Manganese-superoxide dismutase in endothelial cells: localization and mechanism of induction

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Suzuki, Keiichiro, Haruyuki Tatsumi, Shoji Satoh, Takao Senda, Toshiyuki Nakata, Junichi Fujii, and Naoyuki Taniguchi. Manganese-superoxide dismutase in endothelial cells: localization and mechanism of induction *Am. J. Physiol.* 265 (*Heart Circ. Physiol.* 34): H1173–H1178, 1993.—Mechanisms of Mn-superoxide dismutase (Mn-SOD) expression in human umbilical endothelial cells were investigated by Northern blot analysis, enzyme-linked immunosorbent assay, and immunoelectron microscopy. The Mn-SOD in human endothelial cells was markedly induced by the cytokines tumor necrosis factor (TNF), interleukin-1, and lipopolysaccharide as well as by phorbol esters [12-*O*-tetradecanoylphorbol 13-acetate (TPA)]. The induction was partially blocked by dexamethasone and 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine, a potent inhibitor of protein kinase C (PKC). In endothelial cells in which PKC had been desensitized to TPA by pretreatment for 24 h, addition of TNF caused overexpression of Mn-SOD. These facts suggested that at least two separate signal-transducing pathways are involved in expression of the Mn-SOD gene. Immunoelectron-microscopic studies showed that Mn-SOD was localized to the mitochondrial matrix of the capillary vascular endothelial cells of cardiac tissues and cultured endothelial cells. Mn-SOD, which is normally abundant in endothelial cells relative to other cell types, may play an important protective role against stresses such as ischemia and inflammation.

tumor necrosis factor; interleukin-1; dexamethasone; 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; enzyme-linked immunosorbent assay; immunoelectron microscopy; protein kinase C

MUCH INTEREST has developed in the role of superoxide dismutase (SOD) in modifying the toxic effects of superoxide anions. Mammalian cells contain the copper, zinc and manganese forms of the enzyme (Cu,Zn-SOD and Mn-SOD) (15, 27). Mn-SOD, which is localized in the mitochondrial matrix, is known to be induced by tumor necrosis factor (TNF), interleukin (IL)-1, and lipopolysaccharide (LPS) (10, 13, 26, 29). Wong et al. (28, 30) have used expression of sense and anti-sense Mn-SOD RNAs to show that Mn-SOD is one of the most important factors in combating the cytotoxicity of TNF, radiation, and hyperthermic effects in cancer cell lines. We have recently reported that phorbol esters induce Mn-SOD in TNF-resistant cancer cells and that at least two separate signal-transducing pathways are involved in expression of the Mn-SOD gene (3).

In a previous study we found that serum levels of Mn-SOD in patients with acute myocardial infarction were elevated following elevation of TNF-α in the serum (23). This study suggested that Mn-SOD was induced by cytokines secreted from leukocytes and macrophages infiltrating the infarcted myocardium. Mn-SOD levels are also elevated in the serum of patients with other diseases such as leukemia (16), primary biliary cirrhosis (18), ovarian cancer (6), and neuroblastoma (11). In these pathogenic conditions, the Mn-SOD may be induced in the tissues by cytokines and released into the serum. Our preliminary studies have indicated that endothelial cells have a strong potential for induction of Mn-SOD. Endothelial cells play an important role in separating tissues from blood and are considered to inhibit oxidative stresses, ischemia, atherosclerosis, and inflammation. Because endothelial cells are in direct contact with the blood, they are likely play a role in serum Mn-SOD elevations.

The aim of the present study was to investigate the mechanisms by which Mn-SOD is expressed by cytokines in endothelial cells and released into the bloodstream in patients with acute myocardial infarction. Increases in Mn-SOD protein or mRNA levels after treatment with various cytokines or endotoxins have been observed in cultured bovine pulmonary artery endothelial cells (21), porcine pulmonary artery endothelial cells (25), and bovine and mouse endothelial cells (20). Changes in Cu,Zn-SOD levels in bovine pulmonary artery endothelial cells have also been reported (12), but the mechanisms of Mn-SOD induction in endothelial cells have remained unclear. In this paper, Mn-SOD induction in human umbilical endothelial cells was evaluated by Northern blot analysis, enzyme-linked immunosorbent assay (ELISA), and immunoelectron microscopy. We will show that overexpression of Mn-SOD in endothelial cells is induced by various biofactors and that this may relate to the release of the enzyme into the bloodstream.

MATERIALS AND METHODS

Materials. 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and *Escherichia coli* LPS were obtained from Sigma. The protein kinase inhibitor 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) was obtained from Seikagaku Kogyo. Recombinant human TNF-α (sp act 20×10^6 U/mg protein) and IL-1α (sp act 2×10^7 U/mg protein) were obtained from Ube Industries and Otsuka Pharmaceutical, respectively. Human Mn-SOD cDNA was a kind gift from Mitsui Toatsu Chemicals. [α-^{32}P]dCTP was obtained from Amersham. Dispase, a neutral protease from *Bacillus polymyxa*, was purchased from Godo-shusei. Other reagents were of the highest analytical grade available.

Endothelial cells. Human endothelial cells were isolated from human umbilical veins by the method of Jaffe et al. (7), with the exception that dispase was employed instead of trypsin. Endothelial cells were grown at 37°C in 100-mm Petri dishes with modified MCDB131 medium containing fetal bovine serum (2%), recombinant human epidermal growth factor (10 ng/ml), hydrocortisone (1 μg/ml), gentamicin sulfate (50 μg/ml), amphotericin B (0.25 μg/ml), and bovine brain extracts (0.4%) in a humid atmosphere containing 5% CO_2.

Subconfluent cells were stimulated with TNF-α, TPA, IL-1α, or LPS in fresh medium and incubated for an additional 4 h for

Northern blotting and 24 or 48 h for ELISA. Small samples of human cardiac tissues were obtained from the left ventricles of five patients during repair of mitral valve stenosis and two patients with ventricular septal defects. These patients demonstrated no left ventricular hypertrophy, and the myocardial tissues taken were found to be normal.

Immunohistochemical methods. After incubation of endothelial cells with TNF-α for 24 h, the medium was removed and each dish was washed with phosphate-buffered saline (PBS). A solution of 0.025% trypsin and 0.02% EDTA in PBS was added, and the cells were incubated for 5–10 min before being removed by centrifugation at 3,000 rpm for 5 min.

Specimens of cardiac tissue were fixed for 20 min with 4% paraformaldehyde, and endothelial cells were frozen by pressing them rapidly against a copper block cooled to 4°K with liquid helium. After this, the blocks were freeze substituted at −80°C for 3 days in a vial containing 0.3% glutaraldehyde in acetone followed by incubation for 2 h each at −20 and 4°C and room temperature (19). After being rinsed with acetone five times, the blocks were infiltrated with a mixture of Lowicryl solution and ethanol (1:1) at −20°C for 2 h and then with pure Lowicryl solution overnight. The blocks embedded in the above solution were polymerized at −20°C with ultraviolet light. Ultrathin sections mounted on nickel grids were incubated in 0.1% bovine serum albumin dissolved in pH 7.4 PBS for 1 h at room temperature and then treated for 1 h with a polyclonal rabbit immunoglobulin (Ig) G antibody to Mn-SOD diluted 1:100 or 1:300 with PBS. The sections were washed with PBS and then treated for 1 h with colloidal gold-conjugated goat anti-rabbit IgG (1:100; AuroProbe EM GAR G10, Janssen Life Sciences). All procedures were carried out at room temperature. After being washed with distilled water and dried, the sections were stained with 3% uranyl acetate solution and Reynold's lead citrate for 3 and 1 min, respectively.

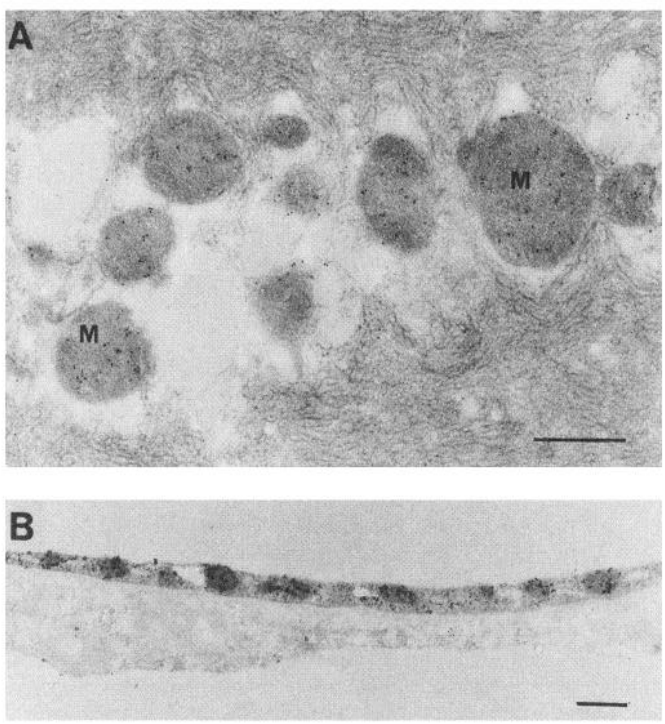

Fig. 1. Immunoelectron microscopy of cardiac muscle and endothelial cells treated with anti-Mn-superoxide dismutase (SOD) immunoglobulin (Ig) G. *A*: immunoelectron microscopy of cardiac muscle treated with anti-Mn-SOD IgG. Myofibrils contain many mitochondria upon which immunogold is deposited. *B*: immunoelectron microscopy of endothelial cells of blood capillaries in cardiac tissue treated with anti-Mn-SOD IgG. Immunogold is seen on mitochondria. Bars = 500 nm. M, mitochondrion.

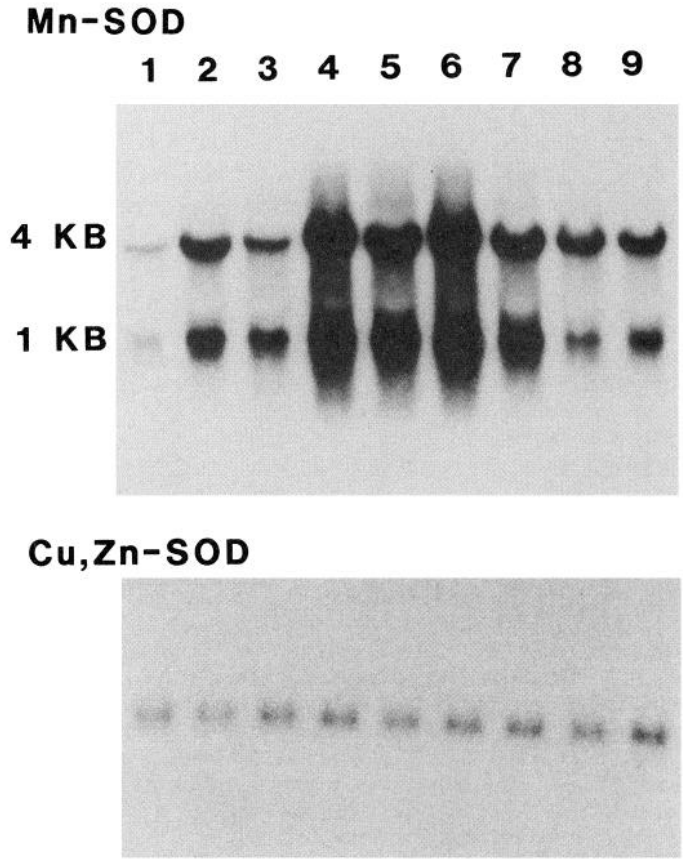

Fig. 2. Effect of 12-*O*-tetradecanoylphorbol 13-acetate (TPA), tumor necrosis factor (TNF)-α, interleukin (IL)-1α, and lipopolysaccharide (LPS) on Mn-SOD and Cu,Zn-SOD mRNA expression in endothelial cells. Endothelial cells were incubated for 4 h under control conditions (*lane 1*), with 100 ng/ml TPA (*lanes 2* and *3*), with 10 ng/ml TNF-α (*lanes 4* and *5*), with 10 ng/ml IL-1α (*lanes 6* and *7*), or with 10 ng/ml LPS (*lanes 8* and *9*). *Lanes 3*, *5*, *7*, and *9* contained samples also incubated with 10 μmol of H-7. Northern blotting was then carried out using a human Mn-SOD probe (*top*) or a human Cu,Zn-SOD probe (*bottom*).

Northern blotting. Total cellular RNA was prepared from the cultured cells using RNA Zol (Biotecx Laboratories). RNA (20 μg) was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde. The size-fractionated RNA was transferred onto a Zeta-Probe membrane (Bio-Rad) by capillary action. After hybridization with a ^{32}P-labeled human Mn-SOD probe or a human Cu,Zn-SOD probe (3) at 42°C in the presence of 50% formamide, the membrane was washed at 55°C in 2× SSC (1× SSC: 150 mM NaCl and 15 mM sodium citrate, pH 7.5) and 0.1% sodium dodecyl sulfate for 80 min. The Kodak XAR films were exposed for 1 day with an intensifying screen at −80°C. The mRNA experiments were repeated at least three times.

ELISA for Mn-SOD and Cu,Zn-SOD. After incubation with a stimulant, the medium was removed and each dish of cultured endothelial cells was washed with PBS. One milliliter of PBS containing 1 mM EDTA was added to each dish, and the endothelial cells were removed with a rubber policeman and pooled. After sonication, the Mn-SOD and Cu,Zn-SOD contents of each sample were measured.

The Mn-SOD level was determined using an ELISA method previously described (8, 9). For the Cu,Zn-SOD measurement, we employed a Cu,Zn-SOD ELISA kit, which was kindly sup-

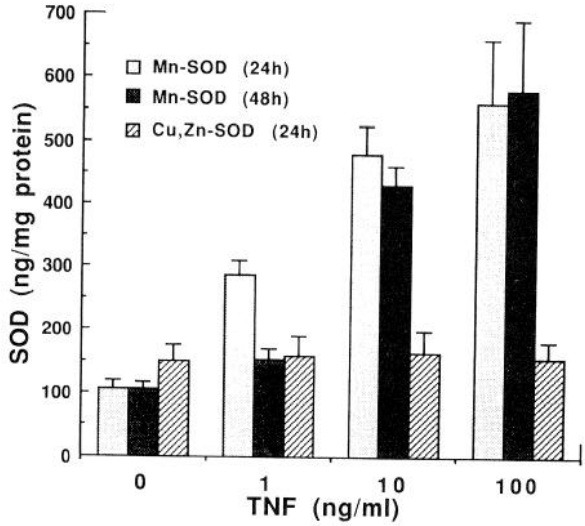

Fig. 3. Induction of Mn-SOD and Cu,Zn-SOD proteins by TNF-α. Endothelial cells were incubated under control conditions or with TNF-α (1, 10, and 100 ng/ml) for 24 or 48 h. Mn-SOD and Cu,Zn-SOD levels were measured by specific enzyme-linked immunosorbent assay (ELISA) methods using monoclonal antibodies. Mn-SOD levels of TNF-α-treated cells were significantly higher than that of control group. Means ± SD; $n = 5$.

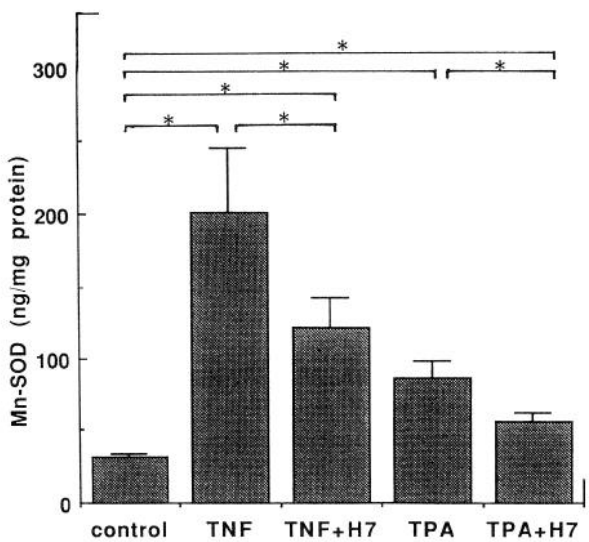

Fig. 4. Effect of H-7 on induction of Mn-SOD by TNF-α and TPA. Endothelial cells were incubated under control conditions or with TNF-α (10 ng/ml), TNF-α and H-7 (10 μM), TPA (100 ng/ml), or TPA and H-7 (10 μM) for 24 h. Mn-SOD protein levels were measured by a specific ELISA. Means ± SD; $n = 5$. * Significant difference between each pair.

plied by Ube Industries (17). Protein was determined with a BCA assay kit (Pierce).

Statistical analysis. The difference between two means was tested by an unpaired two-tailed t test. For comparison of more than two groups, a one-way analysis of variance was performed. $P < 0.05$ was considered significant.

RESULTS

Immunohistochemical studies of cardiac tissues and blood capillaries. Immunoelectron microscopy revealed that immunogold was specifically deposited on the mitochondria among the myofibrils as well as on the mitochondria beneath the sarcoplasmic membrane (Fig. 1*A*). Some immunogold was also seen on the secondary lysosomes and on lipofuscin, which contained heterogeneous substances and appeared in irregular shapes.

The mitochondria of the endothelial cells of blood capillaries were more reactive with the antibody employed than those of heart muscle (Fig. 1*B*). It was anticipated that the endothelial cells would contain more Mn-SOD than the cardiac muscle tissues, and this relationship was found in all the tissue specimens taken.

Mn-SOD induction in cultured human endothelial cells. Several groups including our own have reported that TNF induced synthesis of Mn-SOD but not of Cu,Zn-SOD in

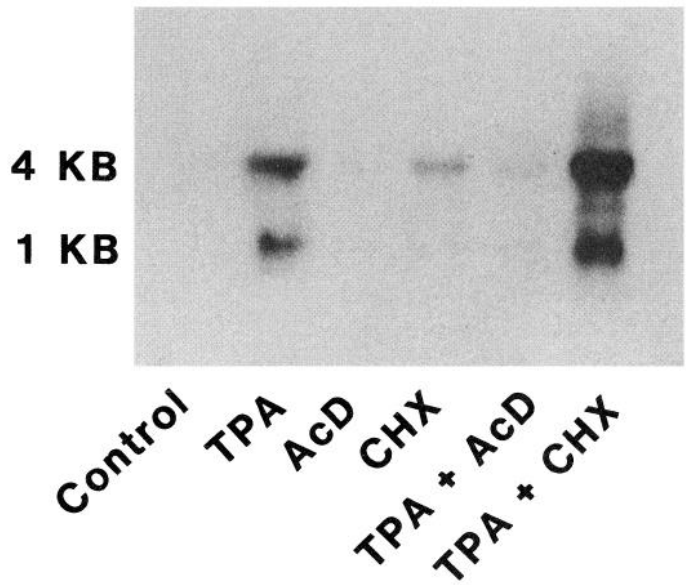

Fig. 5. Effect of actinomycin D (AcD) and cyclohexamide (CHX) on Mn-SOD mRNA induction by TPA. Endothelial cells were incubated under control conditions for 4 h with 100 ng/ml TPA, 4 μM AcD, 50 μM CHX, or combinations indicated.

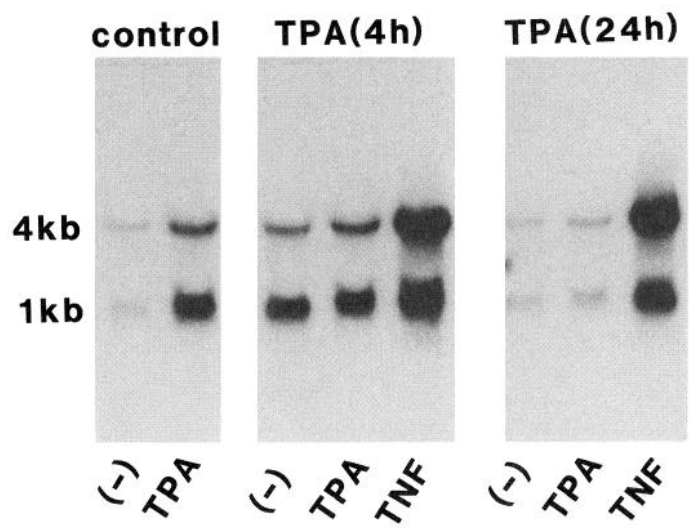

Fig. 6. Effect of TPA pretreatment on Mn-SOD mRNA induction by TPA and TNF. Endothelial cells were incubated under control conditions or treated with 100 ng/ml TPA for 4 or 24 h. Total RNA was prepared after further incubation with medium only (−), 100 ng/ml TPA, or 10 ng/ml TNF-α. Northern blot analysis was carried out using a human Mn-SOD probe.

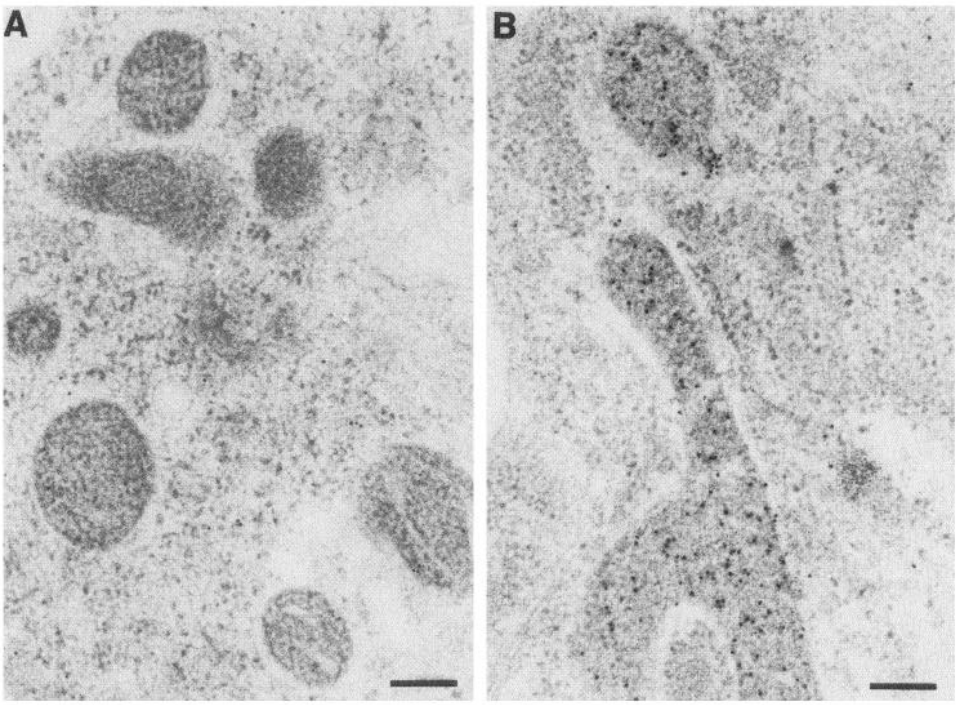

Fig. 7. Immunoelectron microscopy of endothelial cells incubated with TNF-α. Immunoelectron microscopy of endothelial cells treated with anti-human Mn-SOD IgG. *A*: cultured under control conditions; *B*: cultured with TNF-α (10 ng/ml) for 48 h. Bars = 200 nm.

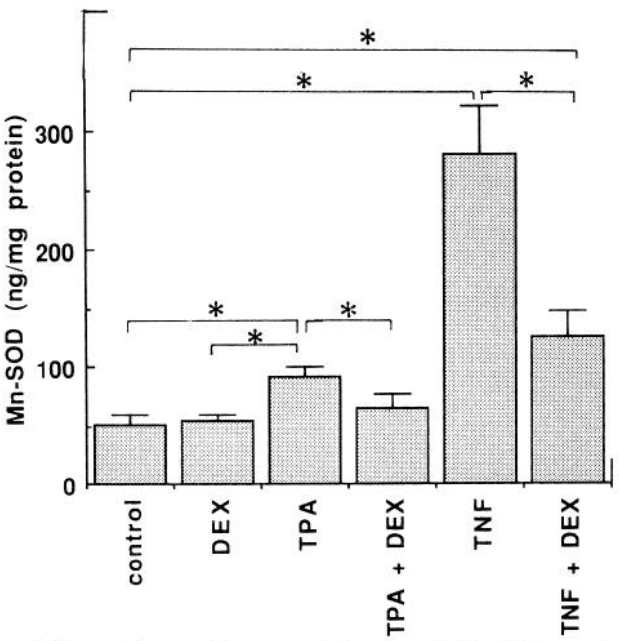

Fig. 8. Effect of dexamethasone on induction of Mn-SOD protein by TPA and TNF. Endothelial cells were incubated for 24 h under control conditions or with 10 μm dexamethasone (Dex), 10 ng/ml TNF, 100 ng/ml TPA, both TNF and Dex, or both TPA and Dex. Mn-SOD was measured by an ELISA method using a monoclonal antibody. * Significant difference between each pair.

TNF-resistant cancer cell lines. We have recently reported that TPA induced Mn-SOD mRNA and that two different induction mechanisms, one of which involves protein kinase C (PKC), could exist in TNF-resistant cancer cells (3).

Figure 2 shows Northern blot analysis of Mn-SOD mRNA from cultured human endothelial cells treated with several cytokines. TPA, TNF-α, IL-1α, and LPS induced both the 1- and 4-kb mRNAs of Mn-SOD. H-7, a potent PKC inhibitor, partially blocked Mn-SOD induction by TPA, TNF-α, and IL-1α, but the effect of H-7 on Mn-SOD induction by LPS was not as clear as its effect with the other cytokines. On the other hand, none of these treatments significantly altered the mRNA levels of Cu,Zn-SOD.

Figure 3 shows protein levels of Mn-SOD and Cu,Zn-SOD in endothelial cells after stimulation with TNF. The level of Mn-SOD was increased by TNF in a dose-dependent manner over fivefold. No increase of Cu,Zn-SOD was observed. As shown by Fig. 4, H-7 also partially blocked induction of Mn-SOD protein by both TNF-α and TPA.

Figure 5 shows the effects of actinomycin D, an inhibitor of RNA synthesis, and cycloheximide, an inhibitor of protein synthesis, on TPA-stimulated Mn-SOD induction in endothelial cells. Mn-SOD mRNA induction by TPA was completely abolished by actinomycin D. On the other hand, superinduction by TPA occurred in the presence of cycloheximide. These data indicate that Mn-SOD mRNA induction by TPA in endothelial cells results from the transcriptional activation of this gene rather than from stabilization of the mRNA and that Mn-SOD mRNA induction by TPA is independent of new protein synthesis.

PKC is known to be desensitized by TPA. As in shown in Fig. 6, we investigated the effects of TPA pretreatment on Mn-SOD induction by TPA and TNF-α in endothelial cells. After a 24-h pretreatment with TPA, further incubation with TPA did not enhance accumulation of Mn-SOD mRNA. In contrast, TPA pretreatment enhanced the induction of Mn-SOD mRNA by TNF-α.

Immunohistochemistry of cultured endothelial cells. Figure 7 shows the immunohistochemistry of Mn-SOD in

cultured human endothelial cells using rapid-freezing and freeze-substitution techniques. Figure 7*A* shows the control section (no stimulation); Fig. 7*B* shows the localization of Mn-SOD after stimulation with TNF-α (48 h, 10 ng/ml). These immunoelectron micrographs revealed that immunogold was deposited only on mitochondria and that over a fivefold increase in Mn-SOD occurred after stimulation with TNF-α.

Effect of dexamethasone on Mn-SOD induction. The effect of dexamethasone (10 μM) on Mn-SOD induction by TNF-α and TPA is shown by the data in Fig. 8. Dexamethasone alone did not change the level of Mn-SOD in endothelial cells but did partially inhibit the induction of Mn-SOD by TNF-α and TPA.

DISCUSSION

Our studies have demonstrated mechanisms of Mn-SOD induction in normal human cells as well as the localization of the induced Mn-SOD. The cytokines TNF-α, IL-1α, TPA, and LPS induced Mn-SOD both at the mRNA and protein levels. The Mn-SOD protein levels in endothelial cells stimulated by TNF-α were five- to sixfold higher than those in unstimulated cells. Such an increase is comparable to that found in TNF-resistant cancer cell lines (10). In fact, Fig. 1 shows that unstimulated endothelial cells of human cardiac tissue contained much higher levels of Mn-SOD than myocytes did. This points to a role for such cells in Mn-SOD-linked activities.

PKC activation can enhance Mn-SOD gene transcription. Endothelial cells in which PKC had been desensitized by pretreatment with TPA did not accumulate Mn-SOD mRNA in response to additional incubation with TPA. As shown in Fig. 6, however, TPA-pretreatment doubled the expression of Mn-SOD message with additional incubation with TNF-α. H-7, a PKC inhibitor, partially blocked Mn-SOD mRNA induction by TPA, TNF-α, and IL-1α but had little effect on the induction by LPS. These facts indicate that Mn-SOD is induced by at least two different mechanisms, one of them through the activation of PKC. TNF-α and IL-1α appear to stimulate both pathways.

Induction of Mn-SOD by TPA is a consequence of transcriptional activation of the gene; actinomycin D completely abolished the mRNA accumulation (Fig. 5). Mn-SOD induction by TPA does not appear to require synthesis of new protein. Indeed cycloheximide treatment seemed to enhance the induction.

Dexamethasone, a strong anti-inflammatory drug, also partially inhibited Mn-SOD induction by TNF-α. The reason for the effect of dexamethasone on Mn-SOD induction by cytokines requires clarification. It is well known that dexamethasone inhibits TNF-mediated cytolysis and, moreover, that glucocorticoids may affect the signal transduction pathway in the induction of Mn-SOD by TNF-α or other cytokines (1, 24). One possible mechanism would be competition for the TPA-responsive element, the AP-1 site, which has been identified in the Mn-SOD promotor region (5). In rat hepatocytes, however, dexamethasone has been reported to induce Mn-SOD mRNA (2). Further study is required to clarify how Mn-SOD induction is blocked by dexamethasone in endothelial cells.

Several explanations may be advanced to explain why Mn-SOD is overexpressed in endothelial cells in response to treatment with TNF-α, IL-1α, or TPA. One is that endothelial cells may produce a large amount of reactive oxygen species via xanthine oxidase. Human endothelial cells have, however, been reported to contain less xanthine oxidase than the endothelial cells of other species (14). Another is that Mn-SOD plays a protective role against various stresses. Mn-SOD, which is located in mitochondria, scavenges superoxide anions. In cancer cell lines, the enzyme plays an important role in protecting cells from radiation, chemotherapy, and hyperthemic effects (28, 30). Endothelial cells are positioned between tissues and blood and are stressed by factors such as ischemia, inflammation, flow shear, and atherosclerosis-induced tissue modifications. Activation of PKC under ischemic conditions has been reported (22). This could lead to the induction of Mn-SOD in endothelial cells. Many reactive oxygen species may be produced under such conditions of stress. Mn-SOD will scavenge these toxic entities and thereby protect the mitochondria and the endothelial cells.

Very recently Hiraishi et al. (4) reported that cellular Cu,Zn-SOD plays a critical role in preventing hydrogen peroxide damage by scavenging superoxide and consequently inhibiting the generation of the toxic species resulting from the so-called "Haber-Weiss reaction." In the current study, however, Cu,Zn-SOD levels in human endothelial cells showed little or no response to cytokine administration, whereas Mn-SOD levels increased dramatically. Mn-SOD appear to protect endothelial cells under various pathogenic conditions by preventing mitochondrial damage.

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