Inducible Expression of Manganese Superoxide Dismutase by Phorbol 12-Myristate 13-Acetate Is Mediated by Sp1 in Endothelial Cells

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Abstract—The expression of manganese superoxide dismutase (Mn-SOD), an important component of the cellular defense system against oxidative stress, is induced in response to a variety of stimuli, including cytokines and phorbol esters, in endothelial cells. To define the molecular mechanisms regulating the expression of Mn-SOD, we have characterized the promoter of the human Mn-SOD gene. In calf pulmonary artery endothelial cells, phorbol 12-myristate 13-acetate (PMA) gradually increased Mn-SOD mRNA levels, with a peak at 6 to 12 hours after stimulation. The increase in Mn-SOD mRNA was significantly inhibited by a protein kinase C (PKC) inhibitor (calphostin C) but not by a mitogen-activated protein kinase kinase-1 inhibitor (PD98059) or a p38 mitogen-activated protein kinase inhibitor (SB203580). By reporter gene transfection experiments of a series of promoter deletions and site-directed mutation constructs, we found 2 consensus Sp1 binding sequences located at -97 and at -77 to play an important role in PMA-induced Mn-SOD transcription. Electrophoretic gel mobility shift assays have indicated that this sequence serves as an Sp1 binding site. Northern and Western blot analysis has revealed that PMA-induced promoter activity of Mn-SOD correlates with an increased expression of Sp1. Nuclear proteins from PMA-treated calf pulmonary artery endothelial cells displayed an increased DNA binding to the Sp1 site. Furthermore, the Mn-SOD promoter was activated either by overexpression of Sp1 or the constitutively activated form of PKCβ in an Sp1 site–dependent manner. These results suggest that PMA stimulates transcription of the Mn-SOD gene through an increase in Sp1 expression and thus implicate Sp1 as an effector mediating the PKC-signaling pathway elicited by extracellular signals. (Arterioscler Thromb Vasc Biol. 2000;20:392-401.)

Key Words: manganese superoxide dismutase ▪ protein kinase C ▪ Sp1 ▪ endothelial cells

Superoxide dismutase (SOD) is an antioxidative enzyme that specifically scavenges superoxide radicals. Three isoenzymes, cytosolic copper/zinc SOD (Cu/Zn-SOD), mitochondrial manganese SOD (Mn-SOD), and an extracellular SOD, are distinguished on the basis of separation by physiochemical methods, subcellular distributions, and enzymatic properties. Mn-SOD is unique among the members of the SOD family of antioxidant enzymes in that Mn-SOD expression is induced by a variety of stimuli, including proinflammatory cytokines, growth factors, UV irradiation, and phorbol esters. Mn-SOD functions as a primary antioxidative enzyme that removes superoxide radicals in mitochondria. Because several lines of evidence suggest that oxidative stress is an early and critical event leading to cell proliferation or cell death, the identification of the factors regulating the expression of Mn-SOD may be among the key approaches that will elucidate the biochemical pathway for cellular response to oxidative stress under various conditions, including cancer, cardiovascular disease, and aging.

The importance of oxidative stress in the development of vascular disease has been underscored by the fact that many conditions known to generate oxidative stress influence the development of atherosclerosis, and antioxidant therapy appears to delay the development of atherosclerosis. In addition, reactive oxygen species play an important role in the signal transduction pathways by which proinflammatory cytokines and other reactive oxygen species–generating agents induce endothelial activation and smooth muscle cell proliferation. Takahashi and Berk have demonstrated that hydrogen peroxide activates Src kinase and mitogen-activated protein (MAP) kinase and accelerates the proliferation of endothelial cells.

It has become clear that the integrity of endothelial function is a prerequisite for preventing atherosclerosis. Because endothelium is constantly exposed to humoral factors, inflammatory mediators, and physical forces, endothelial cells participate actively and directly in maintaining the homeostatic balance by producing vasoactive substances, coagulants, inflammatory mediators, and growth promoters and inhibitors. Increasing numbers of studies have implicated endothelial activation of protein kinase C (PKC) in the pathogenesis of atherosclerosis.
13-acetate (PMA), a well-known tumor-promoting phorbolester that activates PKC and a number of protein kinases, activates endothelial cells by enhancing morphological changes and cell motility as well as by inducing the expression of many genes whose function is implicated in cellular proliferation, coagulation protease cascade, endothelial permeability, and inflammation.21–23 Thus, PMA elicits the genetic response similar to or partly identical to the response induced by pathophysiological stimuli, including humoral factors and mechanical stress in vivo.

Among many genes whose expression levels are elevated in activated endothelial cells are genes for antioxidant enzymes, including Mn-SOD.24 The human Mn-SOD gene has been isolated, and its 5’-flanking region has been structurally characterized.25,26 Sequence analysis revealed that the Mn-SOD gene contains neither the TATA box nor the CAAT box in the promoter region. As with many TATA-less genes, the 5’-flanking region contains many GC residues and potential Sp1 binding sites.26 However, the molecular mechanisms underlying the inducible expression of this gene in response to extracellular stimuli have remained underdetermined.

In the present study, we examined the molecular mechanisms of the PMA-induced expression of the Mn-SOD gene in endothelial cells. Deletion or site-specific mutation analysis and electrophoretic mobility shift assays (EMSA) have demonstrated that binding sites for Sp1 are found to be necessary for both uninduced and PMA-induced expression of the Mn-SOD gene. Sp1 mRNA and its protein expression are induced by PMA in endothelial cells. In addition, exogenous Sp1 enhances transcription of the Mn-SOD promoter in an Sp1 site–specific manner. Because Sp1 sites generally exist within the promoter region of a variety of genes whose expression is increased in activated endothelial genes, the findings in the present study support the hypothesis that Sp1 plays a role in inducing transcription in response to the activation of endothelial cells.

Methods

Materials

The calf pulmonary artery endothelial (CPAE) cell line was obtained from the American Type Culture Collection. PMA, tumor necrosis factor-α (TNF-α), calf thymus, 59-[32P]ATP (6000 Ci/mmol), and 59-[32P]dCTP were purchased from Amersham. A-23187 and A-23187 hydrochloride were obtained from Tocris Cookson. Oligonucleotides were obtained from Biosearch Technologies. Antibodies were obtained from Santa Cruz Biotechnology. Expression vectors of Sp1/CMV, which produces rat Sp1 (436 to 1008), and K. Kaibuchi (Nara Graduate University, Japan), and Egr-1, was made by cloning the 1.6-kb fragment of the entire Egr-1 site and additional bases are indicated by lowercase letters). The resulting PCR product was cloned into pGVB, which yielded the reporter plasmid designated –100LUC. Three mutated constructs derived from –100LUC, designed M1, M2, and M3, were generated by PCR from plasmid –119LUC by using the following primers: for M1, the 5’ primer containing KpnI site was 5’-gggattccGGCGGATCCTTCGACCCGGCCCGGGGGGGGGCCGGGGGGGCG-3’ (KpnI site and additional bases are indicated by lowercase letters), and the 3’ primer Xhol site was 5’-ccccctcgagTCTGACTAGTGCCTGGTACCG-3’ (Xhol site and additional bases are indicated by lowercase letters). The resulting PCR product was cloned into pGVB, which yielded the reporter plasmid designated –100LUC. Three mutated constructs derived from –100LUC, designed M1, M2, and M3, were generated by PCR from plasmid –119LUC by using the following primers: for M1, the 5’ primer containing KpnI site was 5’-gggattccGGCGGATCCTTCGACCCGGCCCGGGGGGGGGCCGGGGGGGCG-3’ (KpnI site and additional bases are indicated by lowercase letters), and the 3’ primer Xhol site was the same as that for –100LUC; for M2, the 5’ primer containing the KpnI site was 5’-gggattccGGCGGATCCTTCGACCCGGCCCGGGGGGGGGCCGGGGGGGCG-3’ (KpnI site and additional bases are indicated by lowercase letters), and the 3’ primer Xhol site was the same as that for –100LUC; for M3, the 5’ primer containing the KpnI site was 5’-gggattccGGCGGATCCTTCGACCCGGCCCGGGGGGGGGCCGGGGGGGCG-3’ (KpnI site and additional bases are indicated by lowercase letters), and the 3’ primer Xhol site was the same as that for –100LUC.

Cell Culture and Transfection

CPAE cells were obtained from the American Type Culture Collection and cultured in minimum essential medium alpha modification (MEM) supplemented with 10% FBS and antibiotics at 37°C in 5% CO2. Transfections into CPAE cells were performed with a modified calcium phosphate coprecipitation technique as described previously.21 Cells were transfected with 1 μg of reporter plasmid or, if indicated, 1 μg of expression plasmid. After transfection, cultures were washed twice with PBS and refed with MEM with or without 100 ng/mL PMA. After 2 hours of incubation, cells were harvested.
for luciferase assays. Luciferase activity was measured with a Berthold Lumat LB9501 luminometer and was normalized to cellular protein concentration. Each transfection was repeated, and the mean±SE of the mean was plotted.

**EMSA and Supershift Assay**

Nuclear extracts from CPAE cells were prepared as previously described. The sequences of the sense strand of double-stranded oligonucleotides used as probes or competitors in EMSAs were as follows, with the consensus motif underlined and mutations of wild-type sequences in boldface: SOD102/84, 5'-GGGCCGGGGC CGGCGGT-3'; SOD102/84M, 5'-GGGGGGGGC C CGGCGGT-3'; SOD80/62, 5'-GGGGGGGGT A AADC GGCGGT-3'; SOD80/62M, 5'-GCGGT A AADC GGCGGT-3'; Sp1, 5'- ATTGC ATCGCGGGCGGCCGGACGC-3'; Egr-1, 5' CGCCTCGCCCCGGCGGGG-3'; and activator protein (AP)-2, 5'GATCGAACTGACC GCCGG-3'. All probes were 5'-end-labeled with T4 polynucleotide kinase and [γ-32P]ATP (Amersham). Membranes were prehybridized and hybridized by standard techniques after staining with methylene blue to verify the relative quality and quantity of the RNA. After washing for 1 hour in 0.2% SSC/0.1% SDS at 42°C, blots were exposed to an x-ray film at −80°C with an intensifying screen. Developed films were scanned by an image scanner (ES-800C scanner; Epson America, Inc) and analyzed by a computer program (NIH Image 1.49) to measure the relative intensity of each band.

**Western Blot Analysis**

Nuclear extracts from vehicle- or PMA-treated CPAE cells were directly subjected to immunoblotting for Sp1. After boiling with sample buffer, SDS-PAGE was performed by using a 15% gel according to the standard procedure, and proteins in the gel were transferred electrophoretically to a nitrocellulose membrane at 2 mA/cm² for 2 hours. Sp1 was visualized by using an affinity-purified rabbit polyclonal antibody and a horseradish peroxidase–linked anti-rabbit IgG secondary antibody (Amersham). The complexes were detected by autoradiography by the ECL chemiluminescence detection system (ECL Western Blotting Analysis System, Amersham).

**Results**

**PMA Induces Mn-SOD mRNA in CPAE Cells**

To determine whether PMA regulates Mn-SOD gene expression in CPAE cells, 24-hour serum-starved CPAE cells were stimulated with PMA at 100 ng/mL and harvested at various time points for Northern blot analysis. As shown in Figure 1, Mn-SOD transcripts were detected at low levels in unstimulated cells. Exposure to PMA caused a time-dependent progressive increase in Mn-SOD mRNA levels and resulted in maximal accumulation after 6 to 12 hours. To determine the signal transduction pathways responsible for the induction of Mn-SOD mRNA expression by PMA, we treated CPAE cells with a PKC inhibitor (calphostin C), a tyrosine kinase inhibitor (genistein), a MAP kinase kinase-1 inhibitor (PD98059), or p38 MAP kinase inhibitor (SB203580). The reagent concentration used in the present study has previously been shown to block the effects of the agonist in a number of cell types. As shown in Figure 2, pretreatment of CPAE cells with calphostin C, a tyrosine kinase inhibitor (genistein), a MAP kinase kinase-1 inhibitor (PD98059), or p38 MAP kinase inhibitor (SB203580). The reagent concentration used in the present study has previously been shown to block the effects of the agonist in a number of cell types. As shown in Figure 2, pretreatment of CPAE cells with calphostin C and genistein markedly prevented the induction of Mn-SOD mRNA expression by PMA. In contrast, pretreatment with PD98059 was without discernible effect, and pretreatment with SB203580 had only minimal effects on PMA-induced Mn-SOD expression (Figure 2B).

These data indicate that PMA-induced Mn-SOD expression is mediated through the signaling pathways involving PKC or
tyrosine kinase but not MAP kinase kinase-1 or p38 MAP kinase.

Exposure of CPAE cells to TNF-α (10 ng/mL) induced Mn-SOD mRNA expression at levels that were similar to those observed with PMA stimulation. TNF-α–induced Mn-SOD expression was effectively attenuated by calphostin C and genistein but not by PD98059 or SB203580 (data not shown).

**Isolation of Human Mn-SOD 5′-Flanking Sequence and Identification of the Minimal Promoter**

Several overlapping clones for the 5′-flanking region of the Mn-SOD gene were obtained by screening the human genomic library. Previous analysis of the human Mn-SOD gene demonstrated that it contains a TATA-less 5′-upstream region with a single transcriptional start site at 90 bp upstream from the initiator methionine codon. According to published panels of potential protein binding motifs, a non-canonical nuclear factor-κB (NF-κB) site exists at −1062, an AP-1 site at −815, and AP-2 sites at −405 and −154. A series of potential Sp1 binding sites are scattered throughout the entire sequence. A schematic diagram of the Mn-SOD promoter region (between −1400 and 90) with the described regulatory elements is shown in Figure 3. In an attempt to delineate the DNA elements that are important for basal and stimulated transcription of the Mn-SOD promoter in CPAE cells, a series of 5′-deletion constructs was made with progressively smaller fragments of the 5′-flanking sequence and cloned in front of the luciferase reporter gene. The resultant luciferase constructs were then transiently transfected into CPAE cells and analyzed for basal activity and expression of Mn-SOD mRNA in untreated cells was set to 1.0.

**Figure 2.** Effects of protein kinase inhibitors on PMA-induced Mn-SOD mRNA expression. A, CPAE cells were pretreated with various protein kinase inhibitors, calphostin C (1 μmol/L), genistein (10 μmol/L), PD98059 (50 μmol/L), and SB203580 (10 μmol/L), for 1 hour and then incubated with PMA (100 ng/mL) for 6 hours. Total cellular RNA (20 μg) was analyzed by Northern blotting for Mn-SOD mRNA expression. 28S ribosomal RNA stained by methylene blue indicates that comparable amounts of total RNA actually blotted onto a membrane. B, Bar graphs show Mn-SOD mRNA levels normalized by intensity of 28S RNA. Expression of Mn-SOD mRNA in untreated cells was set to 1.0.

**Figure 3.** Deletion analysis of Mn-SOD promoter activity in CPAE cells. Depicted on the left side of the figure are the Mn-SOD promoter-luciferase deletion constructs. The location of putative nuclear protein binding sites, NF-κB site, AP-1 site, AP-2 sites, and Sp1 sites, are indicated. The 5′ end points of deletion mutants are indicated, and all constructs end at position 90. Each construct was transiently transfected into CPAE cells and measured for luciferase (Luc) activity. Values are mean±SE. Transfection was performed in duplicate, and the results are representative of at least 5 different experiments that used at least 3 different plasmid DNA preparations. Luciferase activity is normalized to micrograms of protein for each sample and is expressed relative to that of pGVB.
transcriptional response to PMA. Transfection of CPAE cells with −1400LUC, which contained the most 5′ sequence, yielded an ≈130-fold increase in promoter activity relative to the transfection with −3LUC, which lacks the almost entire 5′-flanking region. Deletion from −1400 to −207 reduced the Mn-SOD promoter activity by 74%. Deletion from −119 to −86 resulted in a marked reduction in promoter activity (≈84%). These data suggest that the sequence between −1400 and −207 contains positive elements that enhance the basal promoter activity, whereas the sequence between −119 and 90 contains elements that contribute to the basal transcriptional activity of the Mn-SOD gene.

Effects of PMA on Mn-SOD Transcription in CPAE Cells

We next examined the regulation of the Mn-SOD promoter activity by PMA. A series of 5′ deletions was transfected into CPAE cells, and luciferase activity was measured in untreated cells, as well as in cells that had been exposed to PMA for 18 hours. The promoter region between −1400 and 90 conferred PMA responsiveness to the luciferase gene by 3.2-fold (Figure 4A, −1400LUC). The deletion construct that contains the sequence between −86 and 90 of the Mn-SOD promoter could be activated 2.3-fold by PMA. In contrast, a DNA fragment between −3 and 90 was unresponsive to PMA (Figure 4A). Thus, a sequence between −86 and −3 is required for an induced transcription by PMA.

Effects of Activated Form of PKC on Mn-SOD Promoter Activity in CPAE Cells

PKC is the major intracellular receptor for phorbol ester. Our experiments in fact indicate that induction of Mn-SOD mRNA expression by PMA is completely inhibited by a specific inhibitor of PKC (calphostin C). We next directly tested whether the constitutively active form of PKC induces Mn-SOD promoter activity. A plasmid ΔPKCβ, which lacks a regulatory domain and thus exhibits a constitutive PKCβ activity, was cotransfected with each 5′-deletion construct into CPAE cells. As shown in Figure 4B, −1400LUC promoter activity was increased by 8.1-fold by ΔPKCβ, and −207LUC and −119LUC retained significant responsiveness to ΔPKCβ (>4-fold). Deletion of the sequence to −86 had essentially no effect on fold activation by ΔPKCβ, but further deletion to −3 largely eliminated the response to ΔPKCβ. A promoterless plasmid, pGVB, was not responsive to ΔPKCβ (data not shown). These results indicate that the 5′ end of the major sequence determinants of PKC responsiveness in the Mn-SOD promoter resides between −86 and −3.

Effects of Mutation of Sp1 Site on PMA Response

The results of 5'-deletion analysis shown in Figure 3 revealed the minimal Mn-SOD promoter activity within the sequence between −119 and 90. We then examined more precisely the role of the cis elements within this region by introducing site-specific mutations into −100LUC, which contains a sequence from −100 and 90. Because this region contains 2 copies of the 9-bp sequence, which perfectly fit the Sp1 binding motif, 5′-GGGGCGGGG-3′, we made 3 more constructs in which each of 2 elements is mutated individually and in combination as described in Methods and as outlined in Figure 5A. As shown in Figure 5B, mutation of either the distal Sp1 site located at −97 or the proximal Sp1 site located at −77 resulted in a significant decrease in basal promoter activity; compared with the wild-type mutation (−100LUC), a mutation of the distal Sp1 site alone (M1), the proximal Sp1 site alone (M2), or both sites (M3) resulted in 82%, 63%, and 92% decreases, respectively, in luciferase activity. For the response to PMA, the proximal Sp1 site seems to be more important than the distal site because disruption of the proximal Sp1 site caused a more profound decrease in PMA responsiveness (3.2-fold activation in M1 versus 1.6-fold activation in M2). The disruption of both Sp1 sites severely impaired the responsiveness to PMA. These results demonstrate that response to PMA depends on the integrity of both Sp1 sites, although the proximal Sp1 site is more affected by the response to PMA.

We then tested whether activation of PKC has an effect on Sp1 site–dependent transcription. Enhancement of luciferase activity of −100LUC by ΔPKCβ is significantly attenuated by mutation of the potential Sp1 site at −77 but not at −97 (data not shown), thus demonstrating the critical importance of the −77 site in PKC responsiveness.

Identification of Nuclear Factors Involved in PMA Response

To test the ability of the putative Sp1 sites located at −97 and −77 to interact with Sp1 or other related factors, we performed EMSA with nuclear extracts from unstimulated

Figure 4. Upregulation of Mn-SOD promoter activity by PMA or PKCβ expression vector. A, CPAE cells were transfected with the indicated Mn-SOD deletion constructs. At 20 hours after transfection, cells were incubated with vehicle or PMA (100 ng/mL) for 20 hours as described in Methods. The fold induction was calculated by dividing the luciferase activity values of samples treated with PMA by the activity value of untreated control samples. B, CPAE cells were transiently transfected with the indicated 5'–deletion construct of Mn-SOD luciferase reporter plasmids, along with either control empty vector pcDSRα or ΔPKCβ, which expresses the constitutively active form of PKCβ. The fold induction was calculated by dividing the luciferase activity values of samples cotransfected with ΔPKCβ by the activity value of samples cotransfected with pcDSRα. All results represent mean±SE of at least 3 experiments in duplicate.
CPAE cells and the $^{32}$P-labeled double-stranded oligonucleotides containing sequences between $-102$ and $-84$ or $-80$ and $-62$. Results of EMSA with either probe were essentially the same. Either probe gave rise to 2 major DNA-protein complexes, designated C1 and C2, with the upper complex, C1, exhibiting a higher intensity than the lower complex, C2 (Figure 6A). Both complexes proved to be sequence specific because formation of these complexes is inhibited by an excess amount of unlabeled wild-type oligonucleotides (SOD$-102/-84$) but not by SOD$-102/-84M$, which contains mutations within the putative Sp1 binding site. Complexes C1 and C2 can also be competed away by excess

**Figure 5.** Site-specific mutational analysis of the 2 Sp1 binding sites within the minimal Mn-SOD promoter fragment. A, Sequence of the minimal promoter region of the Mn-SOD gene is shown. Putative Sp1 binding sites (GGGGCGGGGC) that conform to consensus Sp1 binding motif are boxed, and mutations introduced into wild-type sequence are indicated below each Sp1 site. B, Depicted on the left side of the panel are the schematic representations of the wild-type $-100LUC$, M1 (in which the Sp1 site at $-95$ was mutated), M2 (in which Sp1 site located at $-73$ was mutated), and M3 (in which both Sp1 sites were mutated). CPAE cells were transfected with the indicated reporter plasmids. At 20 hours after transfection, cells were incubated with vehicle or PMA (100 ng/mL) for 20 hours. Luciferase activity value of samples treated with PMA and the activity value of untreated control samples are expressed relative to that of pGVB. The data represent mean±SE at least 3 experiments in duplicate.

**Figure 6.** EMSA of the potential Sp1 sites. A, Nuclear extracts prepared from CPAE cells were incubated with $^{32}$P-labeled oligonucleotides (SOD$-102/-84$ or SOD$-80/-62$), which contain the wild-type sequence of the potential Sp1 binding site, in the absence or presence of a 100-fold molar excess of indicated unlabeled competitors (Comp.). DNA-protein complexes were separated on 6% polyacrylamide gels under nondenaturing conditions. Positions of the sequence-specific DNA-protein complexes (C1 and C2), nonspecific bindings (NS), and free probe (FP) are indicated. B, Nuclear extracts prepared from CPAE cells were incubated with antibodies (Ab.), Sp1 antibody ($\alpha$Sp1), Sp3 antibody ($\alpha$Sp3), or Egr-1 antibody ($\alpha$Egr-1), and assayed for DNA binding activity to either SOD$-102/-84$ or SOD$-80/-62$ probes. The complexes were separated on 4.5% polyacrylamide gels under nondenaturing conditions. Positions of the sequence-specific DNA-protein complexes (C1a, C1b, and C2) and supershifted complexes (SS) are indicated.
amounts of the unlabeled consensus Sp1 binding sequence but not by the Egr-1 and AP-2 binding sequences, both of which resemble the Sp1 binding sequence. To verify that C1 and C2 complexes contain Sp1 or Sp1-related proteins, we performed EMSA in the presence of Sp1- or Sp3-specific antisera (Figure 6B). EMSA by 4.5% polyacrylamide gels revealed that complex C1 consists of 2 DNA-protein components, designated complexes C1a and C1b. Addition of an Sp1 antibody resulted in a supershift of complex C1a, indicating that Sp1 is a principal DNA-binding component of this complex. An Sp3 antibody completely supershifted complex C2. We also tested the effects of the Egr-1 antibody on complex formation. Addition of the Egr-1 antibody had no effect on the formation of complexes C1a, C1b, and C2. These results provide evidence that Sp1 and Sp3 but not Egr-1 bind to the SOD−102/−84 and the SOD−80/−62 probes.

### PMA Increases Sp1 Expression at mRNA and Protein Levels

To determine whether PMA affects Sp1 expression in CPAE cells, we performed Northern and Western blot analyses. Induced expression of Sp1 mRNA by PMA was studied in serum-starved CPAE cells. On PMA stimulation, Sp1 mRNA levels were decreased at the earliest 2-hour time point, then began to increase, reached maximum levels by 6 hours (1.35-fold increase compared with unstimulated level), and then gradually declined (Figure 7A). The membranes were also hybridized with a probe for Egr-1, serving as a known immediate-early response gene by PMA stimulation. In accordance with the results reported for other cell types,30 Egr-1 mRNA levels were rapidly and markedly increased by PMA in CPAE cells. Figure 7B shows a densitometric evaluation of the Sp1 mRNA expression levels induced by PMA from 3 independent experiments. In accordance with the mRNA accumulation, PMA increased the amount of Sp1 protein, as determined by Western blot analysis with the nuclear protein prepared from unstimulated and PMA-treated CPAE cells (Figure 7C). Equivalent protein loading of lanes was confirmed by Coomassie blue staining of gels (data not shown). To verify that an increase in Sp1 protein is accompanied by an increase in the shifted complexes containing Sp1, EMSA was performed. DNA-protein complexes formed with SOD−102/−84, which carries the distal Sp1 site at −97, were increased slightly when this probe was incubated with the nuclear extracts prepared from PMA-treated CPAE cells. The increase in intensity of the shifted complexes formed with the PMA-treated cells was
observed more convincingly when oligonucleotide SOD−80/−62, which contains the proximal Sp1 site at −77, was used as a probe (Figure 7D). Taken together, these results demonstrate that PMA increased the Sp1 expression at mRNA and protein levels and that, as a consequence, more Sp1 binds to the cognate binding sites.

**Overexpression of Sp1 Expression Increases Mn-SOD Promoter Activity**

To establish the functional importance of an increase in Sp1 expression in the transcriptional regulation of the Mn-SOD gene, we performed cotransfection experiments with Sp1/CMV, which produces rat Sp1 under the control of the CMV promoter. As shown in Figure 8A, the cotransfection of Sp1/CMV resulted in a 4.5-fold increase in luciferase activity of the −1400LUC construct. In contrast, Egr-1/CMV, which expresses the mouse Egr-1 under the control of CMV promoter, had no effect on luciferase activity. Next, the deletion constructs of the Mn-SOD promoter were transiently transfected with or without Sp1/CMV into CPAE cells. Constructs −207LUC and −119LUC but not −86LUC and −3LUC were responsive to Sp1 overexpression, although the shorter promoter was less responsive (Figure 8B). Furthermore, to verify the importance of the Sp1 sites of the Mn-SOD promoter, the wild-type construct −100LUC and site-specific mutation constructs M1, M2, and M3, along with either Sp1/CMV or control empty vector pRC/CMV. The fold induction was calculated by dividing the luciferase activity values of samples cotransfected with Sp1/CMV by the activity value of samples cotransfected with pRC/CMV. C, CPAE cells were transiently transfected with the indicated 5′-deletion construct of Mn-SOD luciferase reporter plasmids, along with either Sp1/CMV or control empty vector pRC/CMV. The fold induction was calculated by dividing the luciferase activity values of samples cotransfected with Sp1/CMV by the activity value of samples cotransfected with pRC/CMV. M, CPAE cells were transiently transfected with wild-type −100LUC and site-specific mutation constructs, M1, M2, and M3, along with either Sp1/CMV or control empty vector pRC/CMV. The fold induction was calculated by dividing the luciferase activity values of samples cotransfected with Sp1/CMV by the activity value of samples cotransfected with pRC/CMV. All results represent mean±SE at least 3 experiments in duplicate.

**Figure 8.** Upregulation of Mn-SOD promoter activity by Sp1 but not Egr-1 expression vector. A, CPAE cells were transiently transfected with −1400LUC along with either Sp1/CMV, Egr-1/CMV, or control empty vector pRC/CMV. The fold induction was calculated by dividing the luciferase activity values of samples cotransfected with Sp1/CMV and Egr-1/CMV by the activity value of samples cotransfected with pRC/CMV. B, CPAE cells were transiently transfected with the indicated 5′-deletion construct of Mn-SOD luciferase reporter plasmids, along with either Sp1/CMV or control empty vector pRC/CMV. The fold induction was calculated by dividing the luciferase activity values of samples cotransfected with Sp1/CMV by the activity value of samples cotransfected with pRC/CMV. C, CPAE cells were transiently transfected with wild-type −100LUC and site-specific mutation constructs, M1, M2, and M3, along with either Sp1/CMV or control empty vector pRC/CMV. The fold induction was calculated by dividing the luciferase activity values of samples cotransfected with Sp1/CMV by the activity value of samples cotransfected with pRC/CMV. All results represent mean±SE at least 3 experiments in duplicate.

Promoter activity was increased 3.7-fold by Sp1/CMV. Mutation of the distal Sp1 site at −95 only modestly reduced the increase in activity. In contrast, mutation of the proximal Sp1 site at −77 almost completely abrogated Sp1/CMV-induced luciferase activity (M2, in Figure 8C). The effects of mutation of both Sp1 sites (M3) were comparable to those of mutation of the proximal Sp1 site (M2). These findings imply that Sp1 overexpression can lead to activation of the Mn-SOD promoter and that the proximal Sp1 site plays a more important role in mediating this response.

**Discussion**

In the present study, we have analyzed the molecular components involved in PMA-induced expression of the Mn-SOD gene in CPAE cells. The increase in Mn-SOD mRNA levels after treatment of these cells with PMA is most likely mediated at the level of transcription, as judged from upregulation of transiently transfected Mn-SOD promoter-luciferase reporter constructs. Mappings of the PMA-responsive region by 5′-deletion and site-directed mutagenesis analyses in combination with nuclear protein binding, competition, and supershift studies have localized the PMA-responsive element to Sp1 sites at −97 and −77.

PMA activates the expression of a variety of genes with different kinetics. Transcriptional regulation by PMA typically involves transcription factors such as AP-1, serum response factor, and NF-κB, which have been established to play a key role in the rapid induction of gene expression in response to PMA.32–34 It is noteworthy that induction of Mn-SOD expression by PMA is delayed compared with the immediate-early response gene represented by Egr-1 in the present study, whose induction reaches maximum as early as 2 hours after stimulation. We almost exclude the possibility that Egr-1 may play role in the induction of Mn-SOD expression because overexpression of Egr-1 failed to enhance Mn-SOD promoter activity. Likewise, overexpression of c-Jun, c-Fos, or the p65 subunit of NF-κB had no effect on Mn-SOD promoter activity (data not shown). These results favor the hypothesis that the molecular mechanism underlying PMA-induced Mn-SOD expression is distinct from that responsible for early-response gene induction and appears to represent a delayed response to PMA.

One of the main conclusions in the present study is that PMA activates Sp1-dependent transcription. The traditionally accepted role for Sp1 is that it regulates the basal level of expression. Little attention has been paid to its regulatory role in an induced transcription. However, there are increasing numbers of studies demonstrating the involvement of Sp1 in mediating the activation of gene expression in response to extracellular stimuli. A regulatory role for Sp1 has been reported for glucose activation of the carboxylase promoter and plasminogen activator inhibitor-1 promoters, TNF-α–induced vascular endothelial growth factor gene expression in human glioma cells,37 and oncostatin M–induced human α2(I) collagen gene expression.38 In addition, the functional role of Sp1 in PMA-induced gene expression has been described in the WAF1/CIP1 gene59 and in the human platelet thromboxane receptor gene.40 Furthermore, Lin et al41 have reported the shear stress induction of tissue factor gene expression in human umbilical vein endothelial cells is mediated through an increased transcriptional activity of Sp1.
with a concomitant hyperphosphorylation of Sp1. These studies strongly support our notion that Sp1 is not only involved in constitutive expression but also can mediate inducible expression of the Mn-SOD gene in response to PMA.

The data in the present study indicate that PMA increases Sp1 expression modestly but reproducibly at mRNA and protein levels. Although an increase in Sp1 expression has not been generally implicated in PMA-inducible gene expression, our finding is not surprising because previous studies involving insulin-like growth factor-2 in colon cancer cells (Caco-2 cells) transfected with the oncogenic H-ras gene have demonstrated increased Sp1 expression mediated through PKC pathways. Furthermore, treatment of K562 erythroleukemia cells with PMA increases Sp1 mRNA levels, which is followed by enhancement of increased transcription of the thromboxane receptor gene. Although the molecular mechanisms underlying regulated expression of Sp1 mRNA by PMA is beyond the scope of the present study, the results of Northern blot analysis suggest that expression of the Sp1 gene is independent of early growth response transcription factors, such as AP-1, serum response factor, and NF-κB, because Sp1 mRNA levels increased as late as 6 hours after PMA treatment.

In the present study, we cannot entirely eliminate the possibility that PMA increases the DNA binding activity by some form of posttranslational modification, such as phosphorylation by DNA-dependent protein kinase as well as by casein kinase II. Nevertheless, we do not favor this possibility because Western blot analysis did not detect a change of the mobility of Sp1 in the presence or absence of PMA stimulation. It represents a sharp contrast to the previous studies described by Lin et al, in which hyperphosphorylation of Sp1 by okadaic acid is associated with changes in the migration of Sp1.

We showed a noticeable increase in Mn-SOD mRNA levels as early as 2 hours after PMA stimulation. Such an early increase of Mn-SOD mRNA levels cannot be ascribed to the increase in Sp1 expression because Sp1 mRNA levels increase at later hours. Rather, it is reasonable to speculate that Sp1-independent induction plays a major role in an early increase in Mn-SOD mRNA levels. However, our recent data suggest that PMA increases the trans-activation function of Sp1 independent of DNA binding. By using a Gal4-reporter gene system, we found that PMA increases the transcription of the Gal4-TATA-luciferase gene driven by the Gal4-Sp1 fusion protein. Thus, we propose that Sp1 can increase Mn-SOD mRNA levels independent of an increase in Sp1 protein and that such a mechanism may be important for the early increase in Mn-SOD mRNA levels by PMA stimulation.

As in the case of the Mn-SOD promoter, the TATA-less promoters generally contain GC-rich sequences in the basal promoter regions in which Sp1 and Egr-1 binding sites are located (either nearby or overlapping each other). Egr-1 has been implicated in the expression of various endothelial genes during vascular injury. Khachigian and Collins have recently reported that on denudation of endothelium, de novo synthesized Egr-1 displaces the prebound Sp1 and occupies the GC box, which consists of overlapping Sp1 and Egr-1 sites, in the PDGF-A promoter. These considerations led us to test whether the GC-rich sequence in the Mn-SOD promoter may also serve as a binding site for Egr-1. We can almost entirely eliminate the possibility that Egr-1 plays a role in PMA-induced activation of Mn-SOD expression for the following 3 reasons: First, EMSA showed that the shifted complexes formed with oligonucleotides SOD−102/−84 and SOD−80/−62 are not competed away by a molar excess of consensus Egr-1 sequence. Second, the Egr-1 antibody had no effect on complex formation. Third, overexpression of Egr-1 had essentially no effect on the Mn-SOD promoter. Thus, these results indicate the Egr-1–independent activation of the Mn-SOD promoter by PMA.

In conclusion, the functional data, taken together with the DNA binding data, support the hypothesis that the response of the Mn-SOD promoter to PMA is mediated by Sp1. The identification of the Sp1 binding site as a PMA response element will expand our knowledge concerning the mechanisms behind the delayed and long-lasting response to extracellular stimuli. The present study raises the issue of what molecular mechanisms support the Sp1 site–dependent response to PMA in endothelium, because not all of the promoters containing Sp1 sites are responsive to PMA. Investigation into these issues will allow us to understand the pathophysiological basis for the endothelial adaptation and dysfunction provoked by various stresses that involve PKC activation.

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References


Inducible Expression of Manganese Superoxide Dismutase by Phorbol 12-Myristate 13-Acetate Is Mediated by Sp1 in Endothelial Cells
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