Induction of manganese superoxide dismutase mRNA by okadaic acid and protein synthesis inhibitors

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We have reported that the phorbol ester phorbol 12-myristate 13-acetate (PMA) enhances the expression of manganese superoxide dismutase (Mn-SOD) mRNA [Fujii and Taniguchi (1991) J. Biol. Chem. **266**, 23142–23146]. Okadaic acid, an inhibitor of type I and type IIa phosphatases, was also found to induce Mn-SOD mRNA at submicromolar concentrations in HeLa cells. Addition of cycloheximide resulted in superinduction of PMA- or tumour necrosis factor-stimulated expression of the mRNA, but not of okadaic acid-stimulated expression. When the effect of cycloheximide on the decay of Mn-SOD mRNA was examined by inhibiting mRNA synthesis with actinomycin D, cycloheximide had virtually no effect on mRNA stability, suggesting that accumulation of the mRNA was caused by activation by this reagent of transcription of the gene. PMA pretreatment of HeLa cells markedly enhanced cycloheximide-dependent superinduction of Mn-SOD mRNA. These data suggest that phosphorylation of several proteins is implicated in the regulation of Mn-SOD gene expression.

INTRODUCTION

Superoxide dismutases (SODs) are antioxidant enzymes that protect cells by scavenging superoxide radicals [1–3]. Mn-SOD is localized in the mitochondrial matrix [4]. Because mitochondria consume most of the oxygen in the cell and are, therefore, a primary target for oxyradicals produced via the respiratory chain reaction, Mn-SOD is assumed to play a crucial role under physiological and hyperoxic conditions.

Serum levels of Mn-SOD protein increase in malignant diseases such as ovarian cancer [5,6]. Several cytokines such as tumour necrosis factor (TNF), interleukin-1 (IL-1) and IL-6 are known to induce expression of the Mn-SOD gene [7–12], and may cause an elevation of Mn-SOD levels in serum [13]. In addition, accumulation of Mn-SOD mRNA is also superinduced by the protein synthesis inhibitor cycloheximide [9,14], which has a similar effect on many inducible genes such as the c-*jun*, c-*fos* and c-*myc* proto-oncogenes and the collagenase gene [15–19]. Recently we showed that phorbol 12-myristate 13-acetate (PMA), a potent tumour promoter and protein kinase C activator, enhanced the expression of the gene [14], and suggested that there are both protein kinase C-dependent and -independent pathways for induction of the Mn-SOD gene.

In the present paper, we show induction of Mn-SOD mRNA by okadaic acid, indicating the involvement of protein phosphorylation, and provide evidence for the transcriptional activation of the mRNA by protein synthesis inhibitors.

EXPERIMENTAL

Chemicals

PMA, okadaic acid and cycloheximide were from Wako Pure Chemicals. Anisomycin was purchased from Sigma. *N*-[2-(Methylamino)ethyl]-5-isoquinoline sulphonamide dihydrochloride (H8) was from Seikagaku Kogyo. Eagle's minimum essential medium, RPMI 1640 medium and Dulbecco's modified Eagle's medium were from Nikken Biomedical Laboratories; fetal bovine serum was from Whittaker M.A. Bioproducts; and kanamycin sulphate was from Nacalai Tesque. Human recombinant TNFα was obtained from Ube Industries. Zeta-Probe was from Bio-Rad. [α-^{32}P]dCTP was from New England Nuclear. Restriction endonucleases were from Takara Shuzo Co. or Toyobo Co.

Cell culture

The cell lines utilized in this work were described previously [14] except for COS-1, which are simian virus 40-transformed monkey kidney fibroblast cells and were obtained from the Japanese Cancer Research Bank. HeLa cells were grown in 100 mm Petri dishes in Eagle's minimum essential medium supplemented with 10% fetal bovine serum and 200 μg/ml kanamycin sulphate at 37 °C, 5% CO_2 in a humid atmosphere. RPMI 1640 was utilized for ME180, Kuramochi, K562 and A549 cells, and high-glucose Dulbecco's modified Eagle's minimum essential medium was used for COS-1 cells.

Quantification of Mn-SOD protein by e.l.i.s.a.

Subconfluent cells were stimulated with various concentrations of TNFα, PMA, cycloheximide or anisomycin in fresh medium and were incubated for an additional 24 h. Cells were washed twice with Tris-buffered saline (TBS) and were scraped off in the presence of 5 mM EDTA. After precipitation in a microcentrifuge, the cells were suspended in low-ionic-strength buffer (10 mM Tris/HCl, pH 7.4, 5 mM $MgCl_2$), frozen, thawed and then sonicated for 10 min at maximal power in a Bioruptor (Cosmo Bio Co.). Protein concentration was determined according to the method of Smith et al. [20] using BSA as a standard. Mn-SOD protein was quantified by a sandwich e.l.i.s.a. using purified human Mn-SOD as a standard [21].

RNA preparation

After 4 h of incubation with the reagents indicated, cells were washed twice with TBS, collected and precipitated as described above. Total RNA was prepared from the cells essentially as

Abbreviations used: SOD, superoxide dismutase; TNF, tumour necrosis factor; PMA, phorbol 12-myristate 13-acetate; IL-1, interleukin-1; H8, *N*-[2-(methylamino)ethyl]-5-isoquinoline sulphonamide dihydrochloride; TBS, Tris-buffered saline (10 mM Tris, 137 mM NaCl and 3 mM KCl, pH 7.4).

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described by Chomczynski and Sacchi [22]. The quantification of RNA was carried out by measuring the absorbance at 260 nm.

Northern blotting

Portions of 10–20 μg of total RNA were heat-denatured at 65 °C for 15 min in the presence of 50% formamide and gel running buffer (40 mM Mops, 10 mM sodium acetate and 1 mM EDTA, pH 7.0), and then run on a 1% agarose gel containing 2.2 M formaldehyde. The size-fractionated RNAs were transferred on to a Zeta-Probe membrane for 20–40 hours by capillary action, and the blotted RNAs were immobilized on the membrane by u.v. irradiation for 3 min. The human Mn-SOD cDNA probe containing the entire coding sequence [23] was labelled with ^{32}P by a random hexamer method [24]. Hybridization and washing conditions were as described previously [14]. All other DNA and RNA manipulations were conducted according to Maniatis et al. [25].

RESULTS

Induction of the Mn-SOD gene by okadaic acid

Since Mn-SOD gene expression was induced by PMA, the involvement of protein phosphorylation by protein kinase C has been proposed [14]. In order to assess the role of protein phosphorylation in the expression of this gene, Northern blot analysis of RNA from HeLa cells treated with okadaic acid (an inhibitor of type I and type IIA protein phosphatases [26]) was carried out. While Cu,Zn-SOD expression was slightly suppressed by okadaic acid at high concentrations, Mn-SOD mRNA was markedly induced at concentrations greater than 30 nM (Figure 1). This is the first report indicating that okadaic acid induces Mn-SOD gene expression, and confirms the involvement of protein phosphorylation in the expression of this gene.

Cycloheximide and anisomycin induce accumulation of Mn-SOD mRNA

Mn-SOD mRNA and protein levels in HeLa cells were investigated in the presence of various concentrations of cycloheximide or of another protein synthesis inhibitor, anisomycin; the effects of TNF and PMA were also studied. The amount of Mn-SOD protein was quantified after 24 h of stimulation. A 4 h incubation was chosen for the mRNA assay, because the level of mRNA decreased after about 10 h of incubation with TNF or PMA. TNF induced both Mn-SOD mRNA and the protein in a dose-dependent manner (Figure 2a), as reported previously [7,9,11,14]. PMA at greater than 1 ng/ml also induced Mn-SOD protein to about twice the control level (Figure 2b), but the level of protein induced by PMA was less than that induced by TNF. When cells were stimulated with both PMA and TNF, the amount of protein produced was additive (results not shown).

Although the maximal accumulation of Mn-SOD mRNA induced by cycloheximide and anisomycin occurred at concentrations of 50 μM and 10 μM respectively, the levels of Mn-SOD protein actually decreased at these concentrations (Figure 2c and 2d), which could be explained by the blocking effect of these reagents on protein synthesis. While cycloheximide superinduced Mn-SOD mRNA in combination with TNF, IL-1 or PMA [8,14], it had virtually no effect on the okadaic acid-stimulated

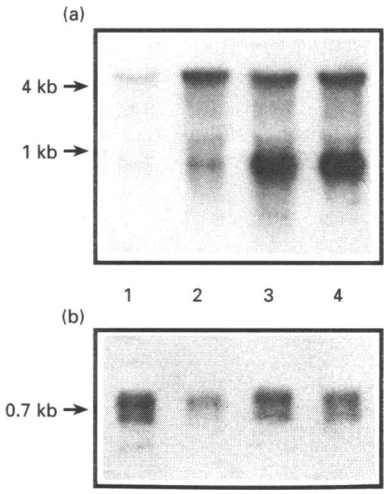

Figure 1 Induction of Mn-SOD mRNA by okadaic acid in HeLa cells

After stimulation of subconfluent HeLa cells with various concentrations of okadaic acid for 8 h, 20 μg samples of total RNA were analysed by Northern blotting using human Mn-SOD cDNA (**a**) and human Cu,Zn-SOD cDNA (**b**) as probes. Lanes: 1, control; 2, cycloheximide; 3, okadaic acid; 4, cycloheximide + okadaic acid.

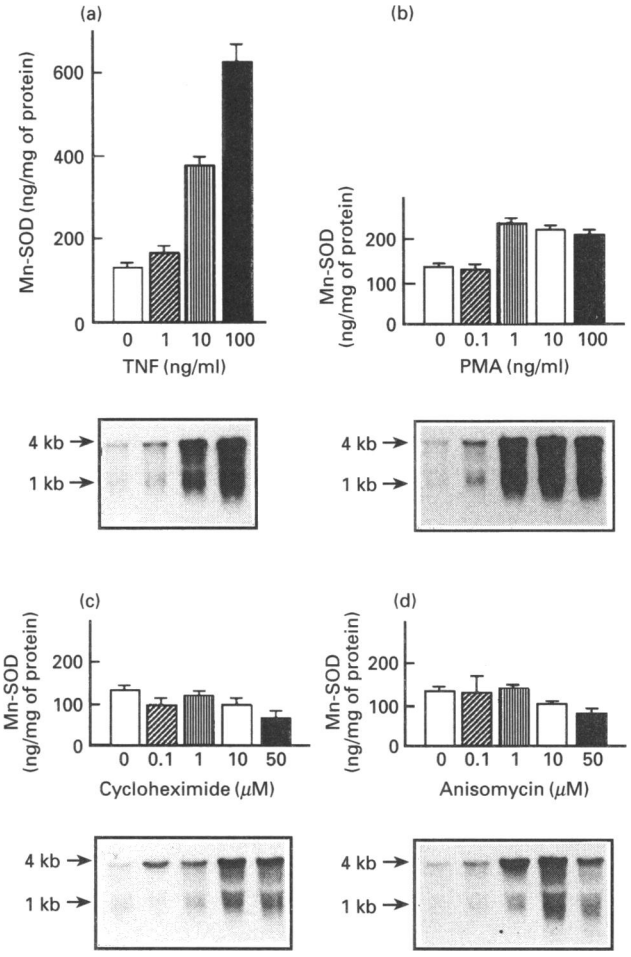

Figure 2 Effect of various stimulants on accumulation of Mn-SOD mRNA and protein in HeLa cells

After stimulation of subconfluent HeLa cells with various concentrations of TNF (**a**), PMA (**b**), cycloheximide (**c**) or anisomycin (**d**) for 24 h, the amounts of Mn-SOD protein in total cell homogenates were quantified by e.l.i.s.a. using purified human Mn-SOD as a standard [19]. Data are means ± S.D. of triplicate experiments. The gels represent typical Northern blots of 20 μg of total RNA prepared from cells which were stimulated for 4 h with the same concentrations of the reagents as described above.

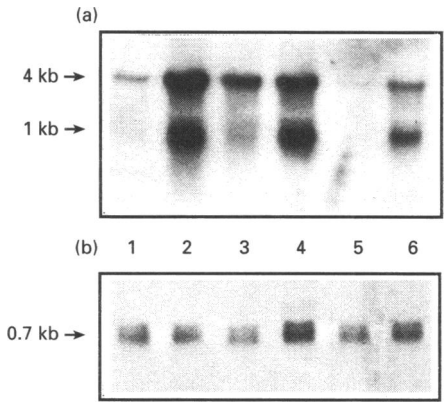

Figure 3 Effect of cycloheximide and H8 on okadaic acid- and TNF-stimulated induction of Mn-SOD mRNA

Samples of 20 μg of total RNA from HeLa cells treated with the reagents indicated were analysed by Northern blotting, using human Mn-SOD (**a**) and human Cu,Zn-SOD (**b**) as probes, as described in Figure 1. Lanes: 1, control; 2, okadaic acid; 3, cycloheximide; 4, cycloheximide + okadaic acid; 5, H8; 6, H8 + okadaic acid.

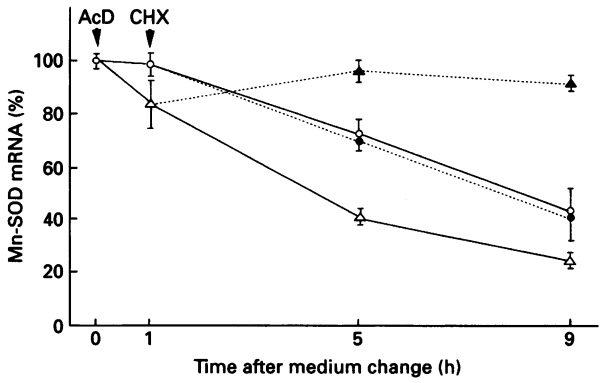

Figure 4 Effect of cycloheximide on the decay of Mn-SOD mRNA

HeLa cells were incubated with 50 ng/ml TNF for 4 h, washed twice with PBS and then incubated with new media with (○, ●) or without (△, ▲) 40 μM actinomycin D (AcD) at time zero (left arrowhead). Cycloheximide (CHX) was added at a final concentration of 50 μM (●, ▲) 1 h after the change in medium (right arrowhead). Northern blotting and quantification of Mn-SOD mRNA was carried at each time point. Results are means ± S.D. of triplicate experiments.

Table 1 Accumulation of Mn-SOD mRNA induced by cycloheximide and anisomycin in various cell lines

Total RNA was prepared from cells stimulated with 50 μM cycloheximide or 10 μM anisomycin for 4 h. Samples of 20 μg of RNA were analysed by Northern blotting. The Mn-SOD mRNA-derived signals were shown relative to the control experiment by scanning the X-ray film. The values are means ± S.D. for triplicate experiments.

	Mn-SOD mRNA (units)		
Cell line	Control	Cycloheximide	Anisomycin
HeLa	1.0 ± 0.2	3.8 ± 0.4	5.3 ± 0.2
A549	1.0 ± 0.2	3.8 ± 0.4	6.1 ± 0.9
Kuramochi	1.0 ± 0.3	2.7 ± 0.2	5.2 ± 0.4
ME180	1.0 ± 0.3	1.6 ± 0.2	1.0 ± 0.1
K562	1.0 ± 0.2	1.3 ± 0.1	1.1 ± 0.1
COS-1	1.0 ± 0.1	1.2 ± 0.1	1.6 ± 0.2

Table 2 Effect of PMA pretreatment on Mn-SOD mRNA accumulation induced by cycloheximide in HeLA cells

HeLa cells were preincubated in the absence or presence of 10 ng/ml PMA for 24 h and then stimulated with 0.1% dimethyl sulphoxide (vehicle), 10 ng/ml PMA or 10 μM cycloheximide for an additional 4 h. The values are means ± S.D. for triplicate experiments as shown in Table 1.

	Mn-SOD mRNA (units)	
	−PMA	+PMA
Vehicle	1.0 ± 0.2	3.3 ± 0.5
PMA (10 ng/ml)	15.6 ± 3.9	2.4 ± 0.3
Cycloheximide (10 μM)	3.6 ± 1.2	18.8 ± 3.3

induction (Figure 3). The protein kinase inhibitor H8 suppressed okadaic acid-induced as well as control expression.

Induction of Mn-SOD mRNA by cycloheximide and anisomycin in various cell lines

We have previously shown that IL-1, TNF, PMA and lipopolysaccharide induced Mn-SOD mRNA in cells that were resistant to the cytotoxic effects of TNF [14]. To examine the cell-type-dependency of the mRNA accumulation, the effects of protein synthesis inhibitors on some cell lines were examined. As shown in Table 1, 10 μM anisomycin or 50 μM cycloheximide induced Mn-SOD mRNA accumulation 5–6-fold in cell lines such as HeLa, A549 (human lung carcinoma cells) and Kuramochi (human ovary undifferentiated carcinoma cells), in which Mn-SOD is also induced by other stimulants [7,11,14]. Since these cells are known to be rather resistant to cytotoxicity of TNF, induction of Mn-SOD mRNA by anisomycin and cycloheximide is seen to occur in TNF-resistant cells.

Effect of cycloheximide on decay of Mn-SOD mRNA

Many inducible genes are known to be superinduced by cycloheximide [15–17]. To elucidate the mechanism of induction of Mn-SOD mRNA accumulation by protein synthesis inhibitors, the effect of cycloheximide on the decay of Mn-SOD mRNA after stimulation by TNF was examined (Figure 4). While actinomycin D itself stabilized Mn-SOD mRNA, cycloheximide had essentially no effect on the decay of mRNA in the presence of actinomycin D, but increased the mRNA level in the absence of actinomycin D, suggesting that cycloheximide exerted a stimulatory effect on the transcriptional stage of gene expression.

PMA pretreatment of HeLa cells enhances induction of Mn-SOD mRNA by cycloheximide

Prolonged incubation of cells with PMA leaves them insensitive to further PMA treatment [14]. As shown in Table 2, the level of Mn-SOD mRNA in cells pretreated with 10 ng/ml PMA for 24 h increased by a 5-fold greater extent in response to cycloheximide compared with that in untreated cells. A similar enhancement of Mn-SOD induction was shown when TNF instead of cycloheximide was used as a stimulant after pretreatment of the cells with PMA [14].

DISCUSSION

We have shown that both PMA and okadaic acid induce Mn-SOD mRNA (Figure 1), confirming the possibility that Mn-SOD gene expression is regulated by protein phosphorylation. The ability of PMA to induce Mn-SOD protein production was less than that of TNF (Figure 2); this was, however, consistent with the total amount of mRNA induced during incubation with the stimulants [14]. mRNA levels induced by PMA returned to the unstimulated level more rapidly than with TNF, probably because protein kinase C–PMA complex would be rapidly degraded by proteases such as calpain [27]. Moreover, we also have shown that two protein synthesis inhibitors, cycloheximide and anisomycin, induce accumulation of Mn-SOD mRNA in cells that are rather resistant to TNF cytotoxicity (Table 1). It has been assumed that the effect of cycloheximide on short-lived mRNA accumulation is attributable to the rapid loss of a specific and unstable nuclease involved in the cleavage of unstable mRNA, although such a nuclease has not yet been identified. In the case of Mn-SOD mRNA, however, superinduction by cycloheximide would not fit this mechanism, since cycloheximide had no effect on the decay of Mn-SOD mRNA when new mRNA synthesis was blocked by actinomycin D (Figure 4).

We previously showed that pretreatment of HeLa cells with PMA enhanced TNF-induced Mn-SOD expression [14]. In the present study, pretreatment of the cells with PMA enhanced the superinduction of Mn-SOD mRNA by cycloheximide (Table 2). This suggests that a protein synthesized during PMA pretreatment functions as a signal transducer for cycloheximide; such a protein has also been implicated in gene induction by TNF. Thus the induction of mRNA accumulation by cycloheximide is also likely to be dependent on protein phosphorylation, and protein kinase C may again be involved. Mahadevan et al. [18] reported that the induction of proto-oncogenes by protein synthesis inhibitors and growth factors was concomitant with rapid phosphorylation of two chromatin-associated proteins, pp33 and pp15. The latter was recently identified as histone H3, and the phosphorylated amino acid residues as serines [19]. It has also been reported that treatment of B cells with cycloheximide resulted in the activation of casein kinase II [28], a serine/threonine kinase localized to both the cytosol and nucleus which phosphorylates many substrates involved in protein, RNA and DNA synthesis [29]. Hence the phosphorylation of chromatin-associated proteins may be an intermediate step in the induction of a family of genes, including the Mn-SOD gene, by protein synthesis inhibitors and tumour promoters. It is known that levels of Mn-SOD in TNF-resistant cells are higher than those in TNF-sensitive cells [7]. H8 suppressed mRNA expression to below the control level (Figure 3). It is, therefore, conceivable that a certain protein involved in the signal transduction pathway is partially phosphorylated and thereby functional in TNF-resistant cells. Okadaic acid would enhance this pathway by blocking the dephosphorylation of the signal molecule. Stimulation of certain cells with either cytokines or other stimulants, which cause oxidative damage to cells, would increase the expression of Mn-SOD through phosphorylation of such a protein. These cells would be thereby afforded protection from oxyradicals produced as a result of increased metabolism in the mitochondria in response to the various stimulations.

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