Overexpression of Manganese Superoxide Dismutase Suppresses Tumor Necrosis Factor-induced Apoptosis and Activation of Nuclear Transcription Factor-κB and Activated Protein-1*

(Received for publication, January 14, 1998, and in revised form, March 19, 1998)

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Several recently identified intracellular proteins are associated with the tumor necrosis factor (TNF) receptor and activate nuclear transcription factor (NF-κB), c-Jun kinase, and apoptosis. However, the mechanism is not understood. In the present report, we investigated the role of reactive oxygen intermediates in TNF-induced signaling. Overexpression of manganese superoxide dismutase (Mn-SOD) in human breast cancer MCF-7 cells completely abolished TNF-mediated NF-κB activation, IκBα degradation, p65 nuclear translocation, and NF-κB-dependent reporter gene expression. Besides TNF, phorbol ester, okadaic acid, ceramide, and lipopolysaccharide-induced activation of NF-κB was blocked by Mn-SOD, indicating a common pathway of activation. H2O2-induced NF-κB activation, however, was potentiated. In addition, Mn-SOD blocked the TNF-mediated activation of activated protein-1, stress-activated c-Jun protein kinase, and mitogen-activated protein kinase kinase. TNF-induced antiproliferative effects and caspase-3 activation, indicators of apoptosis, were also completely suppressed by transfection of cells with Mn-SOD. Suppression of apoptosis induced by okadaic acid, H2O2, and taxol was also inhibited by Mn-SOD but not that induced by vincristine, vinblastine, or daunomycin. Overall, these results demonstrate that in addition to several recently identified signaling molecules, reactive oxygen intermediates play a critical role in activation of NF-κB, activated protein-1, c-Jun kinase, and apoptosis induced by TNF and other agents.

TNF plays a major role in tumorogenesis, inflammation, viral replication, and septic shock (see Ref. 1 and references within). How a single molecule can mediate such a wide array of effects is not known, but these effects are known to be mediated through two different receptors, p60 (type I) expressed on all cell types and p80 (type II) expressed primarily on immune cells. Within the last 5 years, over 15 distinct proteins have been identified that associate with the cytoplasmic domain of the p60 receptor and are involved in activation of apoptosis, NF-κB, N-terminal c-Jun kinase (JNK), and mitogen-activated protein kinase (see Ref. 2 and references within). Similarly, the cytoplasmic domain of the p80 receptor has been reported to associate with four proteins that play a role in activation of NF-κB and apoptosis. How any of these proteins mediate apoptosis or NF-κB activation is less clear. By using metabolic inhibitors, several studies have indicated that TNF-mediated activation of either NF-κB or apoptosis or activation of various kinases requires the production of various intermediates including reactive oxygen intermediates (ROI) (3). We conducted a series of experiments using superoxide dismutase (SOD) to explore the role of ROI.

SOD is a family of antioxidant enzymes that convert harmful superoxide radicals into H2O2, which in turn is metabolized to harmless water and oxygen by catalase and glutathione peroxidase (4, 5). In mammalian cells, two main types of SOD are found; viz., manganese SOD (Mn-SOD), located in the mitochondria, and copper-zinc SOD, found in the cytoplasm. Previous studies have shown that overexpression of Mn-SOD blocks TNF-mediated cytotoxicity (6–8). How overexpression of Mn-SOD affects TNF-induced transcription factors including NF-κB and AP-1 is not known, however. It is also not known how overexpression of SOD affects TNF-induced activation of stress-activated protein kinase, also called JNK, and the growth modulatory kinases of the extracellular signal-regulated kinase family (MAP kinase kinase/MAP kinase (MEK/MAP kinase), implicated in the TNF-induced activation of AP-1 and NF-κB, respectively (9, 10).

In the present report, we demonstrate that overexpression of Mn-SOD in human breast tumor MCF-7 cells blocks TNF-induced cytotoxicity and activation of caspase-3 (indicator of apoptosis), NF-κB, AP-1, JNK, and MEK. The effect of SOD was not specific to TNF, since other agents that activate NF-κB (e.g. PMA, interleukin-1, and okadaic acid) or induce apoptosis (e.g. okadaic acid and taxol) were also inhibited, thus suggesting that the ROI has a critical role in activation of signaling by a wide variety of agents.

EXPERIMENTAL PROCEDURES

Materials—Penicillin, streptomycin, RPMI 1640 medium, and fetal calf serum were obtained from Life Technologies, Inc. Glycine, NaCl, and bovine serum albumin were obtained from Sigma. Bacteria-derived recombinant human TNF, purified to homogeneity with a specific activity of 5 × 10^7 units/mg, was kindly provided by Genetech, Inc. (South San Francisco, CA). Antibody against IκBα and double-stranded oligonucleotide having an AP-1 consensus sequence were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The rat MDR1bCAT plasmid 243RMICAT containing the chloramphenicol acetyl transfer-
ase (CAT) gene with either wild-type or mutated NF-κB binding site was kindly supplied by Dr. M. Tien Kuo of the University of Texas M. D. Anderson Cancer Center (Houston, TX). The characterization of these plasmids has been described previously in detail (11).

Cell Lines—Human breast MCF-7 cells were stably transfected with control (neo) and Mn-SOD genes similarly to what was described in detail previously (8). Two different Mn-SOD cDNAs were examined. Polymorphism in the Mn-SOD gene results in two different amino acids (either Ile or Thr) at position 58 in the human population (12). The replacement of Thr for Ile leads to a protein with about 50% activity and a reduction in protein stability after heat (12). Sense human Mn-SOD cDNA was originally obtained from Dr. Daret St. Clair. After the cDNA was ligated into the pcDNA3 plasmid (Invitrogen) containing a neomycin resistance marker, it was sequenced, which revealed that this cDNA contained an ACA codon for threonine at amino acid 58. Site-directed mutagenesis was performed to change ACA to ATA, the codon for isoleucine. The change was confirmed by cDNA sequencing. The MCF-7 cell line was found to express the isoleucine form of Mn-SOD.

These two types of plasmids were transfected into MCF-7 cells (obtained from ATCC at passage number 148) by a protocol similar to that described previously (8). Briefly, at passage 155, these cells were transfected with pcDNA3 plasmids by themselves (neo control) or plasmids containing sense human Ile 58 Mn-SOD cDNA or sense human Thr 58 Mn-SOD cDNA under the control of a cytomegalovirus promoter. After the cDNA was ligated into the pcDNA3 plasmid (Invitrogen) containing a neomycin resistance marker, it was sequenced, which revealed that this cDNA contained an ACA codon for threonine at amino acid 58. Site-directed mutagenesis was performed to change ACA to ATA, the codon for isoleucine. The change was confirmed by cDNA sequencing. The MCF-7 cell line was found to express the isoleucine form of Mn-SOD.

NF-κB Activation Assays—To determine NF-κB activation, electrophoretic mobility shift assays were carried out essentially as described (13). Briefly, nuclear extracts prepared from TNF-treated cells (2 × 10⁵/ml) were incubated with 32P-end-labeled 45-mer double-stranded NF-κB oligonucleotide (4 μg of protein with 16 fmol of DNA) from the human immunodeficiency virus long terminal repeat, 5′-TTGTTCAAGGGGACCTTCCGCTGGGGACTTTCCAGGGAGGCGTGG-3′ (underline indicates NF-κB binding sites) for 15 min at 37 °C, and the

\[ \text{NF-κB Block by Mn-SOD} \]
DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5'-TTGTTACAACTCACTTTCCGCTGCTCACTTTCCAGG
GAGGCGTGG-3', was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. The dried gels were visualized, and radioactive bands were quantitated by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software.

AP-1 Activation Assay—To determine the activation of AP-1, 4–5 μg of nuclear extract prepared as indicated above were incubated with 16 fmol of the 32P-end-labeled AP-1 consensus oligonucleotide 5'-CGCTTGAGTGACTCGCCGGAA-3' (Santa Cruz Biotechnology, Inc.) (underline indicates AP-1 binding sites) for 15 min at 37 °C and analyzed by electrophoretic mobility shift assay. The specificity of binding was examined by competition with unlabeled oligonucleotide. Visualization and quantitation of radioactive bands was carried out by a PhosphorImager (Molecular Dynamics) using ImageQuant software.

Western Blot for IκBα, p50, and p65—To determine the levels of IκBα, postnuclear (cytoplasmic) extracts were prepared (14) from TNF-treated cells and resolved on 10% SDS-polyacrylamide gels. To determine the levels of NF-κB proteins, p50, and p65, nuclear and postnuclear extracts prepared from TNF-treated cells were resolved on 8% SDS-PAGE. After electrophoresis, the proteins were electrotransferred to nitrocellulose filters, probed with rabbit polyclonal antibodies against IκBα, p50, or p65 and detected by chemiluminescence (ECL, Amersham Pharmacia Biotech) (14). The bands obtained were quantitated using Personal Densitometer Scan version 1.30 using ImageQuant software version 3.3 (Molecular Dynamics).

Cytotoxicity Assays—The cytotoxic effects of TNF on MCF-7 cells were determined by the amount of [3H]thymidine incorporated by the cells as described previously (15). Briefly, cells were plated at 5000/well in 0.1 ml of medium in 96-well flat-bottomed Falcon plates. Different concentrations of TNF were added in an additional 0.1 ml of medium and incubated at 37 °C for 48 h. During the last 6 h before harvesting, [3H]thymidine (5 mCi/mmol; Amersham Pharmacia Biotech) was added to each well (0.5 μCi/well), and then cells were harvested with the aid of a Filtermate 196 harvester (Packard Instruments Co., Meriden, CT). Radioactivity bound to the filter was measured in a liquid scintillation counter (model 1600 TR, Packard Instrument Co.). The cytotoxicity was also measured by the modified tetrazolium salt...
3-(4–5-dimethylthiozol-2-yl)2–5-diphenyl-tetrazolium bromide (MTT) assay (16). Briefly, cells (5000 cells/well) were incubated in the presence or absence of the indicated test sample in a final volume of 0.1 ml for 72 h at 37 °C. Thereafter, 0.025 ml of MTT solution (5 mg/ml in phosphate-buffered saline) was added to each well. After a 2-h incubation at 37 °C, 0.1 ml of the extraction buffer (20% SDS, 50% dimethyl formamide) was added. After an overnight incubation at 37 °C, the optical densities at 590 nm were measured using a 96-well multiscanner autoreader (Dynatech MR 5000), with the extraction buffer as a blank. Percentage of cytotoxicity was determined as follows: percentage of cytotoxicity = (1 – (A570 of test sample)/A570 of control sample)) × 100%.

Immunoblot Analysis of PARP Degradation—TNF-induced apoptosis was examined by proteolytic cleavage of PARP (17). Briefly, cells (2 × 10^6/ml) were treated with TNF for different times at 37 °C and then extracted by incubation for 30 min on ice in 0.05 ml of buffer containing 20 mM HEPES, pH 7.4, 2 mM EDTA, 250 mM NaCl, 0.1% Nonidet P-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μM phenylmethylsulfonyl fluoride, 0.5 μg/ml benzamidine, and 1 μM DTT. The lysate was centrifuged, and the supernatant was collected. Cell extract protein (50 μg) was resolved on 7.5% SDS-PAGE, electrotransferred onto a nitrocellulose membrane, blotted with mouse anti-PARP antibody, and then detected by chemiluminescence (ECL; Amersham Pharmacia Biotech). Apoptosis was represented by the cleavage of 116-kDa PARP into 85- and 41-kDa peptide products (18).

MAP Kinase Kinase Assay—MCF-7 cells, stimulated with different concentrations of TNF for 30 min at 37 °C were washed with Dulbecco's phosphate-buffered saline and then lysed with buffer containing 20 mM HEPES, pH 7.4, 2 mM EDTA, 250 mM NaCl, 0.1% Nonidet P-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μM phenylmethylsulfonyl fluoride, 0.5 μg/ml benzamidine, 1 μM DTT, and 1 μM sodium orthovanadate. A 50-μg aliquot of protein was resolved on 10% SDS-PAGE, electrotransferred onto nitrocellulose membranes, blotted with mouse anti-PARP antibody, and then detected by chemiluminescence (ECL; Amersham Pharmacia Biotech). Apoptosis was represented by the cleavage of 116-kDa PARP into 85- and 41-kDa peptide products (18).

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FIG. 2. Effect of Mn-SOD on different activators (PMA, serum-activated LPS, H_2O_2, okadaic acid, and ceramide) of NF-κB. U-937 or MCF-7 cells (2 × 10^6/ml) were preincubated with PMA (25 ng/ml), SA-LPS (10 μg/ml), H_2O_2 (250 μM), okadaic acid (500 nM), or ceramide (10 μM) for 30 min and then tested for NF-κB activation as described.

FIG. 3. Effect of Mn-SOD on TNF-induced degradation of IκBα and on the levels of p65. MCF-7 cells (2 × 10^6/ml) were incubated for different times with TNF (0.1 nM) and then assayed for IκBα in cytosolic fractions by Western blot analysis (A) and for p65 in cytoplasmic and nuclear extracts by Western blot analysis (B).

FIG. 4. Effect of Mn-SOD on the expression of the CAT gene linked to NF-κB containing the mdr1b promoter. MCF-7 cells were transiently transfected with MDR-NF-κB-CAT (−243RMICAT), both the wild-type and mutant gene, treated with 0.1 nM TNF for 1 h, and then assayed for CAT activity as described under “Experimental Procedures.” Results are expressed as fold-activity of the nontransfected control.
and the bands were detected by chemiluminescence (ECL, Amersham Pharmacia Biotech).

**c-Jun Kinase Assay**—The c-Jun kinase assay was performed by a modified method as described earlier (18). Briefly, after treatment of cells (3 × 10⁵/ml) with TNF for 10 min, cell extracts were prepared by lysing cells in buffer containing 20 mM HEPES, pH 7.4, 2 mM EDTA, 250 mM NaCl, 1% Nonidet P-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml benzamidine, and 1 mM DTT. Cell extracts (150–250 μg/sample) were immunoprecipitated with 0.3 μg of anti-JNK antibody for 60 min at 4 °C. Immune complexes were collected by incubation with protein A/G-Sepharose beads for 45 min at 4 °C. The beads were extensively washed with lysis buffer (4 × 400 μl) and kinase buffer (2 × 400 μl), 20 mM HEPES, pH 7.4, 1 mM DTT, 25 mM NaCl. Kinase assays were performed for 15 min at 30 °C with glutathione S-transferase-Jun-(1–79) as a substrate in 20 mM HEPES, pH 7.4, 1 mM DTT, 10 mM MgCl₂, 1 mM DTT, and 10 μCi of [γ³²P]ATP. Reactions were stopped with the addition of 15 ml of 2 × SDS sample buffer, boiled for 5 min, and subjected to SDS-PAGE (9%). Glutathione S-transferase-Jun-(1–79) was visualized by staining with Coomassie Blue, and the dried gel was analyzed by a PhosphorImager (Molecular Dynamics).

**Transient Transfection and CAT Assay**—To determine the TNF-induced NF-κB-mediated reporter gene transcription, MCF-7 (neo) and Mn-SOD cells were transiently transfected by the calcium phosphate method with the plasmids 243RMICAT (contains wild-type NF-κB binding site) and 2243RMICAT-km (mutated binding site), according to the instructions supplied by the manufacturer (Life Technologies). After 6 h of transfection, the cells were plated for 24 h at 37 °C, stimulated with 100 pM of TNF for 1 h, washed, and examined for CAT activity as described (19).

### RESULTS

In the present study, we investigated the effect of transfection of the Mn-SOD gene on the transcription factors NF-κB and AP-1 and the associated kinases of the MAP kinase family induced by TNF. We also examined the effect of this gene on apoptosis induced by TNF and a wide variety of other agents. For all studies, untransfected cells and those transfected with the neo gene were used as a control. Two different types of clones were studied. Clone SOD18 was transfected with Thr58 Mn-SOD cDNA and was used for the majority of the experiments. SOD18 had an approximately 6-fold increase in Mn-SOD immunoreactive protein as determined by Western blotting compared with wild type or neo lines, but only a 1.8-fold increase in Mn-SOD enzymatic activity. Clones 11, 40, and 59 were obtained from cells transfected with Ile58 Mn-SOD cDNA. The increases in immunoreactive protein over wild type were 13-, 14-, and 8-fold for clones 11, 40, and 59, respectively. The increases in enzymatic activity over wild type and neo controls were 24-, 40-, and 8-fold for clones 11, 40, and 59, respectively. Thus, both types of clones have similar increases in immunoreactive protein, but the Ile58 clones have far more specific increase in Mn-SOD enzymatic activity.
activity than the Thr58 clone. The actual enzymatic activities in units per mg of protein for wild type, neo, SOD18, Mn11, Mn40, and Mn59 were 7 ± 5, 7 ± 3, 12 ± 1, 142 ± 20, 238 ± 56, and 46 ± 13, respectively. Mn-SOD-transfected cells grow more slowly than wild type or neo controls, perhaps because of H2O2 production in these cells. All other clones grow at similar rates to each other, however. To ascertain the stability of the gene, cells were routinely checked for the expression of SOD enzyme activity. When examined for cell surface expression of TNF receptors by radioreceptor assay, both neo- and Mn-SOD-transfected cells were found to show similar specific binding of labeled TNF (3896 ± 100 versus 3975 ± 21 cpm).

Mn-SOD Inhibits TNF-induced NF-κB Activation—Nontransfected and neo- and Mn-SOD-transfected MCF-7 cells were stimulated with different concentrations of TNF, and nuclear extracts were prepared and assayed for NF-κB by electrophoretic mobility shift assay. As little as 10 pg TNF activated NF-κB in both nontransfected and neo gene-transfected cells, whereas in Mn-SOD gene-transfected cells, no significant NF-κB activation was noted, even at 1000 pg TNF concentration (Fig. 1A). Since antibodies against the p50 and p65 subunits of NF-κB supershifted the band on the gel shift (Fig. 1B), NF-κB suppressed by Mn-SOD consisted of p50 and p65 subunits. This supershift was specific, since antibodies to c-Rel or cyclin D1 or preimmune serum (PIS) had no effect. Specificity is also indicated from the observations that this band competed with unlabeled probe and that it did not bind to an oligonucleotide with mutated NF-κB site (Fig. 1B). Since no difference was noted between nontransfected and neo-transfected MCF-7 cells, for all following experiments only neo-transfected cells were used as a control.

In neo-transfected cells, TNF activated NF-κB within 15 min, and its level reached a maximum in 30 min (Fig. 1C). In contrast, no significant NF-κB activation was observed even after 4 h in Mn-SOD-transfected cells. Some reports indicate that the mechanism of NF-κB activation may differ in different cell types, so we transfected human glioblastoma cells. Mn-SOD suppressed TNF-induced NF-κB activation in these cells too (data not shown). This suggests that the effects of Mn-SOD are not cell type-specific.

The effect of Mn-SOD on NF-κB activation was not restricted to a single clone of transfected MCF-7 cells, since three other clones (clones 11, 40, and 59) with medium, high, and low Mn-SOD activity also showed suppressed TNF-induced NF-κB activation (Fig. 1D). These clones expressed the other polymorphic form of Mn-SOD. The inhibitory effect was less pronounced in clone 11 than in clones 40 and 59. As mentioned earlier, when examined for the specific activity of the Mn-SOD, it was found that Mn11 has intermediate overexpression of Mn-SOD activity (20-fold increase). In spite of intermediate Mn-SOD activity, clone 11 showed partial suppression of TNF-induced NF-κB activation, but the reason for this is not clear.

Mn-SOD Inhibits NF-κB Activation Induced by a Wide Variety of Agents—Different agents may activate NF-κB by different mechanisms (20). For instance, TNF-induced NF-κB activation is protein kinase C-dependent, whereas that activated by PMA is protein kinase C-dependent. Therefore, we examined the effect of Mn-SOD on the activation of NF-κB by PMA, LPS, H2O2, okadaic acid, and ceramide (Fig. 2). As we have shown previously, all five agents activated NF-κB in human histiocytic lymphoma U-937 cells (upper panel). Although the overall activation was less pronounced than in U-937 cells, all five activated NF-κB in neo-transfected MCF-7 cells (middle panel). Mn-SOD transfection blocked NF-κB activation by all agents except H2O2 (lower panel). The NF-κB activation by the latter was actually potentiated by Mn-SOD, thus suggesting that H2O2 activates NF-κB by a mechanism that is quite different from that of other agents.
Mn-SOD Inhibits TNF-dependent Degradation of IkBα—The translocation of NF-κB to the nucleus is preceded by the phosphorylation and proteolytic degradation of IkBα. To determine whether Mn-SOD also inhibits TNF-induced IkBα degradation, the cytoplasmic levels of IkBα proteins after treatment of cells with TNF for different times were examined by Western blot analysis. IkBα was degraded within 10 min of TNF treatment and reappeared by 60 min in MCF-7 (neo) cells (Fig. 3A). In Mn-SOD-transfected cells, however, no degradation of IkBα was observed after TNF treatment even up to 60 min. In contrast to neo-transfected cells, levels of p65 remained high in cytoplasmic extracts of Mn-SOD-transfected cells (Fig. 3B). The nuclear levels of p65 protein increased in neo cells but not in Mn-SOD-transfected cells, thus suggesting that in Mn-SOD-transfected cells TNF is unable to dissociate p65 from IkBα.

Mn-SOD Blocks NF-κB-dependent Reporter Gene Expression Induced by TNF—Our results up to now show that Mn-SOD blocks the binding of NF-κB protein to DNA. Whether it also blocks NF-κB-dependent gene expression was also examined. The rat multiple drug resistance gene promoter contains an NF-κB binding site; the promoter was ligated with CAT reporter gene. This plasmid was transfected into neo and Mn-SOD-transfected cells, and then the cells were stimulated with 10 and 100 pM TNF. TNF activated CAT activity by 3–4-fold in neo cells, whereas a minimal increase in CAT activity over the basal level was observed in Mn-SOD-transfected cells (Fig. 4). These effects were specific, since the plasmid with mutated NF-κB binding site was not activated on treatment of cells with TNF. These results thus demonstrate that Mn-SOD also blocks TNF-induced transcriptional activity.

Fig. 8. A, effect of Mn-SOD on cytotoxicity induced by diverse agents. Cells (5 x 10^5/0.1 ml) were incubated with TNF (1 nM), okadaic acid (1 μM), H2O2 (250 μM), vinblastine (25 μM), taxol (25 μM), vincristine (25 μM), or daunomycin (25 μM) for 24 h at 37 °C in a CO2 incubator. Thereafter, cell viability was determined by the MTT method as described under “Experimental Procedures.” All determinations were made in triplicate. B, effect of Mn-SOD on NF-κB activation by different chemotherapeutic agents. MCF-7 cells (2 x 10^6/ml) transfected with neo and Mn-SOD genes were incubated at 37 °C with vinblastine (25 μM), taxol (25 μM), vincristine (25 μM), doxorubicin (25 μM), or daunomycin (25 μM) for 2 h, and then nuclear extracts were prepared and assayed for NF-κB as described under “Experimental Procedures.”

Mn-SOD Inhibits TNF-dependent AP-1 Activation—TNF is a potent activator of another transcriptional factor, AP-1 (9). Several reports indicate that the requirements for AP-1 activation differ from that of NF-κB activation (21). Whether acti-
activation of AP-1 by TNF is through ROI is not known. Therefore, we investigated the effect of Mn-SOD on TNF-induced AP-1 activation. As shown in Fig. 5, TNF activated AP-1 in a dose-dependent manner with 6-fold activation at 100 pM in neo cells. Mn-SOD-transfection, however, resulted in only 2-fold activation of AP-1 even with 10,000 pM TNF. This activation was specific, since it was completely inhibited by the unlabeled probe.

**Mn-SOD Inhibits TNF-induced c-Jun Kinase Activation**—TNF is also a potent activator of JNK. This kinase is activated in response to different kinds of stress and is needed for activation of AP-1. As shown in Fig. 6A, TNF activated JNK in neo cells in a dose-dependent manner, with 27-fold activation noted with 10,000 pM TNF. Mn-SOD transfection, in contrast, completely suppressed TNF-induced JNK activation. These results thus indicate that ROI generated by TNF is also essential for JNK activation.

**Mn-SOD Inhibits TNF-induced Mitogen-activated Protein Kinase Kinase Activation**—It has been reported that TNF activates a serine/threonine kinase, MAP kinase kinase kinase (also called MEKK), which in turn activates a dual specificity kinase, MAP kinase kinase (also called MEK) and that activates another serine/threonine MAP kinase, mitogen-activated protein kinase/extracellular signal-regulated kinase. There are reports that MAP kinase kinase kinase is required for TNF-induced NF-κB activation, so the question of whether Mn-SOD affects TNF-induced activation of MEK was investigated. MEK was activated by TNF in a dose-dependent manner in neo cells but not in Mn-SOD-transfected MCF-7 cells (Fig. 6B). These results indicate that activation of MEK by TNF also occurs through the generation of ROI.

**Mn-SOD Blocks TNF-mediated Cytotoxicity and Activation of Caspase-3**—By cell counting and by clonogenic assay, we have previously shown that Mn-SOD blocks the antiproliferative effects of TNF (8). We now show by thymidine incorporation assay (DNA synthesis) as well as by MTT assay (mitochondrial activity) that Mn-SOD completely protected MCF-7 cells from TNF-induced cytotoxicity (Fig. 7, upper panel). TNF exhibited its cytotoxic effects either by inducing necrosis or apoptosis. One hallmark of apoptosis is the activation of caspase-3 activity, which leads to cleavage of PARP substrate from 116 to 85 kDa. TNF induced cleavage of PARP within 1 h in neo cells (Fig. 7, lower panel), whereas Mn-SOD cells were resistant to PARP cleavage even after 2 h of TNF treatment. These results thus suggest that Mn-SOD also blocks TNF-induced activation of caspase-3.

**Mn-SOD Protects Cells from Taxol-, Okadaic Acid-, and H₂O₂-induced Cytotoxicity and NF-κB Activation**—Besides TNF, MCF-7 cells are sensitive to cytotoxic effects of a wide variety of other agents including chemotherapeutic agents such as vincristine, taxol, and daunomycin and to H₂O₂ and okadaic acid. How Mn-SOD affects the cytotoxicity induced by these agents was also investigated. Mn-SOD completely protected the cells from okadaic acid- and taxol-induced cytotoxicity (Fig. 8A). Unlike these agents, however, Mn-SOD had no effect on cytotoxicity induced by vincristine, vinblastine, and daunomycin. Cells treated with H₂O₂ were only partially protected. These results thus indicate that different agents induce cytotoxicity by different mechanisms. Most chemotherapeutic agents that induce apoptosis also appear to activate NF-κB. Whether this activation is through ROI is not known. Therefore, we also investigated the effect of Mn-SOD on NF-κB activation by various chemotherapeutic agents. The results shown in Fig. 8B indicate that all of the agents that induced cytotoxicity also activated NF-κB, but only taxol-induced activation was suppressed significantly by Mn-SOD. These results suggest that taxol induces cytotoxicity and NF-κB activation through the same free radical species, whereas other agents transduce their signal through a different mechanism.

**DISCUSSION**

The aim of the present report was to investigate the role of ROI in TNF-induced activation of NF-κB, AP-1, JNK, MEK, and caspase-3. To determine this, human breast cancer MCF-7 cells were transfected with the Mn-SOD gene; neo-transfected cells were used as controls. Overexpression of Mn-SOD suppressed TNF-induced IκBα degradation, NF-κB activation, and NF-κB-dependent reporter gene expression. It also blocked TNF-induced activation of AP-1, JNK, MEK, caspase-3, and cytotoxicity (see Fig. 9). The effects of Mn-SOD were not specific to TNF, since NF-κB activation by okadaic acid, PMA, LPS, and ceramide and apoptosis induced by okadaic acid, taxol, and H₂O₂ were also blocked. All of these results suggest that ROI are intermediates for transmission of a wide variety of TNF effects. These effects are not cell type-specific. Moreover, both of the polymorphic forms of Mn-SOD led to similar effects.

Human breast carcinoma MCF-7 cells are known to express only the p60 form of the TNF receptor (22). Recently, over 15 different proteins have been identified that physically associate with the cytoplasmic domain of the TNF receptor and are responsible for mediation of apoptosis and activation of NF-κB, JNK, MEK, and caspase-3 as investigated here. How any of these receptor-associated proteins generate ROI is not clear. By using pyrrolidine dithiocarbamate (a metal chelator) and N-acetyl-l-cysteine (a precursor of glutathione), ROI has been implicated in TNF-induced NF-κB activation (23–26). There are other reports, however, that show that both pyrrolidine dithiocarbamate and N-acetyl-l-cysteine not only fail to inhibit activation of NF-κB but by themselves activate NF-κB (27, 28).
Our results, however, support the notion that ROI are critical for TNF-induced NF-κB activation. It is not possible to ascertain the reactive oxygen species from studies with SOD-transfected cells alone. This is because increased SOD levels certainly lead to decreased superoxide radical levels but may also lead to increased concentrations of hydrogen peroxide and other hydroperoxides (4, 5). Moreover, since the nitric oxide radical effectively competes with SOD for superoxide, increasing SOD will lead to increased levels of NO. Thus, SOD transfection can lead to changed levels of superoxide, hydrogen peroxide, and nitric oxide, as well as any species derived from them. It is not possible with a single transfection to determine the effector species. Double transfection with peroxide-removing enzymes may, however, allow identification of the reactive species involved.

How TNF activates NF-κB is not fully understood. Recently, several kinases have been identified that play a critical role in TNF-induced NF-κB activation. This includes MAP kinase kinase kinase, Ikκ-α, Ikκ-β, and NIK (for a review, see Ref. 29). We found that the overexpression of Mn-SOD blocked the activation of TNF-induced MEK, suggesting that superoxide radical is needed for activation of this kinase. It is possible that the suppression of NF-κB is due to down-regulation of MEK.

Mn-SOD converts superoxide radicals to H₂O₂ and molecular oxygen (30). Interestingly, we found that H₂O₂-activated NF-κB was potentiated by Mn-SOD. These results indicate that ROI species involved in activation of NF-κB by TNF, and most other agents must differ from that of H₂O₂. It is possible that TNF-induced NF-κB activation is through a superoxide radical, whereas that induced by H₂O₂ is through a hydroxyl radical. N-acetyl-l-cysteine, however, has been shown to inhibit both H₂O₂ (24) and TNF-induced NF-κB activation, perhaps because it quenches both the superoxide radical and hydroxyl radical species. A redox-independent mechanism of activation of NF-κB has also been described. For instance, interleukin-1, okadaic acid, and vanadum analogues induces NF-κB activation without involvement of ROI (27, 31–33). Our results based on Mn-SOD overexpression, however, indicate that NF-κB activation by okadaic acid requires superoxide radical. Some of these differences may be due to different cell types as demonstrated by others (26, 27) and also may be due to the lack of specificity of ROI quenchers used as indicated above.

Our results show that various chemotherapeutic agents, including taxol, doxorubicin, daunomycin, vincristine, and vinblastine activate NF-κB. These results confirm recent reports (34, 35). How any of these agents activate NF-κB is not known. Our results show that agents that act by different mechanisms induced both cytotoxicity and NF-κB activation in MCF-7 cells. Mn-SOD, however, blocked only taxol-induced cytotoxicity and NF-κB activation. These results suggest that taxol transduces its signal both for cytotoxicity and NF-κB activation through the superoxide radical, whereas anchracyclines (doxorubicin and daunomycin) and vincaloids (vincristine and vinblastine) act through a ROI-independent mechanism.

We found that Mn-SOD also blocked TNF-induced AP-1 activation. While we examined TNF-inducible activation, other investigators recently showed that overexpression of Mn-SOD abolished the constitutive AP-1 activity in growing fibrosarcoma cells (36). Our finding on the suppression of TNF-induced AP-1 by Mn-SOD was surprising in view of studies that showed that the antioxidants pyroldine dichlorohemate and N-acetyl-l-cysteine activate AP-1 by themselves (21). Since activation of AP-1 requires activation of JNK, we found that Mn-SOD also suppressed TNF-induced JNK activity. There is a recent report (37), however, that indicates that the TNF-receptor-associated kinase NIK is required for activation of NF-κB and AP-1 but not JNK, indicating that TNF-induced AP-1 activation is JNK-independent.

We also demonstrated that Mn-SOD suppresses the TNF-induced activation of caspase-3, an enzyme needed for TNF-induced apoptosis. While there are reports that Mn-SOD can block TNF-induced cytotoxicity (7, 8), ours is the first to show that it is through inhibition of caspase-3 activation. Mn-SOD blocked the cytotoxic effects not only of TNF, but also of taxol and okadaic acid, suggesting that all of these agents kill cells through a superoxide radical-dependent mechanism. Similar to these agents, H₂O₂-induced cytotoxicity was also blocked by Mn-SOD, suggesting that H₂O₂ activates cytotoxicity and NF-κB by different mechanisms. The lack of effect of Mn-SOD on cytotoxicity induced by vincristine, vinblastine, and daunomycin suggests that these agents kill the cells by a mechanism independent of the superoxide radical.

Mn-SOD also blocked TNF-induced NF-κB-dependent gene expression. Previously, it has been shown that TNF-induced interleukin-1α expression in human fibrosarcoma cells is blocked by overexpression of Mn-SOD (38). Since NF-κB activation is needed for interleukin-1 expression, it is possible that suppression of NF-κB as shown in our studies is involved in interleukin-1 inhibition. Mn-SOD has also been reported to inhibit the malignant phenotype of human tumor cells (39). Since several genes involved in tumorigenesis contain NF-κB in their promoter (20), it is possible that suppression of NF-κB by Mn-SOD is linked with its effects on tumorigenesis.

Recent studies showed that TNF-activated NF-κB plays an antiapoptotic role in TNF-induced apoptosis (40–42). How is not clear, but Mn-SOD is one of the genes that is regulated through NF-κB (43), thus suggesting a negative feedback loop. While results from our laboratory and others show that various effects of TNF are mediated through the generation of superoxide radicals, TNF is also known to induce Mn-SOD gene expression in almost all cell types (6). This shows the complexity in the mechanism of action of TNF. The susceptibility of cells to TNF-mediated killing might be influenced (besides by Mn-SOD) by catalase and glutathione peroxidase, which degrade H₂O₂ to form water. Overexpression of catalase has been shown to attenuate TNF and okadaic acid-induced NF-κB activation (44). Overall, it is quite likely that the total cellular balance of prooxidants and antioxidant intermediates determines the signaling mechanism of various responses to TNF and other agents. In the future, we will examine the other antioxidants in the Mn-SOD-overexpressing cells to determine if they are also involved in the mode of action of TNF.

REFERENCES
1. Aggarwal, B. B., and Natarajan, K. (1996) Eur. Cytokine Netw. 7, 93–124
2. Darnay, B. G., and Aggarwal, B. B. (1997) J. Leukocyte Biol. 61, 559–566
3. Goossens, V., Grooten, J., De Vos, K., and Fiers, W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8115–8119
4. Bannister, J. V., Bannister, W. H., and Rotilio, G. (1987) Crit. Rev. Biochem. 22, 111–180
5. Hassan, H. M. (1998) Free Radical Biol. Med. 5, 377–385
6. Wong, G. H. W., and Goeddel, D. V. (1989) Science 242, 941–944
7. Wong, G. H. W., Elwell, J. H., Oberley, L. W., and Goeddel, D. V. (1989) Cell 58, 923–931
8. Li, J. J., and Oberley, L. W. (1997) Cancer Res. 57, 1991–1998
9. Kari, M. (1995) J. Biol. Chem. 270, 16483–16486
10. Lee, F. S., Hagler, J., and Chen, Z. J. (1997) Cell 88, 213–222
11. Zhou, G., and Koo, M. T. (1997) J. Biol. Chem. 272, 15174–15183
12. Bergstahl, G. O. F., Parge, H. E., Hickey, M. J., Boisnault, M., Hallwell, R. A., Lepock, J. R., Cabell, D. E., and Tainer, J. A. (1996) Biochemistry 35, 4297–4297
13. Chaturvedi, M. M., Kumar, A., Darnay, B. G., Chaitny, G. B. N., Aggarwal, S., and Aggarwal, B. B. (1997) J. Biol. Chem. 272, 30129–30134
14. Reddy, S. A. G., Chaturvedi, M. M., Darnay, B. G., Chan, H., Higuchi, M., and Aggarwal, B. B. (1994) J. Biol. Chem. 269, 25369–25372
15. Totpal, K., LaiPuchin, R., Kohn, T., Darnay, B. G., and Aggarwal, B. B. (1994) J. Immunol. 153, 2248–2257
16. Hansen, M. B., Nielsen, S. E., and Berg, K. (1989) J. Immunol. Methods 118, 203–210
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[References not listed in the image]