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Extracellular-Superoxide Dismutase Expression During Monocytic Differentiation of U937 Cells

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ABSTRACT

Leukemic cell lines, such as U937, THP-1, and HL60 cells, can differentiate into macrophages following exposure to various agents including 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in vitro. It is well known that TPA enhances reactive oxygen species (ROS) generation through the activation of NADPH oxidase (NOX), and ROS act as mediators in TPA signaling. Extracellular-superoxide dismutase (EC-SOD) is a major anti-oxidative enzyme that protects the cells from damaging effects of superoxide. Recently, the reduction of Cu/Zn-SOD and the induction of Mn-SOD by TPA in leukemic cells have been reported; however, the regulation of EC-SOD by TPA remains poorly understood. Here, we explored the regulation of EC-SOD during the monocytic differentiation of U937 cells by TPA. We observed the reduction of EC-SOD and Cu/Zn-SOD, whereas the induction of Mn-SOD during the differentiation of U937 cells. The reduction of EC-SOD and Cu/Zn-SOD was attenuated by pretreatments with GF109203X (an inhibitor of protein kinase C, PKC), diphenyleneiodonium (an inhibitor of NOX), and U0126 (an inhibitor of mitogen-activated protein kinase kinase, MEK/extracellular-signal regulated kinase, ERK). Interestingly, pretreatment with BAY11-7082 (an inhibitor of nuclear factor-κB, NF-κB) suppressed the reduction of Cu/Zn-SOD, but not of EC-SOD. Furthermore, we also determined the involvement of newly synthesized protein and the instability of mRNA in the reduction of EC-SOD. Overall, our results suggest that the expression of EC-SOD is decreased by TPA through intracellular signaling consisting of PKC, NOX-derived ROS and MEK/ERK, but not of NF-κB signaling. J. Cell. Biochem. 112: 244–255, 2011.

KEY WORDS: EXTRACELLULAR-SUPEROXIDE DISMUTASE; REACTIVE OXYGEN SPECIES; 12-O-TETRADECANOYLPHORBOL-13-ACETATE; MITOGEN-ACTIVATED PROTEIN KINASE; NUCLEAR FACTOR-κB

Reactive oxygen species (ROS) are known to be generated by activated endothelial cells, macrophages, and leukocytes have been implicated in a variety of pathological processes including asthma, diabetes, and atherosclerosis [Vachier et al., 1994; Wright et al., 2006]. To protect the cells from oxidative stress, mammalians have anti-oxidative enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase [Furukawa et al., 2004; Roberts et al., 2006]. SOD is a major anti-oxidative enzyme that protects cells from the damaging effect of superoxide by accelerating the dismutation reaction of superoxide by approximately 10,000 times. There are three SOD isozymes in mammals; copper and zinc-containing SOD (Cu/Zn-SOD), manganese-containing SOD (Mn-SOD), and extracellular-SOD (EC-SOD) [Faraci, 2003]. EC-SOD is a secretory, tetrameric glycoprotein [Marklund, 1982], whereas Cu/Zn-SOD and Mn-SOD are intracellular enzymes found predominantly in the cytoplasm and mitochondria, respectively. EC-SOD is a major SOD isozyme in the extracellular space but is distributed mainly in blood vessel walls [Ookawara et al., 1998]. After secretion, EC-SOD slowly diffuses and binds to the heparan sulfate proteoglycan in the glycocalyx on the surface of most cell types in the vascular wall. The microenvironment created by neutrophil–endothelial cell interaction plays an important role in the progression of the vascular injury, because the concentration of superoxide released at this site is sufficient to denaturalize the redox homeostasis.

It is well known that leukemic cell lines, such as U937, THP-1, and HL60 cells, can differentiate into macrophages by various reagents, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and all-*trans*-retinoic acid (ATRA) [Miranda et al., 2002; Pathak et al., 2002; Barbieri et al., 2003]. Differentiation into macrophages is

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characterized by morphological changes, which are accompanied by significant increases in the levels of CD11b, CD14, and CD36, surface markers of differentiated macrophages [Greenwalt et al., 1992; Alessio et al., 1996; Miranda et al., 2002; Yamamoto et al., 2009]. During monocytic differentiation and following phagocytosis, ROS are generated by the activated NADPH oxidase (NOX) [Babior, 1999; Ichikawa et al., 2004; Bedard and Krause, 2007]. The excess production of ROS results in oxidative stress, leading to the dysregulation of signal transduction events and biomolecular injury, all of which contribute to pathological changes in cell and tissue function [Vachier et al., 1994; Wright et al., 2006]. Recently, it has been shown that Mn-SOD expression is induced by the activation of protein kinase C (PKC), a family of serine/threonine-specific kinases, extracellular-signal regulated kinase (ERK), a mitogen-activated protein kinase (MAPK), and nuclear factor-κB (NF-κB) signaling cascades during monocytic differentiation of U937 cells by TPA or ATRA [Fujii and Taniguchi, 1991; Kiningham et al., 2008; Traore et al., 2008]. On the other hand, other researchers have shown that the expression of Cu/Zn-SOD and catalase decreased during differentiation [Saito et al., 1989; Yamamoto et al., 2009]. However, the regulation of EC-SOD during monocytic differentiation by TPA remains unclear.

In the present study, we investigated EC-SOD regulation during monocytic differentiation of U937 cells by TPA and examined the role of PKC, ROS, MAPK, and NF-κB signaling cascades in these processes. We found that exposure of TPA induced differentiation of U937 cells into macrophages and reduced the expression of EC-SOD in mRNA and protein levels. Moreover, the reduction of EC-SOD was mediated by PKC, NOX-derived ROS, and MEK/ERK signaling cascades. Interestingly, TPA-triggered reduction of Cu/Zn-SOD was also involved in NF-κB signaling; however, that of EC-SOD was regulated through NF-κB independent mechanisms.

MATERIALS AND METHODS

REAGENTS

TPA was purchased from Sigma–Aldrich Co. (St Louis, MO). MAPK inhibitors (U0126, SP600125, and SB203580), PKC inhibitor (GF103203X), actinomycin D (ActD), cycloheximide (CHX), and nitroblue tetrazolium (NBT) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 5-(and-6)-Carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) was purchased from Molecular Probes (Eugene, OR). Diphenyleneiodonium (DPI) was purchased from Enzo Life Sciences Inc. (Farmingdale, NY). NF-κB inhibitor (BAY11-7082) was purchased from Calbiochem (San Diego, CA). Anti-phospho-PKC (pan) (βII Ser660) rabbit polyclonal antibody, anti-MAPK kinases (MEK) rabbit polyclonal antibody, anti-phospho-MEK rabbit polyclonal antibody, anti-ERK rabbit monoclonal antibody, anti-phospho-ERK mouse monoclonal antibody, anti-c-jun N-terminal kinase (JNK) rabbit polyclonal antibody, anti-phospho-JNK mouse monoclonal antibody, anti-p38 rabbit polyclonal antibody, and anti-phospho-p38 rabbit monoclonal antibody were purchased from Cell Signaling Technology (Danvers, MA). Anti-NF-κB p65 mouse monoclonal antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-actin mouse monoclonal antibody was purchased from Millipore Co. (Billerica, MA). Biotin-conjugated goat anti-rabbit or -mouse IgG (H + L) was purchased from Zymed Laboratories (San Francisco, CA). Phycoerythrin (PE)-conjugated anti-CD11b (CD11b-PE) mouse antibody and PE-conjugated IgG1 κ isotype mouse antibody were purchased from BD Pharmingen (San Diego, CA).

CELL CULTURE

U937, THP-1, and HL60 cells were cultured in RPMI1640 medium containing 10% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator. For differentiation of U937 cells, the cells were seeded at 6×10^5 cells/ml in 3.5 cm dishes and 30 nM TPA was added. After differentiation, the cells and conditioned media were collected. The medium was used to measure the EC-SOD concentration, as described below, and the cells were scraped and washed with cold phosphate-buffered saline (PBS) followed by the extraction of total RNA, flow cytometry analysis, and Western blotting.

ANALYSIS OF CD11b EXPRESSION

Differentiation of U937 cells was assessed by analysis of the expression of CD11b surface marker. After the cells had been treated, they were scraped followed by washing with ice-cold PBS and centrifugation. The pellets were stained with CD11b-PE antibody in PBS containing 1% paraformaldehyde at 4°C for 30 min. After incubation, the cells were washed with PBS containing 1% bovine serum albumin (BSA) three times and resuspended in PBS. Fluorescent intensities were analyzed by FACS Aria™II (Becton Dickinson, Franklin Lakes, NJ). To determine nonspecific binding, PE-conjugated IgG1 κ isotype antibody was used, and its intensities were subtracted from those of cells stained with CD11b-PE antibody.

MEASUREMENT OF ROS GENERATION

Intracellular ROS levels were measured using NBT and carboxy-H_2DCFDA, as previously reported [Pathak et al., 2002; Matsunaga et al., 2008]. For the assay of superoxide production, each cell suspension was mixed with an equal volume of PBS containing 1 mg/ml NBT and 2.5 μg/ml TPA for 30 min in a CO_2 incubator. After incubation, cells containing purple formazan deposits and cells devoid of NBT-reducing activity in each sample were determined by counting 200 cells under a microscope. Data are expressed as a percentage of NBT reduction-positive cells based on the following ratio: purple cells/200 cells. For measurement of intracellular ROS accumulation, the cells were suspended followed by staining with 10 μM carboxy-H_2DCFDA in PBS for 20 min in a 5% CO_2 incubator. After incubation, the cells were washed with PBS three times and resuspended in PBS. Fluorescent intensities were analyzed by FACS Aria™II.

REVERSE TRANSCRIPTIONAL-POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS

After U937 cells had been treated, the cells were lysed in 1 ml TRIzol® reagent (Invitrogen, Carlsbad, CA). The cDNA and RT-PCR were prepared and performed by the methods described in our previous report [Yamamoto et al., 2002], with minor modification.

The primer sequences used in this study are as follows: EC-SOD, sense 5'-AGA AAG CTC TCT TGG AGG AG-3'; antisense 5'-ACC GCG AAG TTG CCG AAG TC-3' (496 bp): Cu/Zn-SOD, sense 5'-GCG ACG AAG GCC GTG TGC GTG-3'; antisense 5'-TGT GCG GCC AAT GAT GCA ATG-3' (348 bp): Mn-SOD, sense 5'-CGA CCT GCC CTA CGA CTA CGG-3'; antisense 5'-CAA GCC AAC CCC AAC CTG AGC-3' (365 bp): TNF-α, sense 5'-GGC GTG GAG CTG AGA GAT AA-3'; antisense 5'-TCG GCA AAG TCG AGA TAG TC-3' (320 bp): CD14, sense 5'-CGA GGA CCT AAA GAT AAC CGG C-3'; antisense 5'-GTT GCA GCT GAG ATC GAG CAC-3' (510 bp): GAPDH, sense 5'-ACC ACA GTC CAT GCC ATC AC-3'; antisense 5'-TCC ACC ACC CTG TTG CTG TA-3' (556 bp). These PCR products were loaded on a 2% (w/v) agarose gel for electrophoresis, and densitometric analysis of the PCR products was performed with Multi Gauge V3.0 (Fuji Film, Tokyo, Japan).

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) ANALYSIS

The EC-SOD contents in concentrated medium were measured by ELISA as described in our previous report [Adachi et al., 2001], with minor modifications. We had confirmed previously that EC-SOD concentration is closely correlated with SOD activity [Adachi et al., 1992]. After U937 cells (8×10^6 cells/6 ml in 10 cm dishes) were treated with TPA for 24 h, the conditioned media were concentrated using Amicon® Ultra ultracel-30K (Millipore Co., Billerica, MA). For the determination of total protein concentration, the pellets were homogenized in PBS containing 1% Triton X-100 for 30 min on ice. The protein concentration of extracts was estimated with protein assay reagent (Bio-Lad Lab., Hercules, CA).

WESTERN BLOTTING

Whole cell extracts were prepared in lysis buffer as described previously [Kamiya et al., 2008]. For phosphorylated protein detection, the cells were scraped and lysed in 200 μl lysis buffer A [20 mM Tris–HCl, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM Na_3VO_4, 20 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 2 μg/ml leupeptin, and 1% Triton X-100]. Cytosolic fractions were prepared in 100 μl lysis buffer B (20 mM Tris–HCl, pH 7.5, 2 mM EDTA, 10 mM EGTA, 1 mM DTT, 100 μg/ml digitonin, 10 mM NaF, 1 mM Na_3VO_4, 20 mM β-glycerophosphate, 1 mM PMSF). After centrifugation at 12,000*g* for 15 min, soluble cytosolic fractions were collected and the remaining pellets were treated for 15 min on ice with 100 μl lysis buffer B supplemented with 1% Triton X-100. The samples were again centrifuged for 15 min and the soluble supernatant containing the membrane fraction was collected. For nuclear extraction, the cells were collected and lysed in 800 μl lysis buffer C (20 mM HEPES–NaOH, pH 7.8, containing 15 mM KCl, 2 mM $MgCl_2$, 0.5 mM PMSF, and 10 μg/ml leupeptin) followed by centrifugation. The pellets were homogenized in 800 μl lysis buffer D (lysis buffer 1 containing 0.5% NP-40) for 30 min on ice. Finally, the pellets were homogenized in 40 μl lysis buffer E (20 mM HEPES–NaOH, pH 7.8, containing 0.4 M KCl, 0.5 mM PMSF, and 10 μg/ml leupeptin). After centrifugation, the protein concentration of extracts was estimated with protein assay reagent. Extracts containing 20 μg protein were boiled with sample buffer (62.5 mM Tris–HCl, pH 6.8, containing 2% sodium dodecylsulfate (SDS), 10% glycerol, 50 mM DTT, and 0.01% bromophenol blue) for 5 min and separated by SDS–PAGE on 10% or 12% (w/v) polyacrylamide gels. After being transferred electrophoretically onto PVDF membranes, nonspecific binding sites were blocked with PBS containing 1% BSA. Subsequently, the membranes were incubated with the respective specific primary antibodies (1:1,000). After the membranes had been washed three times with PBST (PBS containing 0.1% Tween 20), the blots were incubated with biotin-conjugated goat anti-rabbit or -mouse antibody (1:1,000). After the membranes had been washed three times with PBST, the blots were incubated with ABC reagents (Vector Laboratories, Inc., Burlingame, CA; 1:5,000). After the membranes had been washed with PBST three times, the bands were detected using SuperSignal® West Pico (Thermo Scientific, Rockford, IL), and imaged using an LAS-3000 UV mini (Fuji Film).

STATISTICAL ANALYSIS

Data are expressed as the means ± SD of three independent experiments. Statistical evaluation of the data was performed using ANOVA followed by post hoc Bonferroni tests. A P-value <0.05 was considered significant.

RESULTS

SODs EXPRESSION IN HUMAN LEUKEMIC CELLS

First, endogenous expression levels of EC-SOD, Cu/Zn-SOD, and Mn-SOD were analyzed in human U937, THP-1, and HL60 cells by RT-PCR. Cu/Zn-SOD and Mn-SOD were expressed in all cell types, whereas, interestingly, EC-SOD was expressed in only U937 cells (data not

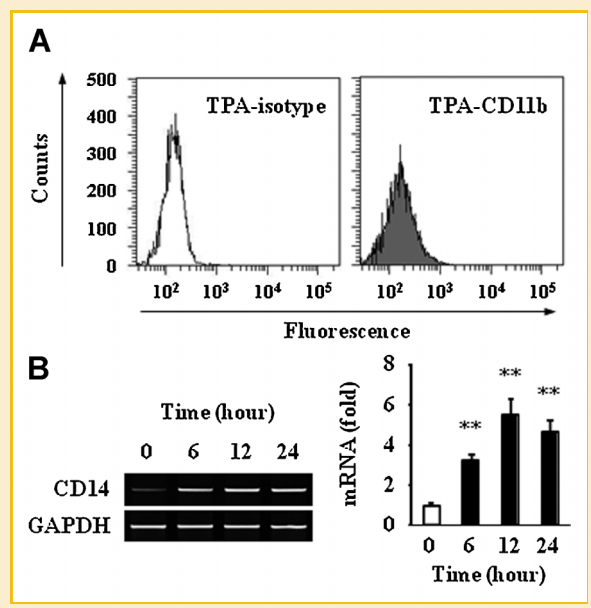

Fig. 1. Monocytic differentiation of U937 cells induced by TPA. U937 cells were treated with 30 nM TPA for 24 h (A) or the indicated time (B). After the cells had been treated, CD11b (A) and CD14 (B) expression were measured by flow cytometry and RT-PCR, respectively. A: Open histogram gives TPA-treated cells stained with isotype antibody, and filled histogram gives TPA-treated cells stained with CD11b antibody. B: RT-PCR data were normalized using GAPDH levels (**$P<0.01$ vs. untreated cells).

shown). According to these results, we studied the regulation of EC-SOD during monocytic differentiation of U937 cells by TPA.

MONOCYTIC DIFFERENTIATION OF U937 CELLS INDUCED BY TPA

It has been reported that the expression of CD11b and CD14, cell-surface markers, is one of the characteristics of monocytic differentiation by TPA [Miranda et al., 2002]. To confirm the potential of TPA as a monocytic differentiation inducer, we measured CDs expression. We observed the increase of CD11b (Fig. 1A) and CD14 (Fig. 1B) expression and morphological changes; floating cells changed to adherent cells by treatment with TPA.

SODs EXPRESSION DURING MONOCYTIC DIFFERENTIATION OF U937 CELLS INDUCED BY TPA

It has been reported that TPA regulates several anti-oxidative enzymes, including Cu/Zn-SOD, Mn-SOD, and catalase in U937 cells [Traore et al., 2008; Yamamoto et al., 2009]; however, the expression of EC-SOD during differentiation into macrophages is still unknown. Treatment with TPA significantly decreased the expression of EC-SOD, similar to Cu/Zn-SOD, in a time-dependent manner (Fig. 2A). In contrast, the expression of Mn-SOD was significantly increased at 6 h and was maintained up to 24 h. Further, we also observed a decrease of EC-SOD at the protein level (Fig. 2B). In this study, we particularly focused on the mechanism of EC-SOD reduction accompanied by that of Cu/Zn-SOD during monocytic differentiation of U937 cells.

INVOLVEMENT OF PKC IN SODs EXPRESSION

It is well known that TPA is a potent PKC activator [Nishizuka, 1992], and activation of PKC is necessary for the induction of Mn-SOD by TPA [Fujii and Taniguchi, 1991]. According to previous reports, we hypothesized that TPA-triggered EC-SOD and Cu/Zn-SOD reduction might be associated with the activation of PKC; therefore, we next investigated the membrane translocation of

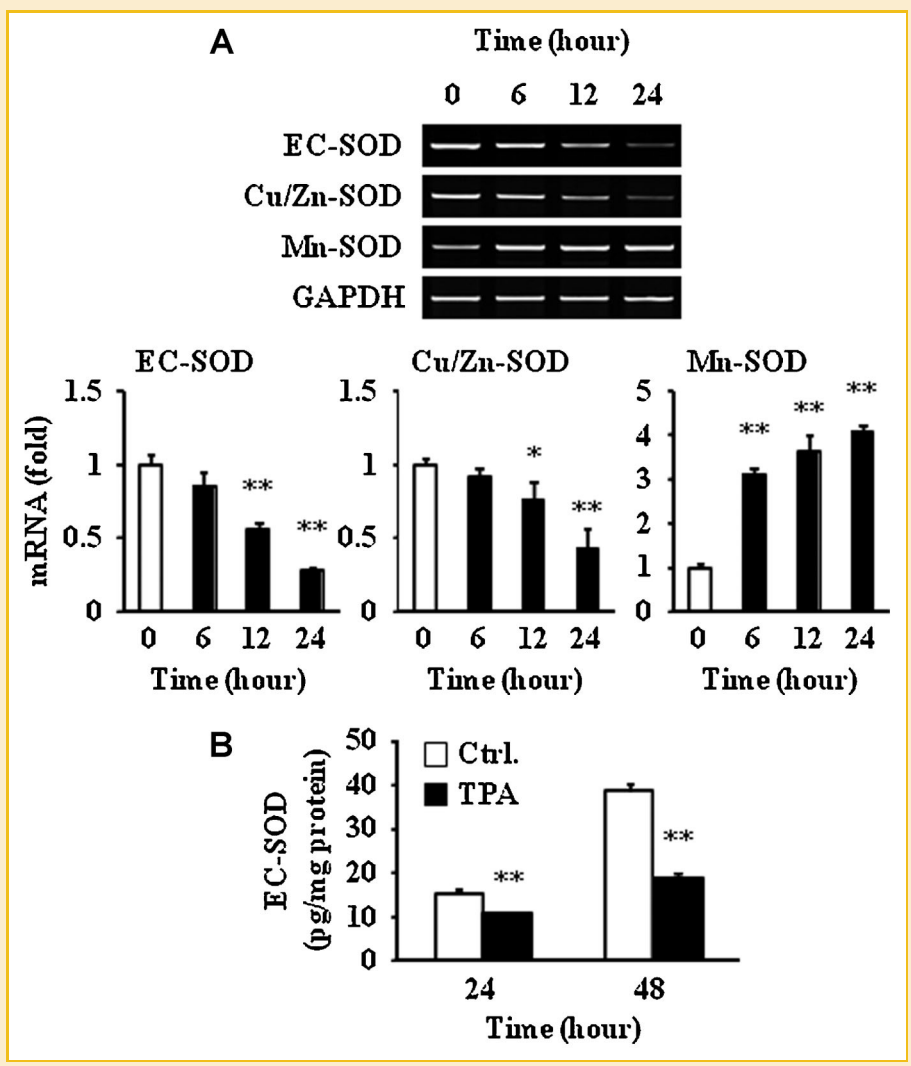

Fig. 2. SODs expression during monocytic differentiation of U937 cells induced by TPA. U937 cells were treated with 30 nM TPA for the indicated time. A: The expression of SODs mRNA was determined by RT-PCR. All RT-PCR data were normalized using GAPDH levels ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. untreated cells). B: The concentration of EC-SOD protein was determined by ELISA ($^{**}P < 0.01$ vs. untreated cells).

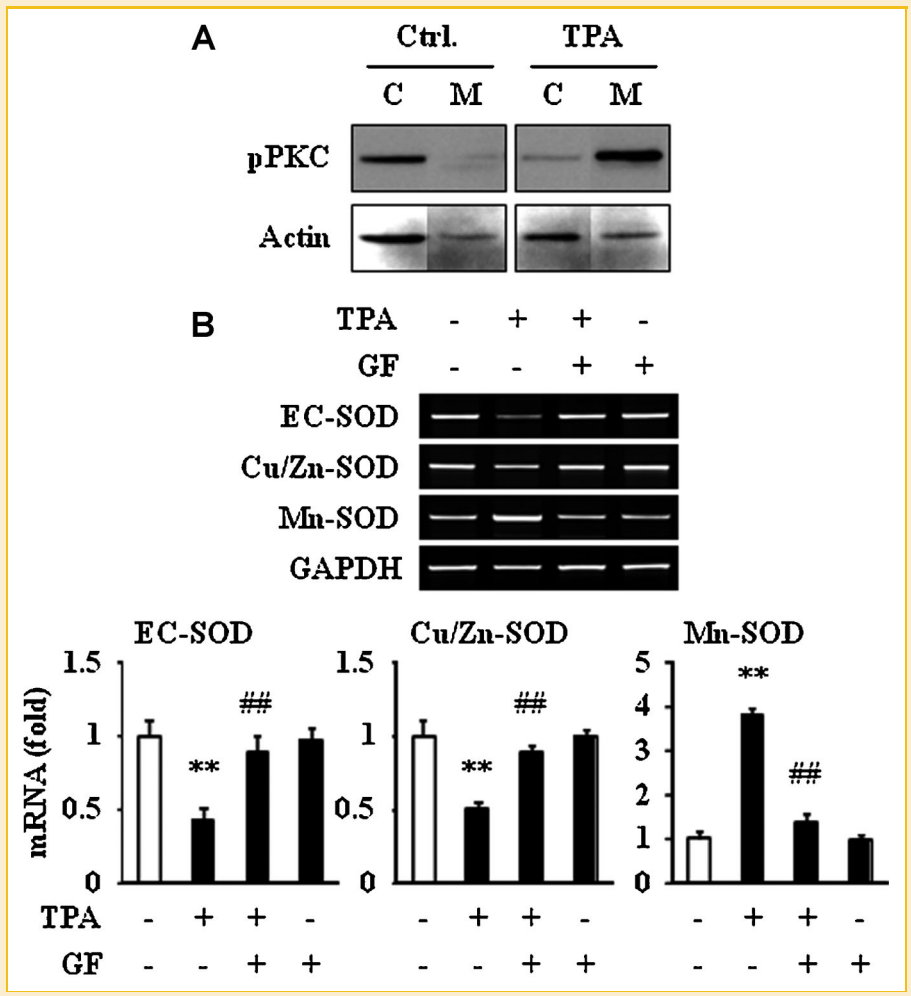

Fig. 3. Involvement of PKC in SODs expression. A: U937 cells were treated with or without 30 nM TPA for 15 min. After the cells were treated, phosphorylated PKC and actin in the cytosolic (C) and membrane (M) fractions were determined by Western blotting. B: U937 cells were pretreated with or without 5 μM GF109203X (GF) for 30 min, and then treated with or without 30 nM TPA for 24 h. After the cells had been treated, RT-PCR was carried out. All RT-PCR data were normalized using GAPDH levels ($^{**}P < 0.01$ vs. untreated cells, $\#\#P < 0.01$ vs. TPA-treated cells).

phospho-PKC and the effect of GF109203X, a PKC inhibitor, on the expression of these genes by RT-PCR. Figure 3A shows that PKC was constitutively phosphorylated in untreated U937 cells in the cytosolic fractions, while phospho-PKC was translocated to the membrane fractions in TPA-treated cells. As shown in Figure 3B, pretreatment with GF109203X significantly blocked the induction of Mn-SOD by TPA. Moreover, the reduction of EC-SOD and Cu/Zn-SOD was also neutralized by this inhibitor, suggesting that PKC plays an important role in the reduction of EC-SOD and Cu/Zn-SOD.

INVOLVEMENT OF ROS IN SODs EXPRESSION

It is well known that the production of superoxide and the accumulation of hydrogen peroxide are increased during differentiation into macrophages [Pathak et al., 2002; Yamamoto et al., 2009]. To confirm the potential of TPA as an inducer of ROS, we measured ROS generation. Treatment with TPA for 24 h significantly increased DCF oxidation (left) and NBT reduction (right) compared to untreated cells (Fig. 4A). It has been reported that TPA-induced monocytic differentiation was mediated by ROS produced through PKC and NOX activation. According to previous reports, we investigated the involvement of ROS in SODs expression by using DPI, a NOX inhibitor. As shown in Figure 4B, pretreatment with GF109203X or DPI significantly blocked TPA-triggered ROS production. Moreover, pretreatment with DPI markedly suppressed TPA-induced EC-SOD and Cu/Zn-SOD reductions and Mn-SOD induction (Fig. 4C), suggesting that TPA-triggered SOD down and upregulation were mediated by NOX-derived ROS.

INVOLVEMENT OF MAPK IN SODs EXPRESSION

Our previous reports showed that MAPK are associated with the regulation of EC-SOD in smooth muscle cells [Adachi et al., 2006] and renal tubular interstitial cells [Kamiya et al., 2008]. Since TPA is known to activate MAPK in many kinds of cells, including U937 cells, we investigated the involvement of MAPK in SODs

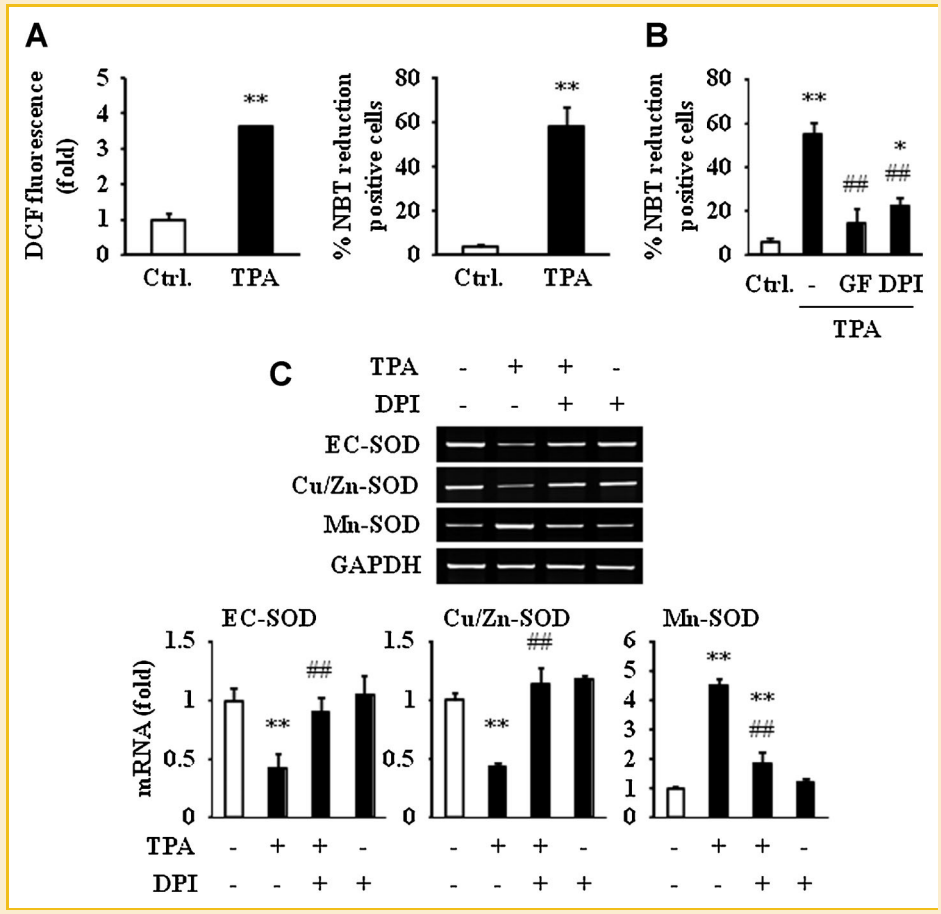

Fig. 4. Involvement of ROS in SODs expression. A: U937 cells were treated with 30 nM TPA for 24 h. After the cells were treated, intracellular ROS generation (left), and the percentage of NBT reduction-positive cells (right) were measured ($^{**}P < 0.01$ vs. untreated cells). B: U937 cells were pretreated with 5 μM GF109203X (GF) for 30 min or 20 μM DPI for 1 h, and then treated with 30 nM TPA for 24 h. After the cells had been treated, the percentage of NBT reduction-positive cells was measured ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. untreated cells, $\#\#P < 0.01$ vs. TPA-treated cells). C: U937 cells were pretreated with or without 20 μM DPI for 1 h, and then treated with or without 30 nM TPA for 24 h. After the cells had been treated, SODs mRNA expression was measured by RT-PCR. All RT-PCR data were normalized using GAPDH levels ($^{**}P < 0.01$ vs. untreated cells, $\#\#P < 0.01$ vs. TPA-treated cells).

expression during differentiation. As shown in Figure 5A, treatment with TPA induced the activation of MEK, ERK, and JNK, but not that of p38. To determine which MAPK is related to SODs regulation, we next investigated the effects of U0126, a MEK inhibitor, and SP600125, a JNK inhibitor, on SODs expression. Pretreatment with U0126 and SP600125 did not affect basal expression of EC-SOD and Cu/Zn-SOD (data not shown), and U0126 significantly blocked TPA-induced reduction of EC-SOD and Cu/Zn-SOD, but SP600125 had no effects on these reductions (Fig. 5B). Moreover, Figure 5C shows that pretreatment with GF109203X or DPI significantly inhibited the activation of ERK by TPA, indicating that TPA-induced activation of ERK is mediated by PKC activation and ROS production.

INVOLVEMENT OF NF-κB IN SODs EXPRESSION

It is well known that Mn-SOD is induced through NF-κB signaling activated by several stimuli, including TPA and ATRA. On the other hand, our previous reports and others showed that TNF-α, which is induced by NF-κB, decreases EC-SOD expression [Adachi et al., 2006; Ueda et al., 2008]. However, the involvement of NF-κB and subsequent increase of TNF-α in the TPA-triggered reduction of EC-SOD and Cu/Zn-SOD remains unknown. To investigate the involvement of NF-κB signaling cascades in these regulations, we first assessed the activation of NF-κB triggered by TPA treatment by detection of the nuclear translocation of NF-κB p65, which is promoted by TPA treatment at 3 h and maintained up to 6 h compared to untreated cells (Fig. 6A). Further, we also observed the induction of TNF-α mRNA expression by TPA in a time-dependent manner (Fig. 6B). Because NF-κB activation is known to be mediated by MAPK [Yang et al., 1999; Lim and Kwon, 2009], we next investigated the involvement of MAPK in the induction of TNF-α by using U0126 and SP600125. Pretreatment with U0126 significantly blocked TPA-induced TNF-α, whereas SP600125 did not affect this change (Fig. 6C), raising the possibility that NF-κB activation might contribute to the reduction of EC-SOD and Cu/Zn-SOD. To explore this possibility, we further studied the effect of BAY11-7082, an NF-κB inhibitor, on the reduction of SODs and induction of TNF-α by RT-PCR. Figure 6D shows that pretreatment with BAY11-7082

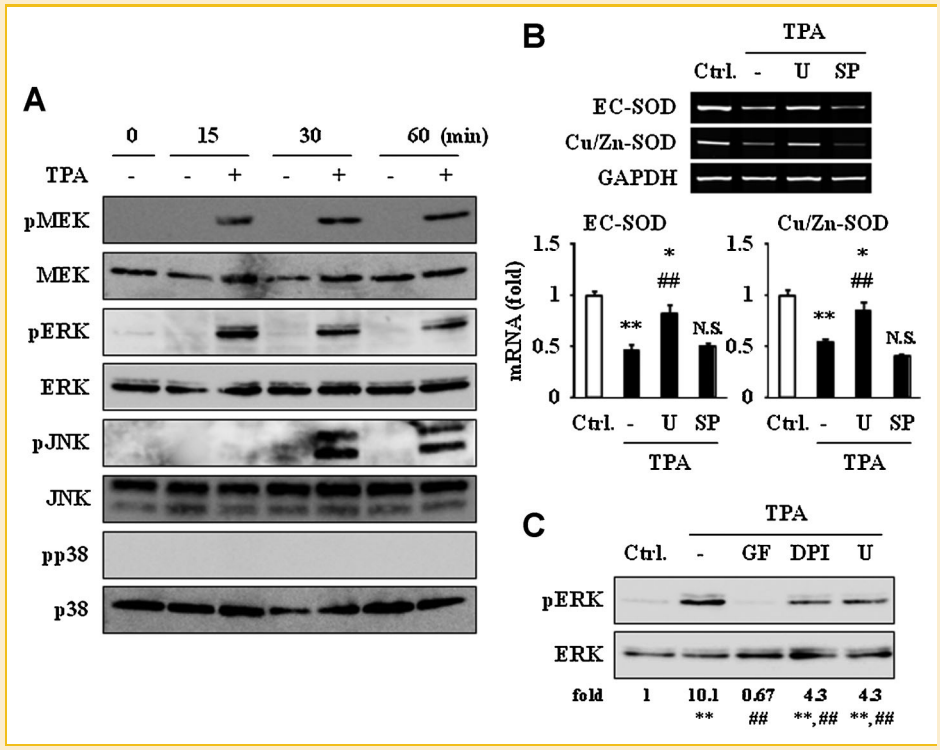

Fig. 5. Involvement of MAPK in SODs expression. A: U937 cells were treated with or without 30 nM TPA for the indicated time. Phosphorylated and total MEK and MAPK levels were determined by Western blotting. B: Cells were pretreated with or without 20 μM U0126 (U) or SP600125 (SP) for 30 min, and then treated with 30 nM TPA for 24 h. SODs mRNA expression was determined by RT-PCR. All RT-PCR data were normalized using GAPDH levels ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. untreated cells, $\#\#P < 0.01$, N.S., not significant vs. TPA-treated cells). C: Cells were pretreated with or without 5 μM GF109203X (GF) for 30 min, or 20 μM DPI for 1 h or 20 μM U0126 (U) for 30 min, and then treated with 30 nM TPA for 15 min. Phosphorylated and total ERK levels were determined by Western blotting. Values are mean of fold change relative to TPA-untreated cells ($n = 3$, $^{**}P < 0.01$ vs. untreated cells, $\#\#P < 0.01$ vs. TPA-treated cells).

significantly blocked TPA-triggered TNF-α induction and Cu/Zn-SOD reduction. On the other hand, this inhibitor did not affect EC-SOD reduction by TPA.

INVOLVEMENT OF NEWLY SYNTHESIZED PROTEIN IN EC-SOD EXPRESSION

We next investigated the involvement of newly synthesized protein in the reduction of EC-SOD by using CHX, an inhibitor of de novo protein synthesis. Pretreatment with CHX significantly blocked TPA-triggered EC-SOD reduction and increase of TNF-α in a CHX-dose dependent manner, whereas this inhibitor did not affect Cu/Zn-SOD reduction (Fig. 7A). To investigate whether newly synthesized protein acts as a mediator of ERK activation by TPA, we further studied the effect of CHX on ERK activation. Figure 7B shows that CHX did not block TPA-induced phosphorylation of ERK, suggesting that newly synthesized protein acts after ERK activation or by an MEK/ERK-independent mechanism.

STABILITY OF EC-SOD MRNA DURING MONOCYTIC DIFFERENTIATION BY TPA

We finally examined the effect of TPA on the stability of EC-SOD mRNA. The remaining amounts of EC-SOD mRNA during monocytic differentiation of U937 cells were determined at various time points after the addition of ActD, a transcriptional inhibitor. As shown in Figure 7C, the remaining amounts of EC-SOD mRNA in TPA-untreated cells were not altered by the addition of ActD. On the other hand, mRNA degradation of EC-SOD was significantly accelerated by TPA compared to untreated cells.

DISCUSSION

Cellular differentiation is the process by which a less specialized cell becomes a more specialized cell type. Leukemic cell lines, such as U937, THP-1, and HL60 cells, have been used to study the induction of monocytic differentiation into macrophages by TPA, ATRA, and 1,25-dihydroxyvitamin D_3 [Miranda et al., 2002; Pathak et al., 2002; Barbieri et al., 2003]. In this study, treatment of U937 cells with TPA resulted in morphological changes from floating cells to adherent cells, and an increase in the level of CD families (Fig. 1), consistent with the results by others [Yang and Shaio, 1994; Datta et al., 2000; Barbieri et al., 2003; Traore et al., 2005]. From these results, we confirmed that TPA-induced monocytic differentiation of U937 cells.

EC-SOD is a major SOD isozyme in the vascular system, and the presence of a high level of EC-SOD throughout the vessel walls might have an important protective role as an anti-inflammatory and anti-arteriosclerosis factor against superoxide and inflamma-

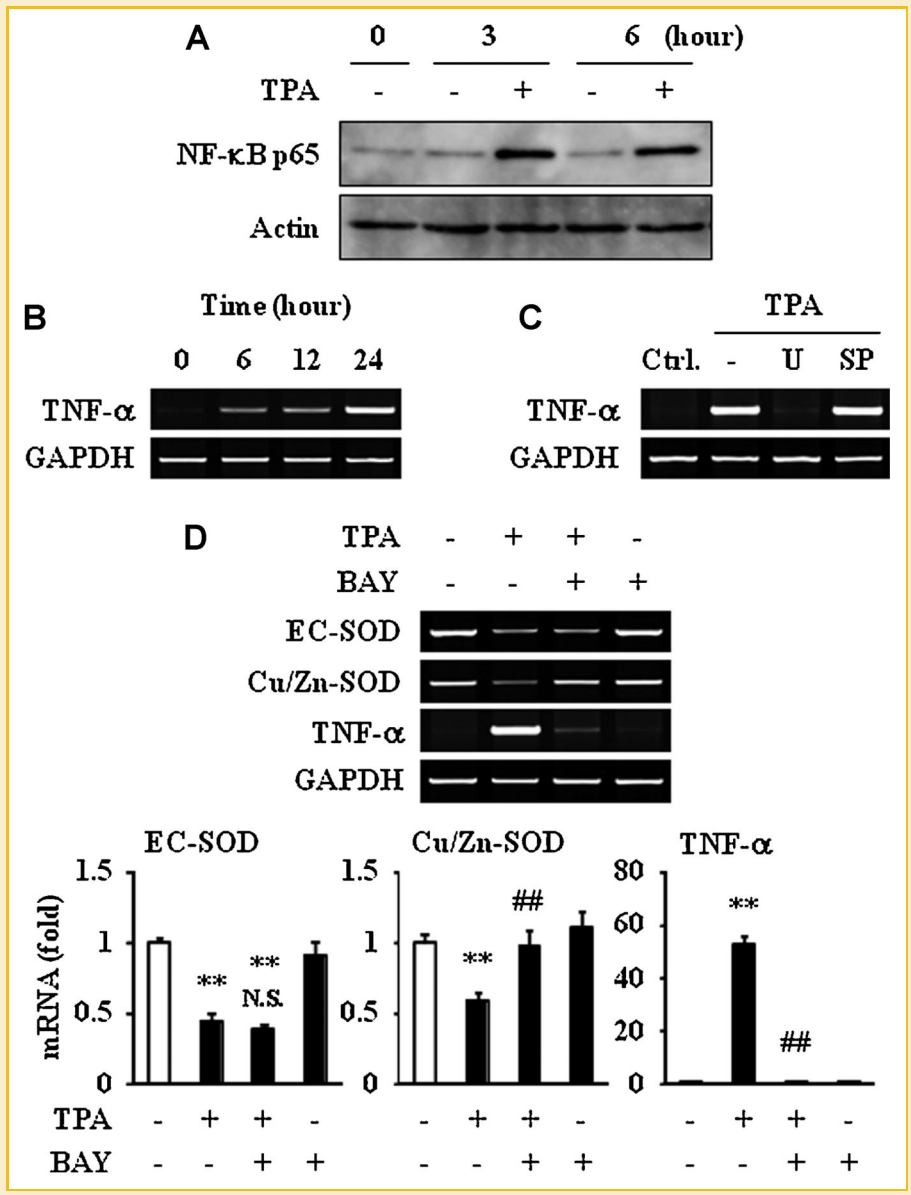

Fig. 6. Involvement of NF-κB in SODs expression. U937 cells were treated with or without 30 nM TPA for the indicated time. The nuclear translocation of NF-κB p65 (A) and the expression of TNF-α (B) were determined by Western blotting and RT-PCR, respectively. C: Cells were pretreated with or without 20 μM U0126 (U) or SP600125 (SP) for 30 min, and then treated with 30 nM TPA for 24 h. The expression of TNF-α mRNA was determined by RT-PCR. D: Cells were pretreated with or without 10 μM BAY11-7082 (BAY) for 1 h, and then treated with or without 30 nM TPA for 24 h. After the cells had been treated, RT-PCR was carried out. All RT-PCR data were normalized using GAPDH levels ($^{**}P < 0.01$ vs. untreated cells, $\#\#P < 0.01$, N.S., not significant vs. TPA-treated cells).

tory cytokines in the vascular system [Takatsu et al., 2001; Heistad, 2006; Laurila et al., 2009]. We have reported that the expression of EC-SOD was increased during the differentiation of 3T3-L1 preadipocytes into mature adipocytes [Adachi et al., 2009]. Therefore, we hypothesized that the expression of EC-SOD is also regulated during monocytic differentiation by TPA, and the change in EC-SOD expression might have an important role in the resistance to oxidative stress. From the results shown in Figure 2, treatment of cells with TPA increased the expression of Mn-SOD, but decreased that of Cu/Zn-SOD, similar to previous reports. Moreover, we first observed the reduction of EC-SOD at mRNA and protein levels during monocytic differentiation.

It is well recognized that ROS act as signaling molecules involved in various physiological processes, including monocytic differentiation of phagocytic cells [Droge, 2002; Finkel, 2003; Nathan, 2003; Kiningham et al., 2008]. Moreover, we have been reported that the excess production of ROS led to a decreased expression of EC-SOD in kidney tubular cells [Kamiya et al., 2008]. We therefore investigated the involvement of ROS in the regulation of SODs during monocytic differentiation. From the results shown in Figures 3 and 4, pretreatment with GF109203X or DPI suppressed TPA-triggered ROS production and alteration of SODs expression. These results have suggested that increased ROS act as modulators of TPA-triggered SODs regulation.

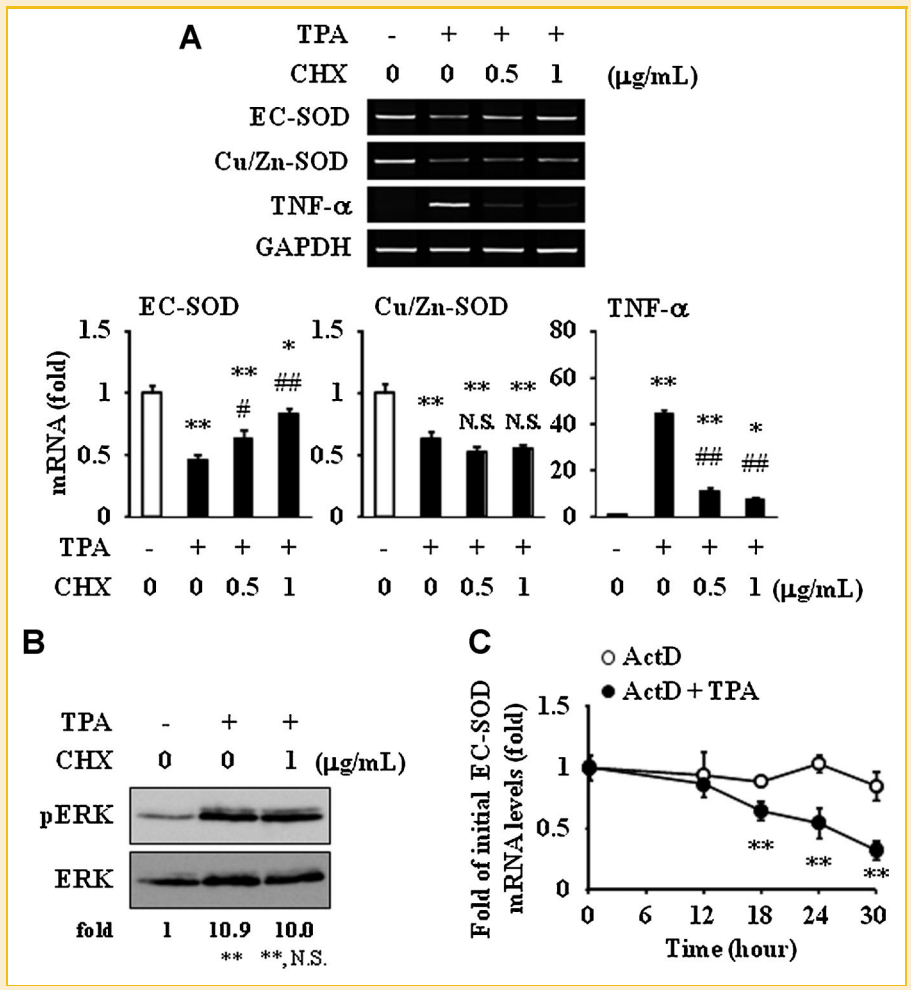

Fig. 7. Involvement of newly synthesized protein in EC-SOD expression. A: U937 cells were pretreated with or without the indicated concentrations of cycloheximide (CHX) for 1 h, and then treated with 30 nM TPA for 24 h. After the cells had been treated, RT-PCR was carried out. All RT-PCR data were normalized using GAPDH levels ($^{*}P < 0.05$, $^{**}P < 0.01$ vs. untreated cells, $\#P < 0.05$, $\#\#P < 0.01$, N.S., not significant vs. TPA-treated cells). B: U937 cells were pretreated with or without 1 μg/ml CHX for 1 h, and then treated with 30 nM TPA for 15 min. After the cells had been treated, Western blotting was carried out. Values are mean of fold change relative to TPA-untreated cells ($n = 3$, $^{**}P < 0.01$ vs. untreated cells, N.S., not significant vs. TPA-treated cells). C: Stability of EC-SOD mRNA during monocytic differentiation induced by TPA. U937 cells were treated with (closed circle) or without (open circle) TPA for the indicated time in the presence of 0.1 μg/ml actinomycin D (ActD). Residual mRNA was determined by RT-PCR. Values represent the fold of residual mRNA level versus mRNA level at time 0. All RT-PCR data were normalized using GAPDH levels ($^{**}P < 0.01$ vs. TPA-untreated cells).

MAPK have been shown to act as central mediators of the regulation of biological response mechanisms, including cell growth, proliferation, differentiation, and death. There are three types of MAPK in many kinds of cells: ERK, JNK, and p38 [Kyriakis and Avruch, 2001; Pearson et al., 2001]. Our previous reports showed that MAPK acts as a regulator of EC-SOD expression in smooth muscle cells [Adachi et al., 2006], renal tubular interstitial cells [Kamiya et al., 2008], and adipose cells [Kamiya et al., 2010]. In this study, we observed the activation of MEK/ERK and JNK at 15 and 30 min after TPA stimulation, respectively (Fig. 5A). Moreover, ERK activation was maintained up to 24 h (data not shown). On the other hand, the activation of p38 was not detected. Numerous studies have shown that PKC- and MEK/ERK-derived signal transduction plays an important role in differentiation processes; inhibition of this activation leads to diminished TPA-induced expression of CD markers [Miranda et al., 2002], Toll-like receptor 2 [Jang et al., 2005], and Mn-SOD [Traore et al., 2008]. On the other hand, the activation of JNK signaling did not directly affect these differentiation-derived processes [Jang et al., 2005]. Concomitant with other cases, the reduction of EC-SOD and Cu/Zn-SOD was mediated by MEK/ERK signaling (Fig. 5B). We further observed that TPA-triggered ERK activation was suppressed by pretreatment with GF109203X, DPI, or U0126 (Fig. 5C). These observations suggested that NOX-derived ROS together with PKC act as modulators of TPA-triggered ERK activation.

It is well known that NF-κB, a transcriptional factor, regulates the release of inflammatory cytokines, such as TNF-α, interleukin-6, and interferon-γ, in some inflammatory diseases, including asthma, rheumatoid arthritis, and atherosclerosis [Papadaki et al., 2002]. NF-κB activation is mediated by the nuclear translocation of NF-κB p65/p50 subunits, following the promotion of transcription by binding κB sites in the target genes. In U937 cells, NF-κB is reported to be activated by TPA and/or lipopolysaccharide, and this process

lead to an increase of TNF-α [Ortiz-Lazareno et al., 2008]. On the other hand, secreted TNF-α is known to suppress the expression of EC-SOD in smooth muscle cells [Adachi et al., 2006] and adiponectin in 3T3-L1 adipocytes [Kim et al., 2005], leading to decreased resistance to oxidative stress and acceleration of inflammatory disorders. In the present study, treatment with TPA induced nuclear translocation of NF-κB p65 and TNF-α expression (Fig. 6A,B). Moreover, Figure 6D shows that pretreatment with BAY11-7082 significantly suppressed the reduction of Cu/Zn-SOD by TPA; however, interestingly, this inhibitor did not affect that of EC-SOD. These observations suggested that NF-κB is a key factor in the reduction of Cu/Zn-SOD, while the reduction mechanism of EC-SOD is different from that of Cu/Zn-SOD. We further observed a difference between the regulations of these SODs; newly synthesized protein is involved in the reduction of EC-SOD, but not Cu/Zn-SOD (Fig. 7A).

mRNA stability is a key factor in the increase and/or decrease of target genes in several biological processes; CCAAT/enhancer-binding protein β and p21 mRNA were stabilized by TPA, but mitochondrial elongation factor Tu mRNA was destabilized [Park et al., 2001; Takeuchi and Ueda, 2003; Goethe et al., 2007]. Moreover, in these reports, these alterations of mRNA stability were mediated by PKC activation. In this study, EC-SOD mRNA degradation was not observed in the early phase (0–12 h) after ActD and TPA treatment; however, degradation was accelerated in the late phase (18–30 h; Fig. 7C). These results indicate that the instability of EC-SOD mRNA by TPA participated in the reduction of that gene. Further, because degradation was observed in only the late phase and TPA slightly decreased the expression of EC-SOD in spite of the presence of U0126 (Fig. 5B), it might be suggested that the degradation mechanism was activated after MEK/ERK activation and/or through MEK/ERK-independent pathways.

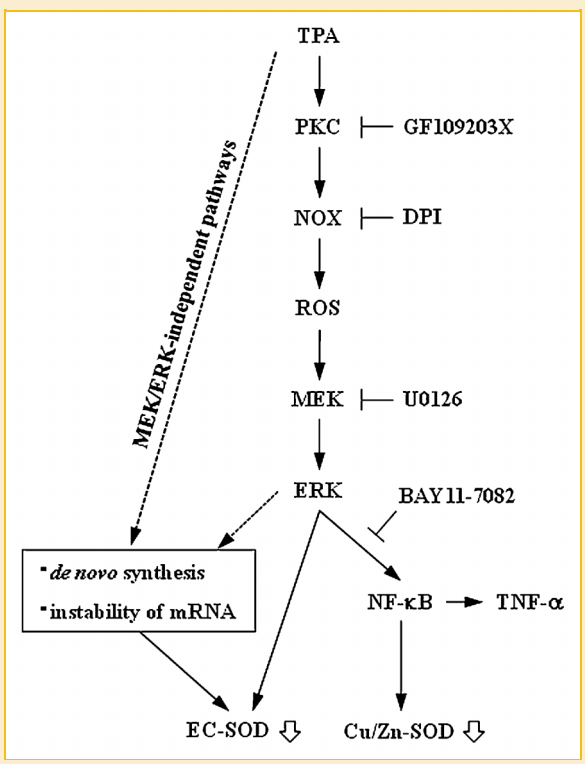

Fig. 8. Hypothesis of the mechanism involved TPA-induced SODs reduction. TPA activates PKC, NOX, MEK/ERK, and NF-κB signaling. Cu/Zn-SOD reduction is mediated by NF-κB activation, whereas that of EC-SOD is not regulated by NF-κB. Further, EC-SOD reduction was regulated by newly synthesized protein and was involved in the alteration of mRNA stability through MEK/ERK-dependent and/or -independent pathways.

In this study, we focused on the regulation of SODs, especially EC-SOD, during monocytic differentiation of U937 cells by TPA. We first revealed that the expression of EC-SOD is decreased during above process. Figure 8 shows that the hypothesis on the mechanism involved TPA-induced SODs reduction. Treatment with TPA activated several signal mediators in the order of PKC, NOX, MEK/ERK, and NF-κB. During monocytic differentiation induced by TPA, MEK/ERK played a critical role of the reduction of both EC-SOD and Cu/Zn-SOD; however, in this study, we detected two differences between the reduction mechanisms of EC-SOD and that of Cu/Zn-SOD. First, the involvement of NF-κB signaling; the reduction of Cu/Zn-SOD was mediated by NF-κB, whereas that of EC-SOD was not affected by NF-κB. Second, the involvement of de novo protein synthesis; the reduction of EC-SOD, but not Cu/Zn-SOD, was mediated by newly synthesized protein. These observations concomitantly indicated that these reduction mechanisms were not regulated by TNF-α. Moreover, in the reduction of EC-SOD, it was possible that the mRNA degradation process was accelerated, and these reactions lead to the reduction of EC-SOD. Overall, the reduction of EC-SOD during monocytic differentiation may contribute to the control of redox homeostasis during monocytic differentiation similar to other SOD isozymes.

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