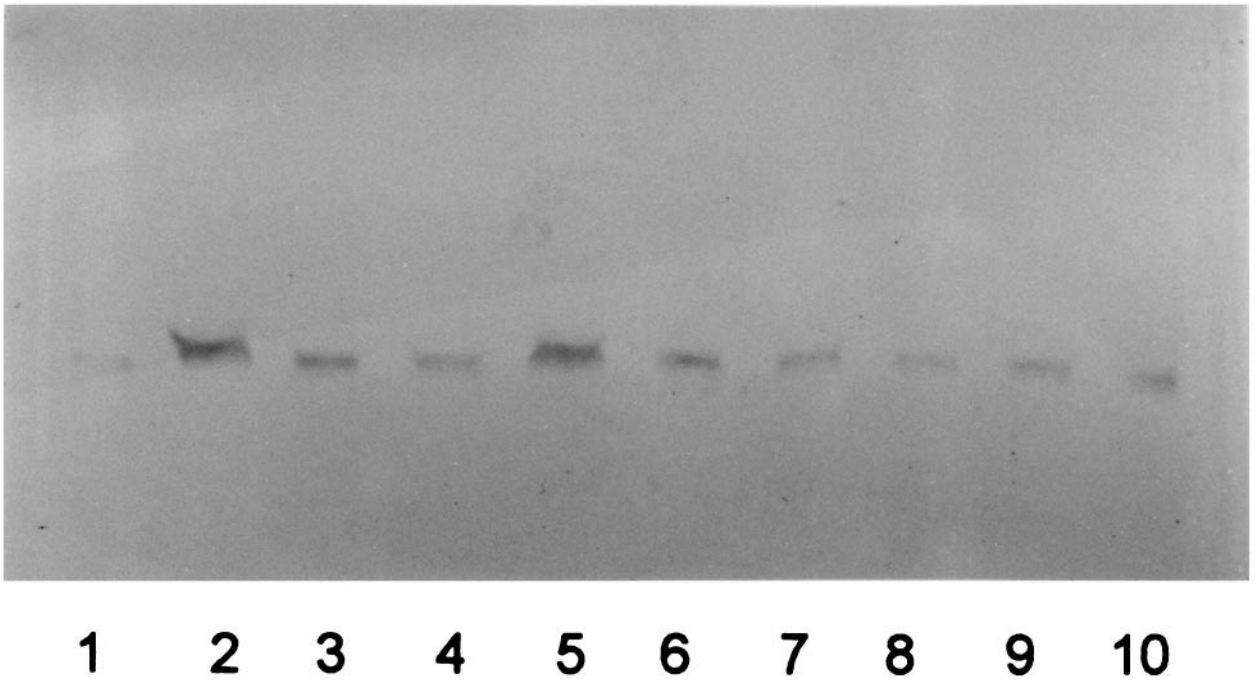

Fig. 1. Western blot analysis demonstrated increased MnSOD expression induced by TNF-α or X/XO and decreased expression in the presence of the oxygen free radical spin trapping reagent DMPO. Twenty micrograms of protein were loaded per lane. Lanes 1 and 10 control human oral carcinoma SCC-25 cells; lane 2, 200 ng/ml TNF-α treated cells; lane 3, 200 ng/ml TNF-α treated cells in the presence of 75 mM DMPO; lane 4, 75 mM DMPO treated cells; lane 5, X/XO treated cells; lane 6, X/XO treated cells in the presence of 75 mM DMPO; lane 7, Xanthine treated cells; lane 8, XO treated cells; and lane 9, 75 mM DMPO treated cells.

was used to analyze MnSOD protein induction. Approximately 10^6 cells were seeded in 100 mm culture dishes in complete medium. Once cells reached 90% confluence, the complete medium was replaced by serum free medium and cultured for 4 h. Then, 200 ng/ml of recombinant human TNF-α was added to the serum free medium and cultured for 6 h before processing for western analysis as described in Materials and Methods. Like many other cell types, treatment of human oral carcinoma SCC-25 cells with recombinant human TNF-α was demonstrated to cause a significant increase in MnSOD protein expression (Fig. 1, lanes 1 and 2).

Previous studies have demonstrated that TNF-α transiently increases intracellular superoxide and other reactive oxygen species production in several cell types [5,7]. To determine if oxygen free radicals can mediate MnSOD induction in SCC-25 cells, DMPO was used to trap oxygen free radicals. In the presence of 75 mM of DMPO, which did not show cytotoxicity to the cultured cells as assayed by trypan blue dye exclusion, the induction of MnSOD protein associated with TNF-α was significantly reduced (Fig. 1, lane 3). In SCC-25 cells treated with xanthine/xanthine oxidase (X/XO: 0.05 mM X, and 20 milliunits/ml of XO), an enzymatic system that generates superoxide, MnSOD protein synthesis was also induced (Fig. 1, lane 5). Moreover, in the presence of DMPO, the induction by X/XO was reduced (Fig. 1, lane 6). Xanthine, xanthine oxidase, or DMPO alone had no effect on MnSOD protein levels (Fig. 1, lanes 7, 8, and 9). These results are consistent with the hypothesis that enhanced oxygen free radical production induced by TNF-α results in the induction of MnSOD.

RT-PCR was used to analyze MnSOD expression at the mRNA level in SCC-25 cells treated with TNF-α and X/XO. Both TNF-α and X/XO increased the steady state MnSOD mRNA levels (Fig. 2A, lanes 2 and 4 compared to control, lane 1). However, in the presence of DMPO, the induction of mRNA levels by both TNF-α and X/XO was greatly reduced (Fig. 2A, lanes 3 and 5). Xanthine, xanthine oxidase, or DMPO had no effect on MnSOD mRNA levels (Fig. 2A, lanes 6, 7, and 8). Moreover, the steady state level of mRNA for GAPDH was not significantly affected by treatment with either TNF-α or X/XO (Fig. 2B). These results are consistent with the hypothesis that oxygen free radicals are, at least, in part, involved in the induction of MnSOD expression at the mRNA level by TNF-α.

The steady-state level of mRNA in the cells is dependent on the rates of both transcription and degradation [15]. To investigate the mechanisms of MnSOD expression by TNF-α and X/XO, nuclear run-on assays were performed. Cells were cultured in T-75 flasks and treated

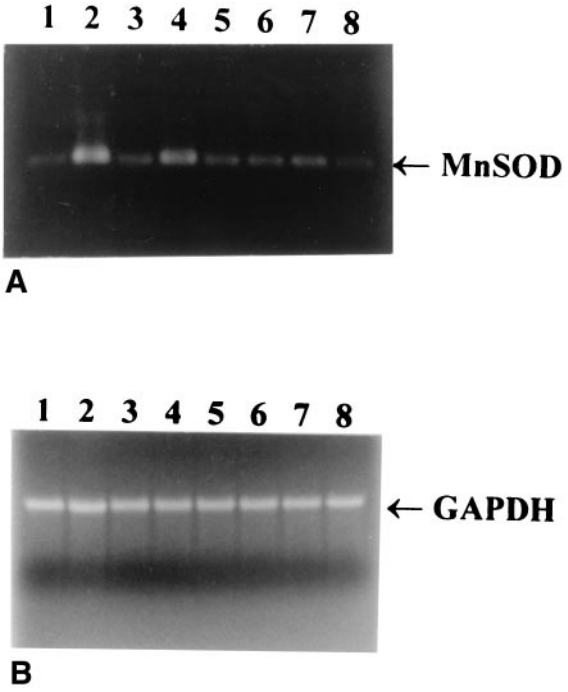

Fig. 2. RT-PCR analysis demonstrated increased MnSOD expression induced by TNF-α, or X/XO and decreased expression in the presence of the oxygen free radical spin trapping reagent DMPO (A), and GAPDH calibration of RT-PRC analysis appears in (B). Lane 1 controls human oral carcinoma SCC-25 cells; lane 2, TNF-α treated cells; lane 3, TNF-α treated cells in the presence of 75 mM DMPO; lane 4, X/XO treated cells; lane 5, X/XO treated cells in the presence of 75 mM DMPO; lane 6, Xanthine treated cells; lane 7, XO treated cells; and lane 8, 75 mM DMPO treated cells.

with 200 ng/ml TNF-α or 50 μM xanthine and 20 mU/ml of xanthine oxidase. Nuclei from control, TNF-α, and X/XO treated cells were isolated, and nuclear run-on experiments were carried out as described in Materials and Methods. The results are shown in Fig. 3. MnSOD was transcribed at low levels under normal culture conditions; however, when cells were treated with TNF-α, or X/XO, the transcription was greatly increased (Fig. 3A). When cells were pretreated with 75 mM DMPO, the induction of MnSOD transcription by both TNF-α and X/XO was greatly decreased (Fig. 3B). Thus, we conclude that induction of MnSOD expression by TNF-α is at least partially at the transcriptional level, and oxygen free radicals may be the second messengers that function downstream of the TNF-α effect, thereby inducing MnSOD expression.

These results strongly suggest that the induction of MnSOD expression by TNF-α is mediated by the formation of oxygen free radicals. To examine this possibility in more detail, EPR spin trapping was used to detect cellular production of oxygen free radicals. 5×10^5 cells were cultured in 60 mm diameter dishes until 90% confluent. After washing with 5 ml of sterile PBS twice, cells were cultured with 200 ng/ml of TNF-α for 2 h. Then, 75 mM DMPO was added as described in Material and Methods. After an additional 30 min incubation with 75 mM DMPO and 200 ng/ml of TNF-α in PB buffer, cells were scraped off the dishes in the same solution, and the cell mixture was immediately frozen in liquid nitrogen [16,17]. Upon thawing, 500 μl of the cell mixtures were rapidly examined by EPR. The spectrum from the TNF-α treated cells showed the presence of both the superoxide and hydroxyl radical adducts of DMPO (Fig. 4, spectrum A). These signals were not observed in control cells without TNF-α (Fig. 4, spectrum C) or cells pretreated with 100 units/ml of *E. coli* MnSOD and 100 units/ml of bovine catalase (Fig. 4, spectrum B). Thus, we conclude that TNF-α induces the production of superoxide radical in human SCC-25 cells, and the hydroxyl radical formation is via a superoxide- or hydrogen-peroxide dependent pathway.

DISCUSSION

The data presented here demonstrate that MnSOD expression in human oral carcinoma SCC-25 cells is modulated by oxygen free radicals produced from the X/XO system or TNF-α (Figs. 1 and 2). X/XO directly produces oxygen free radicals, whereas, the production of oxygen free radicals by TNF-α requires interaction with cells. The induction of MnSOD expression by both X/XO and TNF-α is at least partially at the transcriptional level (Fig. 3). Furthermore, we have demonstrated that the induction of MnSOD by TNF-α is at least partially mediated by the formation of intracellular oxygen free radicals (Figs. 1, 2, and 3). Thus, we propose that the increased production of oxygen free radicals induced by TNF-α serves as intracellular second messengers to regulate MnSOD gene expression. The induction of MnSOD by X/XO may come from reaction of reactive oxygen species with the cell surface or may result from entry of these species into the cells. It is possible that the effects of X/XO are due to the uric acid produced rather than the oxygen free radicals, but this seems unlikely because TNF-α produced similar effects and has not demonstrated to directly produce uric acid.

Regulation of MnSOD expression

SODs are metalloenzymes that catalyze the dismutation of superoxide to hydrogen peroxide; these enzymes are thought to play an important role in the cellular defense against ROS [18,19]. MnSOD is a mitochondrial protein that dismutates superoxide produced by the mitochondrial electron transport chains during normal cell metabolic processes [20]. Reduction or a deficiency of

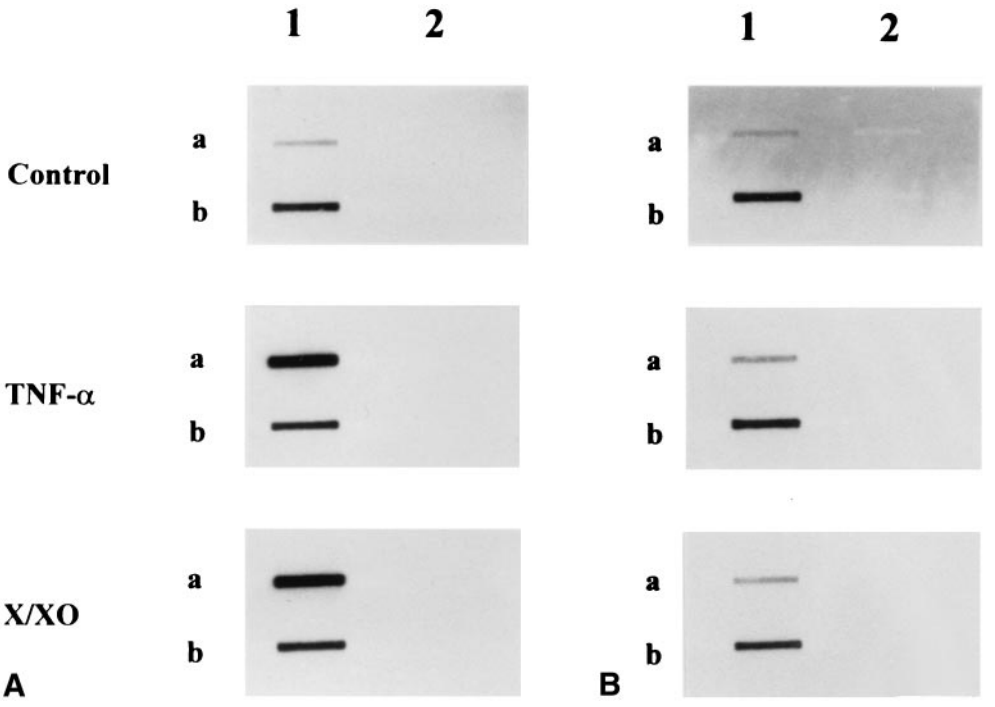

Fig. 3. Nuclear run-on experiment demonstrated the increased transcription of MnSOD induced by TNF-α or X/XO and decreased transcription in the presence of the spin trapping reagent DMPO. In the absence of DMPO (A); in the presence of DMPO (B); plasmid DNA with MnSOD cDNA insert (a); plasmid DNA with GAPDH cDNA insert (b); plasmid DNA with MnSOD or GAPDH cDNA inserts (1); and plasmid DNA control (2).

MnSOD or CuZnSOD activity has been demonstrated to be associated with several diseases, such as familial amyotrophic lateral sclerosis [21], aging [22], and cancer [23,24]. Recently, it has been demonstrated that many cytokines, chemical reagents, and ionizing radiation can induce MnSOD expression. For example, TNF, IFN-γ, and IL-1 induced MnSOD expression in a variety of cell types [7,25,26]. Ionizing radiation induced MnSOD gene expression as well as MnSOD activity in mouse heart tissues and in human fibroblast cells [27,28]. In the present study, we have shown that TNF-α, and oxidative stresses from superoxide can induce MnSOD expression as detected by western analysis and RT-PCR analysis (Figs. 1 and 2). Furthermore, the effects of TNF-α and other oxidative stress on MnSOD expression in human oral carcinoma SCC-25 cells are at least partially at the transcriptional level, as demonstrated by nuclear run-on assay (Fig. 3). The molecular pathways and nature of the induction of MnSOD by TNF-α are not known at the present time. Cloning of the MnSOD gene by Wan et al. [29] has shown that there are several copies of NF-κB, AP-1, and SP-1 binding elements in the promoter region. Very recently, it was shown that an intron 2 enhancer sequence containing a NF-κB element is necessary for induction of MnSOD by TNF-α [30]. Since the transcription factors involved in MnSOD induction are redox sensitive, oxidative stress may be an important factor in determination of MnSOD expression at the transcriptional level. Our data are consistent with these observations, and further suggest that oxygen free radicals may act as second messengers to regulate MnSOD expression induced by TNF-α.

Superoxide and other reactive oxygen species act as intracellular second messengers

Like other cytokines in general, the first event in triggering a cellular response by TNF-α is a specific high-affinity interaction with membrane receptor molecules, thereby initiating a cascade of signal transduction inside cells [31]. Unlike other cytokines, the receptors for TNF-α do not possess intrinsic tyrosine kinase activity in their intracellular domains. The biochemical purification of TNF membrane receptors has revealed structural heterogeneity with two major binding proteins p60 and p75 [32]. Upon binding to its receptor, the TNF-α effect was transduced to the inside of the cell. Guanine nucleotide binding proteins (G-protein) and phospholipase A_2 have been reported in the cascade of TNF-α signals of intracellular reactions that resulted in cellular responses [33]. In addition, cytoplasmic protein kinase A and C have been suggested to participate in the TNF-α signal transduction [34]. The downstream signal transducing molecules have not been identified. However,

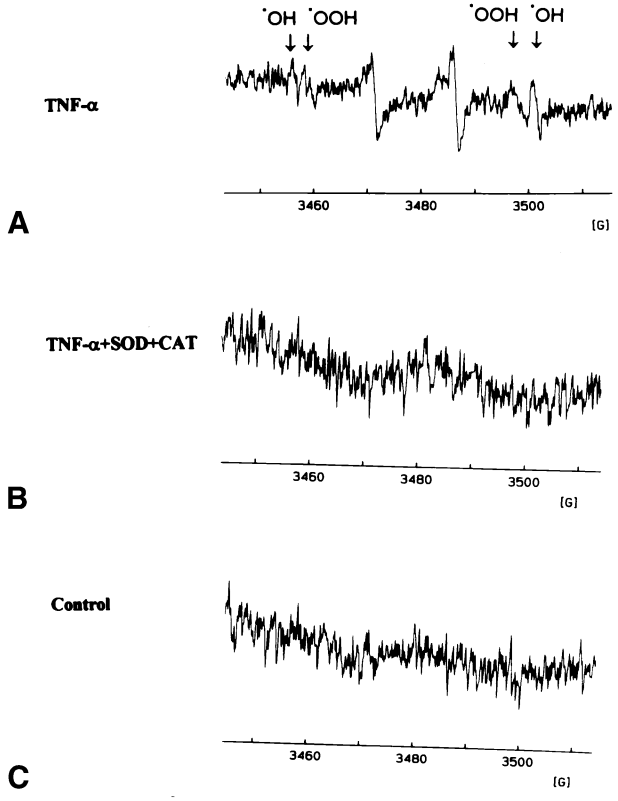

Fig. 4. TNF-α induces oxygen free radical production as seen by EPR spin trapping. TNF-α (200 ng/ml) treated cells produced both DMPO/·OOH (a^N = 14.1G, a^H = 11.3 G, a^H_β = 1.3 G) and DMPO/·OH (a^N = a^H = 14.9 G; the four-line spectrum with an approximate 1:2:2:1 intensity ratio) (A); 200 ng/ml TNF-α treated cells preincubated with 100 units/ml SOD and 100 units/ml CAT (B); and control SCC-25 cells (C). In spectra B and C, the vertical display was increased by a factor of two compared to A.

oxygen free radicals or their derivatives have been suggested to participate in the signal relay processes to regulate gene expression by TNF-α [5,7]. Thus, it is known that TNF-α activates proteins in the plasma membrane to produce ROS [5] and that this activation is somehow passed to the mitochondria, which at later times, are also activated to produce ROS [6].

In the present study, we have shown that human oral carcinoma SCC-25 cells produce oxygen free radicals and/or other reactive oxygen species upon stimulation with TNF-α as evidenced by EPR spin trapping (Fig. 4). These oxygen free radicals appear to be mediators in the induction of MnSOD expression, because upon their elimination by DMPO, the induction of MnSOD both at the mRNA and protein levels was greatly reduced (Figs. 1 and 2). Thus, we hypothesize that oxygen free radicals serve as second messengers involved in gene regulation. This hypothesis is based on DMPO having the ability to enter eukaryotic cells. Samuni et al. [35] found that DMPO "partitioned roughly equally between the cellular and exocellular domains." Thus, in this work, we hypothesize that DMPO enters the cells and then scavenges intracellular oxygen radicals. Of course, it is possible that the DMPO spin adduct has an effect by itself, rather than the scavenging of oxygen free radicals.

Oxygen free radicals or ROS serving as second messengers in cells has some distinct advantages. First, because the half-life for oxygen free radicals is generally short, they will be transiently active; thus, their signals will be delivered only at specific times [36]. Second, because the intracellular concentration of oxygen free radicals is low and their reactions in general are fast, their signals will in general be localized and targeted. Third, oxygen free radicals can react with many cellular molecules, such as small molecular weight antioxidants and antioxidant enzymes; thus a radical signal can be easily insulated, thereby minimizing its effects on other pathways. Much evidence has demonstrated that oxygen free radicals or ROS mediate cellular activity and regulate gene expression. For example, superoxide mediates cell growth and motility in tumor cells and endothelial cells [24,37,38]. More recently, it has been found that oxygen free radicals and their derivative species are involved in the activation of MAP kinase activity, which cascades phosphorylation and dephosphorylation leading to the conversion of extracellular effects to intracellular action [39]. In the present investigation, we have found that oxygen free radicals are intracellular mediators regulating MnSOD expression in SCC-25 cells upon stimulation by TNF-α. Our data are consistent with Meier and colleagues' [5] observation that superoxide is transiently produced in TNF-α treated cells. More recently, it has been demonstrated that TNF-α can activate NF-κB activity. However, if free radical scavengers were present, the induction was reduced, suggesting a role for oxygen free radicals in intracellular signal transduction [40]. A separate report has demonstrated that hydrogen peroxide mediates the effects of platelet derived growth factor (PDGF) [41]. It has also been demonstrated that oxidants mediate the mitogenic signaling induced by the Ras oncogene [42]. In this investigation we have shown that oxygen free radicals participate in the TNF-α signal relay to mediate MnSOD expression. Taken together, we propose that oxygen radicals and other reactive oxygen species act as intracellular second messengers mediating signal transduction to affect cellular activity upon stimulation by some cytokine or growth factors.

In conclusion, our results have demonstrated for the first time that TNF-α induces MnSOD expression in human oral carcinoma SCC-25 cells and that this induction is mediated, at least in part, by the formation of intracellular oxygen free radicals. Thus, the hypothesis that oxygen free radicals serve as second messengers to regulate MnSOD gene expression has been examined for the first time. In the future, it would be of interest to

examine whether other scavengers of intracellular superoxide, such as adenoviral*CuZnSOD*, as well as extracellular scavengers, such as CuZnSOD protein, affect the induction of MnSOD by TNF-α or X/XO.

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ABBREVIATIONS

CAT—catalase
CuZnSOD—copper- and zinc-containing superoxide dismutase
TNF—tumor necrosis factor
Dig—digoxygenin
DMEM—Dulbecco's modified Eagle's medium
DMPO—5,5-dimethylpyrroline-*N*-oxide
EPR—electron paramagnetic spectroscopy
FBS—fetal bovine serum
GADPH—glyceraldehyde phosphate dehydrogenase
MMLV—Moloney murine leukemia virus
MnSOD—manganese-containing superoxide dismutase
PB—phosphate buffer
PBS—phosphate-buffered saline
PDGF—platelet derived growth factor
ROS—reactive oxygen species
RT-PCR—reverse transcriptase polymerase chain reaction
SOD—superoxide dismutase
TBS—Tris-buffered saline
UTP—uridine-5′-triphosphate
X/XO—xanthine, xanthine oxidase