Phorbol Ester Induces Manganese-Superoxide Dismutase in Tumor Necrosis Factor-resistant Cells*

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The effects of phorbol ester (TPA) and other known stimulators such as tumor necrosis factor (TNF), interleukin-1, and lipopolysaccharide on induction of mRNA for manganese-superoxide dismutase (Mn-SOD) were investigated in various cell lines. TPA enhanced Mn-SOD mRNA expression in TNF-resistant cell lines including HeLa cells, in which the other reagents also induced expression of the gene, but did not affect TNF-sensitive cells, in which the other stimulators did not alter expression of the gene. HeLa cells which had been desensitized to TPA by pretreatment with TPA for 24 h expressed Mn-SOD mRNA at a slightly higher level than the cells without TPA treatment. TPA-pretreated cells stimulated with TNF, however, expressed Mn-SOD mRNA at about twice the level of TNF-stimulated, TPA-untreated cells. When protein synthesis was inhibited by cycloheximide during TPA pretreatment, TNF no longer enhanced the Mn-SOD mRNA accumulation. These data suggest that at least two separate signal-transducing pathways are involved in expression of this gene. One is triggered by protein kinase C activation itself in the absence of new protein synthesis. The other can be activated by stimulation with TNF, interleukin-1, or lipopolysaccharide and in which a protein factor that can be induced by TPA treatment is involved.

Mammalian cells contain both copper, zinc- and manganese-superoxide dismutases (Cu,Zn- and Mn-SODs) to protect them against oxygen radicals (1, 2). Mn-SOD is localized in the mitochondria matrix (3) and is known to be induced by tumor necrosis factor (TNF), interleukin-1 (IL-1), and lipopolysaccharide (LPS), while Cu,Zn-SOD is localized in the cytoplasm and is constitutively expressed (4-9). It is believed that active oxygens may participate in tumor cell killing by TNF. Recently Wong et al. (5) showed that cell killing by TNF could be modulated by expression of sense and antisense Mn-SOD RNAs, indicating that Mn-SOD is a determinant of cellular resistance to TNF. However, the mechanism by which TNF enhances Mn-SOD expression in TNF-resistant cells is not yet understood.

In this paper, we show that phorbol ester (TPA), a protein kinase C activator, also enhanced Mn-SOD gene expression in TNF-resistant cells but not in TNF-sensitive cells. Possible intracellular signal-transducing mechanisms for these stimulators are discussed.

EXPERIMENTAL PROCEDURES

Materials—Human cell lines utilized in this research were HeLa cervical carcinoma cells, Jurkat, and MCAS ovarian undifferentiated carcinoma cells, and MRC5 human diploid fibroblasts. MRC5 cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in Eagle’s minimum essential medium containing 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT) supplemented with 2% L-glutamine and 1% sodium pyruvate. Other cell lines including HeLa cells, which were cultured under the same conditions except that RPMI 1640 was used instead of Eagle’s minimum essential medium. Subconfluent cells were stimulated with TNF, TPA, IL-1, or LPS in fresh medium and were incubated for an additional 4 h. Viability of the cells was measured after 22 h of incubation by the method described by Mayerl et al. (12).

Cell Culture—HeLa cells and MCAS cells were grown in 100-mm Petri dishes with Eagle’s minimum essential medium (Nihonbo Biomedical Laboratories) containing 10% or 20% fetal bovine serum (Whittaker M. A., Bioproducts, Inc., Walkersville, MD), respectively, and 200 μg/ml kanamycin sulfate (Nakarai Tesque, Inc.) in a humid atmosphere of 5% CO₂ at 37°C. ME180, Jurkat, A549, HL60, and K562 cells were cultured under the same conditions except that RPMI 1640 was used instead of Eagle’s minimum essential medium. Subconfluent cells were stimulated with TNF, TPA, IL-1, or LPS in fresh media and were incubated for an additional 4 h. Viability of the cells was measured after 22 h of incubation by the method described by Yamazaki et al. (12).

Human Cu,Zn-SOD cDNA Cloning—Two 20-mer oligodeoxyribonucleotide primers corresponding to the sequence of nucleotides 83-102 and to the complementary sequence of nucleotides 403-422 of human Cu,Zn-SOD cDNA (13) were synthesized by Mitsui Toatsu Chemicals, Inc. Complementary DNA to total RNA from human leukocytes were synthesized by reverse transcriptase from Rous-associated virus 2 (Takara Shuzo Co.). The 140-base pair cDNA encoding human Cu,Zn-SOD was amplified from this cDNA pool by polymerase chain reaction (14) in a Thermal Cycler Reactor TC-100 (Hoei Science Co.) using Taq DNA-polymerase (Perkin-Elmer Cetus). The DNA fragment was purified by electrophoresis on a 5% polyacrylamide gel and was labeled with [32P] using a Multiprimer DNA Labeling System (Amersham Corp.). Three positive clones were obtained by screening 10⁶ phages from the human liver cDNA library (Clontech Laboratories, Inc.). The cDNA inserts were subcloned into a Bluescript plasmid vector (Stratagene) and were sequenced using T7 polymerase (Pharmacia LKB Biotechnology, Inc.) and [α-32P]dCTP. These clones encoded full-length human Cu,Zn-SOD with the same sequences as reported previously (13). A 300-base pair DNA...
Northern Blotting—Total cellular RNA was prepared from the cultured cells using RNA Zol (Biotecx Laboratories, Inc.) according to Chomczynski and Sacchi (15). Seven to 20 μg of total RNA were run on 1% agarose gel containing 2.2 M formaldehyde. The size-fractionated RNA was transferred onto Zeta-Probe membrane (Bio-Rad) by capillary action. After hybridization with 32P-labeled, human Mn-SOD probe (5' EcoRI linker to PsI, Ref. 16) or the human Cu,Zn-SOD probe described above at 42 °C in the presence of 50% formamide, the membrane was washed at 55 °C in 2 x SSC (1 x SSC, 15 mM sodium citrate and 150 mM NaCl, pH 7.5) and 0.1% sodium dodecyl sulfate for 80 min. The Kodak XAR films were exposed for 1–3 days with an intensifying screen at ~80 °C. The intensities of bands on x-ray film were quantitated by gel scanner CS 9000 (Shimadzu).

All other DNA and RNA manipulations were conducted according to Maniatis et al. (17).

RESULTS

Several groups (4–6, 8) including us (7) showed previously that Mn-SOD expression but not Cu,Zn-SOD expression could be enhanced by TNF. In order to determine whether protein kinase C participates in Mn-SOD gene expression, the effect of TPA, a well-known tumor promoter which induces many genes through protein kinase C activation, on Mn-SOD and Cu,Zn-SOD mRNA expression in ME180 cells and HeLa cells was examined. As shown in Fig. 1, TPA as well as TNF dramatically enhanced Mn-SOD mRNA expression in HeLa cells but not in ME180 cells. The Cu,Zn-SOD mRNA level, however, was not changed by either of these stimulators (Fig. 2). Although both cell lines originate from human cervical carcinoma and are sensitive to TPA at high concentrations, HeLa cells are resistant to TNF while ME180 cells are sensitive to TNF-killing. Wong and Goeddel (4) suggested that the protective effect of Mn-SOD against O2− might make cells overexpressing Mn-SOD resistant to killing by TNF. As shown in Fig. 3, the accumulation of Mn-SOD mRNA reached maximum levels within 6 h after TPA or TNF treatment, sustained its high level for about 9 h, and then gradually decreased. The profiles of Mn-SOD mRNA accumulation after administration of TPA or TNF were similar. We chose 4 h for stimulation with various reagents in subsequent experiments because the mRNA induction was high enough for clear comparison and the incubation time short enough to minimize secondary effects of the stimulators on the cells.

It has been reported that Mn-SOD mRNA induction by TNF or LPS occurs independent of new protein synthesis (4–6). We therefore evaluated the effects of actinomycin D, a RNA synthesis inhibitor, and cycloheximide, a protein synthesis inhibitor, on Mn-SOD mRNA induction by TPA treatment (Fig. 4). HeLa cells constitutively express a low level of Mn-SOD mRNA which was changed little by actinomycin D treatment. However, the Mn-SOD mRNA induction during treatment with TPA was completely abolished by coincubation with actinomycin D. Actinomycin D caused a slight decrease in the Cu,Zn-SOD mRNA level both in the presence and absence of TPA. Thus the accumulation of Mn-SOD mRNA during TPA-treatment results from activation of transcription of this gene rather than from an increase in the stability of the mRNA. As Visner et al. (6) showed, cyclohex-
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The effects of IL-1, LPS, and TNP on Mn-SOD mRNA induction in various cell lines were also investigated (Table I). With respect to Mn-SOD mRNA induction, the cells could be divided into two groups: HeLa, A549, Kuramochi, and MCAS cells expressed enhanced amounts of Mn-SOD mRNA upon treatment with any of these reagents and are all TNF-resistant; ME180, HJ60, and K562 cells showed no induction by these reagents and are TNF-sensitive. Although 50 μM H8, an inhibitor of protein kinases (10), inhibited the inductions with TPA and with TNF by 60% and 80%, respectively, the same concentration of H7, a fairly specific inhibitor of protein kinase C (10), had no effect on them. Moreover, dibutylr cAMP and forskolin had no effect on Mn-SOD induction in HeLa cells (data not shown), indicating that cAMP and cAMP-dependent protein kinase are not alone responsible for this gene expression.

It has been suggested that TNF and IL-1 induced responsive gene expression through phosphorylation of enhancer-binding proteins such as NF-κB and AP-1 (18, 19) by protein kinase C. Since protein kinase C is down-regulated by persistent treatment with TPA (20), we investigated the effects of TPA and TNF on TPA-pretreated HeLa cells (Fig. 5). Following a 24-h pretreatment with 10 ng/ml TPA, further incubation with either 16 ng/ml or 30 ng/ml TPA did not enhance accumulation of Mn-SOD mRNA. In contrast, TPA pretreatment doubled the level of Mn-SOD mRNA induction by TNF (Fig. 6). A similar enhancement was seen when TPA-pretreated cells were stimulated with IL-1 or LPS (data not shown). Although cycloheximide itself induced accumulation of Mn-SOD mRNA, neither TPA nor TNF altered the level of mRNA in cells pretreated with both 10 ng/ml TPA and 50 μM cycloheximide. These data indicate that protein kinase C activation can enhance Mn-SOD gene transcription in the absence of protein synthesis, but another protein factor which mediates the stimulation by TNF to the Mn-SOD gene can be synthesized during TPA pretreatment.

**DISCUSSION**

Although mature Mn-SOD protein is located at the mitochondrial matrix, the Mn-SOD gene is encoded by nuclear DNA and its expression exhibits the characteristics of other nuclear genes. TNF (4, 8), IL-1 (4, 6, 9), and LPS (6) have been reported to enhance Mn-SOD expression in some cell lines, but there is almost no description of how these stimulants induce expression of the gene.

Here we showed that TPA, a well-known tumor promoter and a protein kinase C activator (20), also enhanced Mn-SOD mRNA expression in TNF-resistant cells. Although Wong and Goeddel (4) showed no Mn-SOD induction in lung carcinoma-derived A549 cells by either TPA or LPS, both of these reagents induced Mn-SOD expression in several cell lines including A549 cells in our experimental system (Table I). This discrepancy could be explained by desensitization of protein kinase C (20) in the former experiment. In our hands, A549 cells were less reactive to TPA than to TNF or IL-1 (Table I), and prolonged incubation of the cells with a high concentration of TPA desensitizes protein kinase C to TPA.

TPA-induced accumulation of Mn-SOD mRNA is the consequence of activation of transcription of the gene since actinomycin D treatment completely abolished the mRNA accumulation (Fig. 4). This transcriptional activation does not require new protein synthesis, in similarity to gene activation by TNF, IL-1, and LPS (4–6). TPA is known to produce active oxygen and induce genes such as heme oxygenase (21) in a certain cells. Induction of the Mn-SOD gene does not appear to occur by this mechanism, however, because other active oxygen-producing agents such as H2O2, menadione, and methylviologen did not induce Mn-SOD (data not shown).

TNF, IL-1, and LPS first bind to receptors at the cell surface and then produce intracellular messengers to transduce the signals to genes in the nucleus. TPA, on the other hand, directly binds and activates protein kinase C so that induction by TPA is independent of membrane receptors. All four reagents induced Mn-SOD expression in TNF-resistant cells but were unable to induce it in TNF-sensitive cells (Table I). This is not likely due to defective expression of TNP...
Yielded essentially the same result are shown.

Protein kinase C resulting in phosphorylation of some target either 4 h induction by TPA and TNF.

As shown in Fig. 5, however, while TPA-pretreated receptors such as diacylglycerol (24) which might have been generated by treatment with TPA, additional mRNA accumulation was seen in the TPA-pretreated cells in response to TNF (Fig. 6). Indeed TPA-pretreatment doubled the level of induction of Mn-SOD mRNA by TNF. Similar enhancement of Mn-SOD mRNA accumulation was seen in TPA-pretreated cells after stimulation with IL-1 and LPS (data not shown). One possible explanation is that protein kinase C, which was desensitized to TPA, could still be activated by other mediators such as diacylglycerol (24) which might have been generated by treatment with TNF or other stimulators. There is evidence, however, that calpain I cleaves protein kinase C in the presence of TPA, resulting in depletion of the enzyme from the cell (20). An alternative possibility is that the mediator of the signal from TPA directly activates the protein which is the substrate for protein kinase C. One likely candidate for this substrate is nuclear factor \( \kappa B \) (NF-\( \kappa B \)). HeLa cells have an inactive form NF-\( \kappa B \) (25) which can be activated by TPA as well as by TNF, IL-1, and LPS (18, 19, 26). Mn-SOD gene expression might also be regulated by NF-\( \kappa B \) through protein kinase C-dependent phosphorylation, and thereby inactivation of inhibitory protein I\( \kappa B \).

Yet another possibility is that protein kinase C-dependent phosphorylation of AP-1 (28) is responsible for Mn-SOD gene

### Table I

**Relative stimulation of Mn-SOD mRNA expression by TPA, TNF, IL-1, and LPS in various cell lines**

Total RNA was prepared from various cells treated with 10 ng/ml TPA, 100 ng/ml TNF, 1000 units/ml IL-1, or 10 \( \mu g/ml \) LPS for 4 h. The amount of Mn-SOD mRNA was evaluated by scanning x-ray film exposed to Northern blot membrane filters. The mRNA levels relative to the control are presented as the means \( \pm \) S.D. for three experiments.

| Cell line       | Control       | TPA \( \beta \) | TNFa | IL-1\( \beta \) | LPS  |
|-----------------|---------------|----------------|------|----------------|------|
| HeLa*           | 1.0 \( \pm \) 0.1 | 18.7 \( \pm \) 1.0 | 7.8 \( \pm \) 2.5 | 8.1 \( \pm \) 1.2 | 3.0 \( \pm \) 0.7 |
| A549*           | 1.0 \( \pm \) 0.2 | 9.0 \( \pm \) 1.2 | 18.5 \( \pm \) 1.4 | 25.3 \( \pm \) 3.8 | 1.0 \( \pm \) 0.1 |
| Kuramochi*      | 1.0 \( \pm \) 0.1 | 6.7 \( \pm \) 0.1 | 2.7 \( \pm \) 0.5 | 19.8 \( \pm \) 2.7 | 1.4 \( \pm \) 0.1 |
| MCAS*           | 1.0 \( \pm \) 0.1 | 11.2 \( \pm \) 1.3 | 1.4 \( \pm \) 0.8 | 6.0 \( \pm \) 1.4 | 16.5 \( \pm \) 0.7 |
| ME180*          | 1.0 \( \pm \) 0.3 | 0.2 \( \pm \) 0.1 | 0.8 \( \pm \) 0.4 | 1.3 \( \pm \) 0.2 | 0.4 \( \pm \) 0.2 |
| HL60*           | 1.0 \( \pm \) 0.2 | 0.4 \( \pm \) 0.1 | 0.6 \( \pm \) 0.1 | 0.7 \( \pm \) 0.1 | 0.6 \( \pm \) 0.4 |
| K562*           | 1.0 \( \pm \) 0.1 | 0.9 \( \pm \) 0.1 | 1.0 \( \pm \) 0.3 | 1.3 \( \pm \) 0.1 | 1.0 \( \pm \) 0.1 |

*TNF-resistant cell.
*TNF-sensitive cell.

### Figure 5

**Effect of TPA pretreatment on Mn-SOD mRNA induction by TPA and TNF.** HeLa cells were incubated under control conditions (lanes 1, 3, 5, and 7) or treated with 10 ng/ml TPA for either 4 h (panel A) or 24 h (panel B) (lanes 2, 4, 6, and 8). Total RNA was prepared from the cells after further incubation with medium only (lanes 1 and 2), 10 ng/ml TPA (lanes 3 and 4), 30 ng/ml TPA (lanes 5 and 6), or 100 ng/ml TNF (lanes 7 and 8) for 4 h. Northern blot analysis of the RNA was carried out using \( 32P \)-labeled Mn-SOD probe. Data from one of the duplicate experiments which yielded essentially the same result are shown.

### Figure 6

**Quantitative representation of Mn-SOD mRNA levels induced by TPA and TNF after 24-h TPA-pretreatment.** The mean units of Mn-SOD mRNA relative to control are shown for the four experiments identical in design to those described in Fig. 5. HeLa cells were stimulated with medium only (columns 1-3), 10 ng/ml TPA (columns 4-6), or 100 ng/ml TNF (columns 7-9) after incubation with medium only (columns 1, 4, and 7), 10 ng/ml TPA (columns 2, 5, and 8), or 10 ng/ml TPA and 50 \( \mu M \) cycloheximide (CHX, columns 3, 6, and 9) for 24 h before stimulation.
expression because Ho et al. (27) found the consensus sequence for AP-1 enhancer-binding protein in the 5′-flanking region of the rat Mn-SOD gene. TPA acts both to activate AP-1 protein through protein kinase C and to enhance protooncogene Jun/AP-1 expression (29), it is conceivable that the AP-1 synthesized during TPA pretreatment enhances Mn-SOD expression after stimulation with TNF to a level above that seen upon TNF stimulation of untreated cells.

Recent reports suggested that several pathways may exist for gene activation by TNF and that the depletion of protein kinase C can be fully compensated for by pathways in which protein kinase C is not involved (24, 30). Our data show that stimulators which activate protein kinase C also enhance synthesis of Mn-SOD mRNA in certain cells but that protein kinase C activation may not be prerequisite for this gene induction by TNF as shown in Fig. 6. In vitro analysis of the regulation of the Mn-SOD gene will likely be necessary to determine the precise mechanism.

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