Phororb Ester-induced Generation of Reactive Oxygen Species Is Protein Kinase Cβ-dependent and Required for SAPK Activation*

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Treatment of human U-937 myeloid leukemia cells with 12-O-tetradecanoylphorbol-13-acetate (TPA) is associated with protein kinase C (PKC) βII-mediated activation of the stress-activated protein kinase (SAPK) pathway. The present studies demonstrate that the TPA response of U-937 cells includes the generation of reactive oxygen species (ROS). By contrast, the TPA-resistant U-937 cell variant (TUR), which is deficient in PKCβII expression, failed to respond to TPA with the induction of ROS. Moreover, we show that TPA-induced ROS production is restored in TUR cells stably transfected to express PKCβII. The results also demonstrate that TPA-induced ROS production is required for activation of the MEK kinase-1 (MEKK-1)→SAPK pathway. In concert with this observation, treatment of U-937 with H2O2 as a source of ROS is associated with activation of the MEKK-1→SAPK cascade. These findings indicate that PKCβII is required for TPA-induced ROS production and that the MEKK-1→SAPK pathway is activated by a ROS-mediated mechanism.

Human myeloid leukemia cells respond to 12-O-tetradecanoylphorbol-13-acetate (TPA)1 and other agents that activate protein kinase C (PKC) with growth arrest and induction of a differentiated monocytic phenotype (1). These findings have indicated that factor-independent growth of myeloid leukemia cells is reversible by activation of PKC-mediated signaling. The PKC family of serine/threonine kinases consists of at least 12 isoforms that are involved in diverse cellular responses (2, 3). The available evidence indicates that PKCβII is essential for the TPA response of myeloid leukemia cells. In this context, TPA-resistant U-937 and HL-60 myeloid leukemia cell variants exhibit defects in PKCβII expression (4–9). Defective translocation of PKCβII from the cytosol to the cell membrane has also been identified in TPA-resistant variants (4, 10). Moreover, defects in the TPA response of myeloid leukemia cell variants are reversed by transfection of the PKCβII gene (9, 11).

The response of myeloid leukemia cells to TPA is associated with induction of the stress-activated protein kinase (SAPK), also known as Jun kinase or c-Jun NH2-terminal kinase (11). SAPK phosphorylates and thereby activates the c-Jun, ATF2, and Elk-I transcription factors (12–15). SAPK-mediated activation of c-Jun, ATF2, and Elk-I, in turn, contributes to induction of the c-Jun and Egr-I early response genes associated with the response of myeloid leukemia cells to TPA (16–19). Other work has demonstrated that PKCβII is necessary for TPA-induced activation of SAPK and that PKCβII associates directly with MEK kinase-1 (MEKK-1), an upstream effector of the SAPK cascade (11). MEKK-1 preferentially activates SEK1 (20–22) and consequently SAPK (23).

The present studies demonstrate that TPA treatment of myeloid leukemia cells is associated with the generation of reactive oxygen species (ROS). The results demonstrate that PKCβII is required for TPA-induced ROS production. We also show that ROS production is necessary for TPA-induced activation of the MEKK-1→SAPK pathway.

MATERIALS AND METHODS

Cell Culture and Reagents—Human U-937 myeloid leukemia cells (American Type Culture Collection, Manassas, VA) and the TPA-resistant TUR clone (4) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. TUR cells overexpressing PKCβII were prepared as described (11). The cells were treated with 32 nM TPA, 30 mM N-acetyl-L-cysteine (NAC), 1 mM pyrrolidinedithiocarbamate (PDTC), or 50 mM H2O2 (all from Sigma).

Measurement of ROS Production—Cells were incubated with 10 μM 2′,7′-dichlorofluorescein diacetate (DCF-DA) (Sigma) for 15 min at 37 °C to assess ROS-mediated oxidation of DCF-DA to the fluorescent compound DCF (24). Fluorescence of oxidized DCF was measured at an excitation wavelength of 480 nm and an emission wavelength of 525 nm using a flow cytometer (Becton Dickinson and Co., Lincoln Park, NJ). Generation of ROS was also measured by superoxide dismutase-inhibitable reduction of cytochrome c as described by Pick and Mizel (25). Briefly, cells (1×106/ml) suspended in Hank’ balanced salt solution with 10 mM HEPES and 50 μM cytochrome c (Sigma) were incubated with TPA in the presence or absence of superoxide dismutase. Superoxide dismutase-inhibitable reduction of cytochrome c reduction was assayed as described (25).

Isolation of Cytosolic and Membrane Fractions—Cytosolic and membrane fractions were prepared as described (10). Cells were suspended in TEM lysis buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM sodium-mercaptoethanol) and sonicated. After sedimentation of nuclei, the extracts were centrifuged at 55,000 × g for 30 min. The supernatant was used as the cytosolic fraction. The pellets were solubilized in TEM buffer containing 1% Nonidet P-40.

Immunoblot Analysis—Cell fractions were subjected to electrophoresis in 10% SDS-polyacrylamide gels, and the proteins were transferred to nitrocellulose paper. Residual binding sites were blocked by incubating the filters with 5% dry milk in PBST (phosphate-buffered saline, 0.05% Tween 20). The filters were incubated with anti-PKCβII (Santa Cruz Biotechnology, Santa Cruz, CA). After washing twice with PBST, the blots were incubated with anti-rabbit IgG peroxidase conjugate (Amersham Pharmacia Biotech). The antigen-antibody complexes were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

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1 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; SAPK, stress-activated protein kinase; ROS, reactive oxygen species; NAC, N-acetyl-L-cysteine; PDTC, pyrrolidinedithiocarbamate; DCF-DA, 2′,7′-dichlorofluorescein diacetate; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MEKK-1, MEK kinase-1; GST, glutathione S-transferase; SEK1, SAPK/extracellular signal-regulated kinase kinase 1.
visualized by chemiluminescence (ECL detection system, Amersham Pharmacia Biotech).

Immune Complex Kinase Assays—MEKK-1 kinase assays were performed as described (11). Briefly, lysates were incubated with anti-MEKK-1 (provided by Dr. Gary Johnson, University of Colorado, Denver, CO) for 1 h at 4 °C and then for 30 min after addition of protein A-Sepharose. The immune complexes were washed and resuspended in kinase buffer (50 mM HEPES, pH 7.4, 10 mM MgCl₂, 20 μM ATP/γ-32P]ATP) containing 5 μg of glutathione S-transferase (GST)-SEK1(K-R) (Lys-129 to Arg mutant) (provided by Dr. Leonard Zon, Harvard Medical School, Boston, MA). The reaction mixtures were incubated for 5 min at 30 °C and terminated by the addition of SDS sample buffer. SAPK kinase assays were performed as described (11). Samples were analyzed by 10% SDS-polyacrylamide gel electrophoresis and autoradiography. Autoradiograms were scanned, and intensity of signals was quantitated by laser densitometry.

RESULTS

To determine whether treatment of myeloid leukemia cells with TPA is associated with ROS generation, U-937 cells were incubated with DCF-DA, and ROS-mediated oxidation of the fluorochrome was assayed by flow cytometry. Compared with control cells, treatment with TPA was associated with increases in ROS that were detectable at 5 and 15 min (Fig. 1A, upper panel). To extend these findings, ROS generation was also assessed by monitoring the reduction of cytochrome c. Using this approach, the results confirmed that TPA induces the production of ROS (Fig. 1A, lower panel). As additional controls, cells were preincubated with NAC, a precursor of glutathione and scavenger of reactive oxygen intermediates (26, 27). As assessed by both oxidation of DCF-DA and cytochrome c reduction, NAC treatment was associated with abrogation of TPA-induced ROS production (Fig. 1B and data not shown). Similar findings were obtained with the ROS scavenger PDTC (Fig. 1C). These results demonstrate that the response of myeloid leukemia cells to TPA includes the generation of ROS.

Treatment of myeloid leukemia cells with TPA is associated with translocation of PKCβII from the cytosol to the cell membrane (11). To determine whether ROS generation is essential for TPA-induced translocation of PKCβII to the cell membrane, cells were pretreated with NAC or PDTC. TPA treatment of U-937 cells resulted in the detection of PKCβII in the cell membrane fraction at 5–30 min (Fig. 2A). Pretreatment with NAC had no effect on the distribution of PKCβII in control cells (Fig. 2B). Moreover, NAC had no detectable effect on TPA-induced translocation of PKCβII to the cell membrane (Fig. 2B).
Similar results were obtained with cells pretreated with PDTC and then exposed to TPA (Fig. 2C). These findings indicate that TPA-induced ROS production is dispensable for translocation of PKCβII from the cytosol to the cell membrane.

To determine whether PKCβII is essential for TPA-induced ROS production, we used the U-937 cell variant, designated TUR, which is deficient in PKCβII expression (4), and TUR cells stably transfected to express the PKCβII gene (TURβII) (11). Immunoblot analysis with anti-PKCβII confirmed that, although PKCβII levels were decreased in TUR cells, expression of PKCβII in TURβII cells was comparable with that in U-937 cells (Fig. 3A). In contrast to wild-type U-937 cells, treatment of the TUR variant with TPA resulted in no detectable induction of ROS production (Fig. 3B). However, the results demonstrate that expression of PKCβII in TUR cells restores TPA-induced ROS production (Fig. 3B). Using the reduction of cytochrome c as an additional assay system, the results confirmed that TURβII cells, but not TUR cells, respond to TPA with generation of ROS (Fig. 3C). These findings demonstrate that TPA induces ROS production by a PKCβII-dependent mechanism.

Previous studies have demonstrated that PKCβII interacts with MEKK-1 as an upstream effector of SAPK activation in the TPA response (11). To determine whether ROS generation contributes to the activation of MEKK-1, we assayed anti-MEKK-1 immunoprecipitates for phosphorylation of GST-Jun. The results demonstrate that, as shown previously (11), TPA treatment is associated with induction of MEKK-1 activity (Fig. 4A). To determine whether ROS production is involved in activation of MEKK-1, the cells were pretreated with NAC and then assayed for MEKK-1 activity. The results demonstrate that NAC blocks TPA-induced activation of MEKK-1 (Fig. 4A). The activation of MEKK-1 by TPA was also inhibited by PDTC (Fig. 4A). To further assess involvement of ROS in induction of MEKK-1 activity, the cells were treated with H2O2 as a source of reactive oxygen intermediates. The results demonstrate that H2O2 induces the activation of MEKK-1 (Fig. 4A). The finding that NAC blocks H2O2-induced MEKK-1 activity provided further support for involvement of ROS (Fig. 4A). Cell lysates were also subjected to immunoprecipitation with anti-SAPK, and the immunoprecipitates were assayed for phosphorylation of GST-Jun. The results demonstrate that treatment with NAC blocks TPA-induced SAPK activity (Fig. 4B). Similar findings were obtained with PDTC (Fig. 4B). As a control, the treatment of U-937 cells with H2O2 was associated with SAPK activation and, like induction by TPA, this response was sensitive to NAC and PDTC treatment (Fig. 4B and data not shown). These findings indicate that TPA activates the MEKK-1→SEK1→SAPK pathway by a ROS-dependent mechanism.

**DISCUSSION**

The finding that human myeloid leukemia cells respond to inducers of differentiation with growth arrest and the induction of a differentiated phenotype has indicated that their growth factor-independent phenotype is reversible. The induction of myeloid leukemia cell differentiation by TPA is depend-
TPA Induces SAPK by ROS-dependent Signaling

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