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Redox-Regulation of ERK 1/2-Directed Phosphatase by Reactive Oxygen Species:

Role in Signaling TPA-Induced Growth Arrest in ML-1 Cells

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Abstract

Extracellular signal-regulated kinase (Erk)1/2 activity signals myeloid cell differentiation induced by 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Previously, we reported that Erk1/2 activation (phosphorylation) induced by TPA required reactive oxygen species (ROS) as a second messenger. Here, we hypothesized that ROS generated in response to TPA inhibit Erk1/2-directed phosphatase activity, which leads to an increase phosphorylation of Erk1/2 to signal p21^{WAF1/Cip1}-mediated growth arrest in ML-1 cells. Incubation of ML-1 cells with TPA resulted in a marked accumulation of phosphorylated Erk1/2, and is subsequent to H₂O₂ generation. Interestingly, post-TPA-treatment with N-acetylcysteine (NAC) stimulated a marked and a rapid dephosphorylation of Erk1/2, suggesting a regeneration of Erk1/2-directed phosphatase activity by NAC. ROS generation in ML-1 cells induced by TPA was suggested to occur in the mitochondrial electron transport chain (METC) based on the following observations: (i) undifferentiated ML-1 cells not only lack p67-phox and but also express a low level of p47-phox key components required for NADPH oxidase enzymatic activity, (ii) pretreatment with DPI, an inhibitor of NADH- and NADPH-dependent enzymes, or rhein, an inhibitor of complex I, blocked the ROS generation, and (iii) examination of the microarray analysis data and Western blot analysis data revealed an induction of MnSOD expression at both mRNA and protein levels in response to TPA. MnSOD is a key member of the mitochondrial defense system against mitochondrial-derived superoxide. Together, this study suggested that TPA stimulated ROS generation as a second messenger to activate Erk1/2 via a redox-mediated inhibition of Erk1/2-directed phosphatase in ML-1 cells.

Reactive oxygen species (ROS) are generated from partial reduction of molecular oxygen. Superoxide (O₂^{•-}), the first member of ROS, is generated when a single electron interacts and partially reduces molecular oxygen (Reid and Durham, 2002). Dismutation of O₂^{•-} forms hydrogen peroxide (H₂O₂) which is then reduced to water by the enzymatic actions of catalase, glutathione peroxidase, or other peroxidases. Following the discovery of ROS, the primary focus was on the oxidative damage elicited by ROS within cells (Agarwal and Sohal, 1994a, 1994b). However, in the last several years, physiological roles have been attributed to ROS generation in cells (Chen et al., 1995; Sundaresan et al., 1995; Bae et al., 1997; Bogoyevitch et al., 2000; Rinna et al., 2006). In a recent study, we demonstrated that TPA-induced ROS generation signaled expression of p21^{WAF1/Cip1} in human acute myeloid leukemia cells THP-1 (Traore et al., 2005). p21^{WAF1/Cip1}, a 21-kDa protein and inhibitor of cyclin-dependent kinases

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is a critical downstream effector in the p53-specific pathway of cell cycle control. However, p21 can also be induced to express by p53-independent pathways during initiation of terminal differentiation, and has been implicated in mediating cell cycle arrest in response to extracellular factors including phorbol esters, irradiation, H₂O₂, tumor necrosis factor- α , and transforming growth factor- β (TGF- β) (Michieli et al., 1994; Akashi et al., 1995; Datto et al., 1995; Biggs et al., 1996; Droge, 2002; Traore et al., 2005).

Potential sources of ROS in phagocytic cells include NADPH oxidase and the mitochondrial electron transport chain (METC) (Forman and Torres, 2002; Ichikawa et al., 2004). Mitochondria-generated cellular energy, ATP, is synthesized through oxidative phosphorylation, a process during which the METC accepts electrons from NADH and FADH₂ (Weiss et al., 1991; Devin and Rigoulet, 2006). As electrons are passed down the METC, molecular oxygen is eventually converted to water (Ichikawa et al., 2004). O₂^{-•} is generated when a single electron prematurely leaves the METC and partially reduces molecular oxygen. ROS generated in the METC during the oxidative phosphorylation activity has been shown to modulate multiple molecular processes, signaling pathways, and transcription factor activities (Kim et al., 1999; Ichikawa et al., 2004; Kim et al., 2005).

The concept that ROS can stimulate cellular signal transduction cascades has been continuously advancing in recent years (Chen et al., 1995; Sundaresan et al., 1995; Yoshizumi et al., 2000; Traore et al., 2005; Traore et al., 2007). Numerous studies have indicated that ROS play a role as messengers or effector molecules to activate members of a large family of enzymes known as the mitogen-activated protein kinases (MAPKs) including JNK and p38 in response to various extracellular stimuli including TNF- α and lysophosphatidic acid (Chen et al., 1995; Yoshizumi et al., 2000; Kamata et al., 2005). In our previous studies, we have shown that ROS was required for TPA-induced extracellular signal-regulated kinase (Erk)1/2 activation in ML-1 and THP-1 cells (Traore et al., 2005; Traore et al., 2007). Furthermore, studies have shown that JNK, P38, and Erk1/2 are phosphorylated by treatment with exogenous H₂O₂ and this event can be inhibited by pretreatment with anti-oxidants (Abe et al., 1996; Lo et al., 1996; Guyton et al., 1996b). Despite these well-documented findings, the mechanism by which ROS activates MAPKs is not well understood. However, one well-accepted model is that ROS transiently inactivates regulatory phosphatases via interaction with catalytically active, redox sensitive cysteines in the active site (Denu and Tanner, 1998; Kamata et al., 2005).

While our previous studies identified the importance of ROS-mediated signaling in ERK-dependent THP-1 cell growth inhibition and ICAM-1 expression in ML-1 cells, we did not identify the mechanism of Erk1/2 activation in response to TPA (Traore et al., 2005; Traore et al., 2007). In this study, we investigated the mechanism of ROS-mediated Erk1/2 activation induced by TPA in human myeloid leukemia cells ML-1. We found that TPA-induced PKC-stimulated ROS generation inactivates Erk1/2-directed phosphatases and promotes a transient and reversible activation of Erk1/2 cells to signal growth inhibition in ML-1 cells.

Materials and Methods

Reagents and antibodies

The pharmacologic inhibitors for MEK (PD98059 and U0126) were purchased from Sigma (St. Louis, MO). The pharmacologic inhibitor for PKC (GF109203X) was obtained from ALEXIS Biochemicals (San Diego, CA). The pharmacologic inhibitor for mitochondria complex I and NADPH oxidase diphenyleneiodium (DPI), the competitive inhibitor of mitochondria complex I (rhein) (Kean et al., 1971; Floridi et al., 1989), the inhibitor for mitochondria complex I (rotenone), and the ROS scavenger N-acetylcysteine (NAC) were purchased from Sigma. Anti-human phospho-Erk1/2 antibodies were purchased from Cell

Signaling Technology, Inc. (Beverly, MA). Anti-human p21^{WAF1/Cip1} antibodies were purchased from BD Pharmingen (San Diego, CA). Anti-human p47-phox antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-human p67-phox antibodies were from BD-Transduction Laboratories (Lexington, KY).

Cell culture

Human myeloid leukemia ML-1 cells were obtained from Dr. Ruth Craig, Dartmouth School of Medicine. Human myeloid leukemia THP-1 cells were purchased from American Type Cell Collection (ATCC). All cell culture media and supplements were obtained from Invitrogen (Carlsband, CA). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin) in 150 cm² tissue culture flasks, and maintained at 37°C in a humidified 5% CO₂ atmosphere. Experiments were routinely carried out using cells in the log phase of growth. 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO to obtain a 100 µM stock solution and further diluted before use. For all experiments, cells were cultured at an initial density of 5×10^5 cells/ml and treated with TPA for various concentrations and times as indicated. For negative controls, the cells were incubated in the absence of TPA, in medium containing an equivalent 1% (v/v) DMSO. Viability was determined by hemocytometer counts of trypan blue-impermeable cells.

Microarray analysis

ML-1 cells were grown at density of 10^5 cells/ml in 150 ml dish and treated with vehicle (DMSO) or 5 nM TPA (n = 3) 6 h, 3 and 6 days. Total RNA was extracted using Trizol reagent and purified with the Qiagen Rneasy minikit. The purified RNA was subjected to Affymetrix oligonucleotide microarray analysis using Human Genome U133 2.0 Plus Array Chip. Six replicates, including three controls and three TPA treated ML-1 cells samples were examined.

RT-PCR

RNA samples representing ML-1 cells incubated in the presence or absence of TPA for 6 h. Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Total RNA (2 µg) was used for cDNA synthesis. RT-PCR analyses were performed using assay probe sets from Applied Biosystems (Foster City, CA). Assays were performed by using the ABI 7000 Taqman system (Applied Biosystems). GAPDH was used for normalization. The cycle threshold (C_T) value indicates the number of PCR cycles that are necessary for the detection of a fluorescence signal exceeding a fixed threshold. The fold change (FC) was calculated by using the following formulas: $\Delta C_T = C_T(\text{GAPDH}) - C_T(\text{target gene})$ and $FC = 2^{-(\Delta C_{T2} - \Delta C_{T1})}$, in which ΔC_{T1} represents the highest C_T value among all the samples and ΔC_{T2} represents the value of a particular sample.

Western-blot analysis

Cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 50 mM sodium fluoride, 5 mM EDTA, 40 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10⁻⁴ M phenylmethylsulfonyl fluoride, 10⁻⁶ M leupeptin, 10⁻⁶ M pepstatin A, 1% (v/v) Triton X-100. Lysates were clarified by centrifugation at 13,000g for 10 min. Samples, each containing 40 µg of cell lysates in loading buffer, were subjected to SDS/PAGE (12% gel) electrophoresis and proteins were transferred to a PVDF membrane (Immobilon; Millipore, Bedford, MA). Immunoblotting was performed using anti-human p47-phox, p67-phox, MnSOD and p21^{WAF1/CIP1} diluted 1:1,000. After washing the blots, horseradish-conjugated anti-IgG, diluted 1:2,000, was added to the blots. Immunoreactivity was detected by chemiluminescence (CL) (Perkin-Elmer, Life Sciences, MA).

Determination of phospho-Erk1/2 by FACS analysis

The levels of phospho-Erk1/2 were determined using FACS-Scan analysis. Cells (1×10^6) were washed twice in PBS, fixed in 2% formaldehyde in PBS and permeabilized with ice cold 90% methanol. Cells were resuspended in PBS containing 0.1% sodium azide and 5% FBS for 30 min, incubated with (Alexa-fluor 488)-conjugated anti-phospho-Erk1/2 antibodies for 1 h, washed twice in PBS and analyzed by FACS-Scan analysis. Negative controls were stained with isotype-matched (Alexa-Fluor 488)-conjugated IgG and compensation was adjusted using the single-stained cell samples. The fluorescence intensities were determined using Cellquest software (Becton Dickinson, Bedford, MA).

Measurement of ROS generation by

Luminol-derived chemiluminescence (CL)—The generation of cellular ROS, in particular H_2O_2 , was assessed using luminol-derived CL as previously described (Traore et al., 2005; Traore et al., 2007). One million cells were preincubated with luminol (10 μ M) and horseradish peroxidase (10 μ g/ml) in complete PBS (PBS with 0.5 mM $MgCl_2$, 0.7 mM $CaCl_2$, 0.1% glucose) for 10 min followed by TPA treatment. The CL was measured continuously in a Berthold LB9505 luminometer (Pforzheim, Germany) at 37°C for 50 min.

FACS analysis using fluorephore hydroethidine—To detect intracellular ROS, hydroethidine was used as probe. ML-1 cells were first incubated for 5 min with a 0.5 μ M hydroethidine Sigma at 37°C followed by 30 min incubation with TPA or DMSO as control. The reaction was stopped by incubating cells for 10 min on ice followed by two washes with PBS. ROS was determined within 1 h on a FACSCaliber flow cytometry (BD Biosciences, San Jose, CA) using fluorescent intensity of excitation at 490 nm and emission at 520 nm.

Protein phosphatase activity

The cellular phosphatase activity was assessed using the 3, 6-fluorescein diphosphate (FDP) dependent fluoregenic phosphatase substrate assay (AnaSpec, San Jose, CA). ML-1 cells were incubated in media in the presence or absence of TPA for 1 h. Cells were harvested, washed twice with PBS and lysed in buffer containing 1% Triton X-100, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 100 μ g/ml PMSF, and 10 μ g/ml pepstatin. Phosphatase activity assay was conducted according to manufacturer protocol using phosphatase assay buffer without DTT (AnaSpec).

Results

TPA treatment inhibits ML-1 cell proliferation by upregulating p21^{WAF1/cip1} expression

One of the required cellular processes necessary for differentiation to occur is the inhibition of cell growth. In our previous report, we demonstrated that incubation of human myeloid THP-1 cells with TPA resulted in growth arrest at G₁ phase of the cell cycle (Traore et al., 2005). In this study, in order to better understand the mechanism of TPA-induced growth arrest, we examined the effect of TPA on another human myeloid cell line, ML-1 cells. The cell proliferation assay data shown in Figure 1 indicated that TPA inhibits the proliferation of both ML-1 and THP-1 cells for up to 72 h. Examination of microarray analysis data demonstrated that TPA induces the expression of negative regulators and inhibits the expression of positive regulators of the cell cycle (Table 1). In Figure 2, data revealed an increase in p21^{WAF1/Cip1} expression at the mRNA and protein levels in ML-1 cells in response to TPA.

TPA induces p21^{WAF1/Cip1} expression via a PKC, ROS, and MAPK dependent mechanism

ROS-mediated Erk1/2 activation has been implicated in signaling p21^{WAF1/cip1} expression induced by TPA in human myeloid cells THP-1 (Traore et al., 2005). Using GF109203X (a

PKC inhibitor), N-acetylcysteine, NAC (an ROS scavenger), and DPI (an inhibitor of NADH and NADPH dependent enzymes) we assessed the role of PKC and ROS in p21^{WAF1/Cip1} expression induced by TPA in ML-1 cells. Pretreatment with GF109203X, NAC, and DPI effectively blocked p21^{WAF1/Cip1} expression (Fig. 3). Moreover, p21^{WAF1/Cip1} expression was also suppressed by U0126 (a MEK inhibitor) (Fig. 3). These findings indicated the involvement of PKC, ROS, and the MAPK cascade in p21^{WAF1/Cip1} expression induced by TPA in ML-1 cells and support our previous report on the mechanism of TPA-induced growth arrest in THP-1 cells (Traore et al., 2005).

TPA induces ROS generation in absence of p67-phox in ML-1 cells

Previous studies have indicated that TPA induces ROS generation in undifferentiated U937 and THP-1 cells (Datta et al., 2000; Traore et al., 2005). Data shown in Figure 4A illustrates the detection of extracellular H₂O₂ by luminol-dependent CL within minutes following addition of TPA to ML-1 cells and its inhibition by GF109203X and NAC assuring the involvement of PKC and the effectiveness of NAC as scavenger of ROS generated in response to TPA in ML-1 cells. Similarly, increased intracellular ROS was also detected using dihydroethidine-dependent fluorescence in ML-1 cells as a result of the addition of TPA (Fig. 4B).

Major sources of ROS in differentiated myeloid cells include NADPH oxidase (Forman and Torres, 2001) and the METC (Rembish and Trush, 1994). Following the TPA-induced differentiation of ML-1 cells to macrophages, the mitochondria are also a cellular source of ROS (Li and Trush, 1998; Li et al., 1998; Li et al., 1999b). To determine the involvement of NADPH oxidase in the ROS generation induced by TPA, we examined the levels of p47-phox and p67-phox protein (two key components of NADPH oxidase) in undifferentiated (untreated) and 3 days TPA-treated THP-1 and ML-1 cells. Western blot analysis data shown in Figure 5 indicated a low level of p47-phox and a complete absence p67-phox expression in undifferentiated (untreated) ML-1 cells. However, both components p47-phox and p67-phox levels were detectable in undifferentiated THP-1 cells, and were further increased following differentiation in both THP1 and ML-1 cells. The expression of p47-phox and p67-phox observed following addition of TPA first occurs after 24 h of incubation with TPA in ML-1 cells (data not shown). In addition, to determine the involvement of mitochondria as the source of ROS we assessed the effect of DPI, rotenone and rhein (a competitive inhibitor of mitochondria complex I) (Kean et al., 1971; Floridi et al., 1989). As shown in Figure 6, DPI and rhein inhibited H₂O₂-dependent luminol-derived CL in both the THP1 and ML-1 cells following the addition of an equivalent concentration of TPA. Interestingly, rotenone a mitochondria complex I inhibitor increased luminol-derived CL induced by TPA in ML-1 cells, an event that is also blocked by DPI (Fig. 6). While DPI can inhibit both NADPH oxidase and complex I of the METC (Li and Trush, 1998), ML-1 cells lack the p67-phox component necessary for NADPH oxidase enzymatic activity (Fig. 5).

MnSOD, a key member of the mitochondrial defense system against METC-derived O₂⁻ (Kim et al., 2005), expression was induced at both the mRNA and protein levels (Fig. 7). MnSOD expression was further linked to PKC, MAPK, and ROS as pretreatment with GF109203X, PD9059, U0126, and DPI also blocked MnSOD expression (Fig. 8). Taken together, the above findings indicated that TPA-induced ROS generation in ML-1 cells occurred in absence of p67-phox a key component of NADPH oxidase. The data also suggested mitochondria ETC at least in part as the source of the observed low level of ROS generated in ML-1 cells in response to TPA.

Kinetics of Erk1/2 phosphorylation induced by TPA correlates with ROS generation

As we have previously shown, Erk1/2 is important in signaling both the early events (growth inhibition) as well as the overall differentiation of ML-1 cells (He et al., 1999). H₂O₂ has been shown to play a role in the activation of MAPKs (p38, Erk1/2, and JNK) induced by extracellular stimuli including TNF- α , TPA, and lysophosphatidic acid (Lo et al., 1996; Bouaouina et al., 2004; Kamata et al., 2005; Traore et al., 2005; Traore et al., 2007). To understand the mechanism of Erk1/2 activation induced by TPA in ML-1 cells, we first examined the kinetics of Erk1/2 phosphorylation induced by TPA. Several independent experiments were conducted using Western blot and flow cytometry analysis. Treatment with TPA resulted in a marked increase in phosphorylation (activation) of Erk1/2 within 15 min of incubation with TPA (data not shown). The highest level of phospho-Erk1/2 induced by TPA was observed 1 h after incubation with TPA (Fig. 9A and B) subsequent to H₂O₂ generation induced by TPA (Fig. 4), which could be blocked by pretreatment with NAC (Fig. 10A). The level of Erk1/2 phosphorylation 6 h after TPA treatment was much less than at 3 h and correlated with increase in DUSP6 protein levels (Fig. 9A and B). In addition, incubation with exogenous H₂O₂ also induced Erk1/2 phosphorylation (Fig. 9B), assuring the effectiveness of H₂O₂ in mediating of Erk1/2 phosphorylation in ML-1 cells. These observations indicated that Erk1/2 activation (phosphorylation) in response to TPA is rapid and required ROS generation in ML-1 cells. However, the Erk1/2 deactivation (dephosphorylation) is a slow process and occurs 3–6 h after TPA treatment.

TPA and H₂O₂ inactivates Erk1/2-directed phosphatase activity

Erk1/2 activity is controlled by a critical balance between the activities of an activating kinase MEK and inhibitory phosphatases activity (Gronda et al., 2001; Nika et al., 2004). Given the reported sensitivity of protein phosphatases to the cellular redox state (Lo et al., 1996; Lee et al., 1998; Kamata et al., 2005), we examined whether the modulation of Erk1/2-directed phosphatases activity by ROS played a role in TPA-induced Erk1/2 increased phosphorylation (activation) in ML-1 cells. A cell-based assay was utilized to assess Erk1/2 directed phosphate activity by monitoring the kinetics of intracellular Erk1/2 dephosphorylation and in the presence or absence of the anti-oxidant (reducing agent) NAC. ML-1 cells were incubated in media containing TPA for 1 h, to reach maximal levels of intracellular Erk1/2 phosphorylation (Fig. 10). One hour post-TPA-treated cells were then incubated in the presence of 25 mM NAC for various times and the levels of phospho-Erk1/2 were determined by flow cytometry (Fig. 10B). The kinetics of Erk1/2 dephosphorylation in the absence (Fig. 9A) or in the presence (Fig. 10B) of NAC would then be indicative of Erk1/2-directed phosphatase activity. As shown in Figure 10B, the post-TPA-treatment with NAC stimulated a marked and rapid dephosphorylation of Erk1/2 within minutes of treatment with NAC, presumably in response to a rapid regeneration of the phosphatase activity by NAC. In addition, a direct analysis of phosphotyrosine phosphatase activity revealed a dose dependent decrease of cellular phosphotyrosine phosphatase activity in response to TPA and H₂O₂ in ML-1 cells (Fig. 10C). Together, our data suggested that Erk1/2 activation induced by TPA occurred at least in part in response to a ROS-mediated inactivation of Erk1/2-directed phosphatase activity in ML-1 cells.

DUSP5 and DUSP6 expression induced by TPA occurs in response Erk1/2 activation

To further understand the mechanism of Erk1/2 regulation in response TPA, we examined whether Erk1/2 inactivation which occurred 6 h after its activation by TPA was associated with de novo synthesis of delayed Erk1/2-directed phosphatase in ML-1. Examination of microarray analysis data and RT-PCR data revealed a significant induction of DUSP5 and DUSP6 (dual specific phosphatases) expression at mRNA levels 6 h after TPA treatment (Fig. 10D). This event could be blocked by pretreatment with PKC and MEK inhibitors, and by NAC, ROS

scavenger (Fig. 10D), suggesting the role of PKC and Erk1/2 activation in this process. Furthermore, Western blot analysis data shown in Figure 9B indicates that the kinetic of Erk1/2 dephosphorylation (inactivation) which occurs 3–6 h after treatment with TPA correlates with an increase accumulation of DUSP6 protein in ML-1 cells. These observations suggested that the induction of DUSP6 protein expression, 3–6 h following Erk1/2 activation may have occurred to regulate Erk1/2 activity in ML-1 cells.

Discussion

A key role of low-level cellular ROS that has recently developed is in mediating signal transduction systems (Lo et al., 1996; Bouaouina et al., 2004; Kamata et al., 2005; Traore et al., 2005; Traore et al., 2007). For example, H₂O₂ generation in response to extracellular stimuli has been implicated as a second messenger that initiates activation of protein kinase cascades (Robinson et al., 1999). Intracellular ROS has been implicated in playing a role in the signal transduction of various biological processes (Fernandez et al., 1995; Goossens et al., 1999; Li et al., 1999a; Bogoyevitch et al., 2000; Majumder et al., 2000; Forman and Torres, 2001; Droge, 2002; Forman et al., 2002; Butow and Avadhani, 2004; Cadenas, 2004; Darley-Usmar, 2004; Ichikawa et al., 2004; Andreyev et al., 2005; Kim et al., 2005). Quagliaro et al. (Quagliaro et al., 2005) have shown that intermittent high glucose enhances mitochondria-derived ROS to signal ICAM-1, VCAM-1, and E-selectin expression in human umbilical vein endothelial cells, and support our previous finding that TPA-induced ROS signals ICAM-1 expression in ML-1 cells (Traore et al., 2007).

Growth arrest is a critical event in order for myeloid cell differentiation to occur (Tanigawa et al., 1983; Selvakumaran et al., 1996; Adachi et al., 1997; Kovanen et al., 2000; Gazitt et al., 2001). In this study, we demonstrate that TPA stimulates ROS generation to signal growth arrest in ML-1 cells. Our suggestion that mitochondria may be the source of the ROS is based on the observations that undifferentiated ML-1 cells lack a key component of NADPH oxidase, p67-phox; second, this ROS generation is inhibited by agents that effectively inhibit complex I activity, DPI and rhein (Kean et al., 1971; Floridi et al., 1989). In data shown in Figure 6, rotenone increased luminol-derived CL in ML-1 cells in response to TPA was blocked by DPI. This observation is consistent with previous reports that rotenone can enhance mitochondria-derived ROS generation in many cell types including dopaminergic primary culture (Li et al., 2003; Radad et al., 2006). Moreover, MnSOD, a key mitochondrial enzyme against METC-derived O₂^{·-} was induced in response to TPA. The induction of MnSOD expression has previously been reported in other cell lines when stimulated with TPA (Kim et al., 1999; Majumder et al., 2001; Porntadavity et al., 2001). MnSOD expression induced by TPA in ML-1 cells required not only PKC but also was dependent on MAPK activity.

The data presented in this and other previous studies suggest that TPA signaling is transduced via various isoforms of PKC (Darbon et al., 1986; Datta et al., 2000; Traore et al., 2005). Many different isoforms of PKC are known to interact with components of the mitochondria (Fernandez et al., 1995; Majumder et al., 2000; Majumder et al., 2001). We previously reported that PKC could interact with mitochondria in rat alveolar macrophages (Rembush et al., 1994). Our present as well as our previous studies have demonstrated that PKC inhibitors block both ROS generation as well as downstream signaling events in both THP-1 and ML-1 cells (Traore et al., 2005; Traore et al., 2007).

Much of our current understanding of ROS-dependent signaling is based on experiments in which exogenous H₂O₂ has been added to cells (Guyton et al., 1996a, 1996b, 1996c, 1996d). H₂O₂ is a diffusible form of ROS that can transverse from the outside in or the inside out of cells. The H₂O₂-dependent extracellular luminol-derived CL is consistent with PKC-stimulated mitochondrial-derived H₂O₂ being able to diffuse from the mitochondria, passing

through the cytoplasm and crossing the plasma membrane. As this ROS is passing through the cell it can then interact with target molecules. One mechanism by which ROS can regulate cellular signaling is via interaction with critical sites of redox sensitive proteins (Kamata et al., 2005).

Many redox sensitive proteins and enzymes contain redox sensitive cysteine thiolate anions (Cys-S⁻) (Georgiou and Masip, 2003). H₂O₂ can oxidize redox-sensitive cysteine residues to cysteine sulfenic acid or a disulfide that can be readily reduced back to cysteine by various cellular reductants (Lee et al., 1998; Rhee et al., 2000). Protein tyrosine phosphatases (PTPs) contain this essential cysteine residue in their active sites. With a pK_a value between 4.7 and 5.4, this make them susceptible to inactivation by low level of oxidants such as H₂O₂ (Lee et al., 1998; Robinson et al., 1999; Rhee et al., 2000; Bouaouina et al., 2004; Kamata et al., 2005; Rinna et al., 2006). Inactivation of MAPK specific phosphatases in response to H₂O₂ has been shown to stimulate the activation of MAPK family members (JNK, p38, and Erk1/2) induced by various extracellular stimuli (Lee et al., 1998; Robinson et al., 1999; Rhee et al., 2000; Bouaouina et al., 2004; Kamata et al., 2005). In this study, we have demonstrated that ROS (H₂O₂) generated in mitochondria response to TPA transiently inactivates Erk1/2-directed phosphatases activity which stimulates Erk1/2 phosphorylation in ML-1 cells. Similarly, in cardiomyocytes, mitochondrial ROS was suggested to initiate p38 phosphorylation in response to hypoxia (Kulisz et al., 2002). Moreover, in phagocytic cells, ROS was shown to mediate TPA-regulated tyrosine phosphorylation and phospholipase C activation by inhibiting phosphotyrosine phosphatase activity (Zor et al., 1993). Treatment with NAC (reductant) quickly restored the total phosphatases activity resulting to a rapid dephosphorylation of Erk1/2 in ML-1 cells. The restoration of the phosphatase activity by NAC may have occurred by the regeneration of sulfhydryl groups on critical cysteines in these phosphatases previously oxidized by H₂O₂. This observation is further supported by previous report that oxidized and purified PTP1B enzyme could be effectively reactivated by treatment with thioredoxin (Trx) (Lee et al., 1998). Thus, Trx might be a physiological electron donor for PTP1B as well as for other PTPs (Lee et al., 1998). Furthermore, the expression of de novo Erk1/2-directed phosphatase protein DUSP6, 3–6 h following Erk1/2 activation could have occurred in response to lost phosphatase activity to regulate Erk1/2 activity in ML-1 cells. Thus, Erk1/2-directed phosphatases appear to function as critical cellular “molecular switches.”

In conclusion, the findings of this study demonstrate that following the addition of TPA to ML-1 cells that there is a PKC-dependent generation of cellular ROS. This ROS (H₂O₂) can then stimulate Erk1/2 activation via a redox mechanism associated with a reversible inactivation of Erk1/2-directed phosphatases (Fig. 11). This leads to the downstream activation of p21 expression resulting in cell growth inhibition and ICAM1 expression (Traore et al., 2005; Hu and Mivechi, 2006; Traore et al., 2007).

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Abbreviations

TPA, 12-O-tetradecanoyl-phorbol-13-acetate; NAC, N-acetyl cysteine; ROS, reactive oxygen species; Erk, extracellular signal-regulated kinase.

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Fig. 1. Effects of TPA on human myeloid cell proliferation. One million ML-1 or THP-1 cells were incubated in the presence or absence of 5 and 20 nM TPA respectively for the indicated times. Cells were harvested each day for 3 days and counted using a hemocytometer to determine the cell number in each sample.

Fig. 2.

TPA induces p21^{WAF1/Cip1} expression in ML-cells. ML-1 cells were incubated in the presence or absence of 5 nM TPA for indicated times. A: p21^{WAF1/Cip1} mRNA levels were determined by examination of microarray analysis data. B: Total proteins were extracted and 40 µg of total protein were analyzed by Western blot analysis using mouse anti-human p21^{WAF1/Cip1} antibodies. The figure depicts representative results from one of three replicate experiments.

Fig. 3. Effects of GF109203X, U0126, NAC, and DPI on p21^{WAF1/Cip1} protein expression. ML-1 cells were pretreated with 5 μ M GF109203X (GF), 10 μ M U0126, 10 μ M DPI, or 25 mM NAC for 10 min followed by incubation with 5 nM TPA for 6 h. Total proteins were extracted and analyzed by Western blot analysis as indicated above.

Fig. 4.

Role of PKC in TPA-induced ROS generation in ML-1 cells. The generation of ROS in ML-1 cells in response to TPA was assessed by: (A) luminol-derived CL or (B) flow cytometry analysis using fluorephore hydroethidine. Cells were preincubated with GF109203X or NAC for 10 min followed by 5 nM TPA treatment for 50 min. Luminol-derived luminescence of 1 million cells was measured in Berthold luminometer for 1 h. The CL in (A) are CPM $\times 10^4$ over the indicate time period. The data in bar graph is the CL from three cell samples integrated over 50 min (means $\pm$ SD, $n = 3$). Analysis of the data by one way ANOVA indicates that the effect of GF109203X and NAC was significant ($P < 0.01$). The luminol-dependent CL of untreated cells is at the level of the instrument background. B. Cells were preincubated in presence of hydroethidine for 5 min followed by incubated in the presence or in the absence of 5 nM TPA for 30 min. Cells were harvested, washed with PBS and analyzed by flow cytometry as indicated in materials and methods.

Fig. 5. Determination of p47-phox and p67-phox expression in undifferentiated and differentiated ML-1 and THP-1 cells. ML-1 and THP-1 cells were incubated in growth medium in the presence or absence of 5 or 20 nM TPA respectively for 3 days. Total proteins were extracted and 40 μ g of proteins were analyzed by Western blot analysis using anti-human p47-phox and p67-phox antibodies. The figure depicts representative results from one of three replicate experiments.

Fig. 6.

Effects of DPI, rotenone, rhein, and NAC on luminol-derived CL in ML-1 and THP-1 cells. Cells were preincubated with 10 μ M DPI, 10 μ M rotenone, 40 μ M rhein, or 25 mM NAC for 10 min followed by incubation with 5 or 20 nM TPA respectively for 50 min. Luminol-dependent luminescence of 1 million cells was measured in Berthold luminometer for 1 h. The CL are in CPM $\times 10^4$ over the indicated time period. The data is the CL from three cell samples integrated over 50 min (means $\pm$ SD, $n = 3$). The luminol-dependent CL of untreated cells is at the level of the instrument background.

Fig. 7.

TPA induces MnSOD expression at mRNA and protein levels in ML-1 cells. Three million cells were incubated in the presence or in the absence of 5 nM TPA for various times. MnSOD mRNA levels were determined by examination of microarray analysis data. MnSOD protein levels were determined by Western blot analysis using mouse anti-human MnSOD antibodies. The figure depicts representative results from one of three replicate experiments.

Fig. 8.

The effects of GF10920X, PD9059, U0126, and DPI on TPA induced MnSOD expression in ML-1 cells. Three million cells were pretreated with 5 μ M GF109203X, 25 μ M PD98059, 10 μ M U0126, or 10 μ M DPI for 20 min follow by incubation with 5 nM TPA for 24 h. MnSOD protein levels were determined by Western blot analysis as indicated above.

Fig. 9.

Erk1/2 activation induces by TPA and H₂O₂ treatment. Three millions cells were incubated in the presence of TPA for various times, or in the presence of 0.1 or 1 mM H₂O₂ for 1 h. Cells were harvested, and the levels of phospho-Erk1/2 were determined (A) by flow cytometry analysis or (B) by Western blot analysis as described in the Section "Materials and Methods." The figure depicts representative results from one of three replicate experiments.

Fig. 10.

ROS-dependent inactivation of Erk 1/2-directed phosphatases mediates Erk 1/2 activation induced by TPA. Using flow cytometry analysis, a cell based assay was used to assess the levels of intracellular phospho-Erk1/2 in various experimental conditions. **A:** Cells were preincubated in the presence of NAC for 10 min followed by incubation with TPA for 1 h. **B:** Cells were incubated in media containing TPA for 1 h followed by incubation with 25 mM NAC for 15, 30, or 45 min. Cells were then harvested, washed, fixed, permeabilized, and stained using (Alexa-Fluor 488)-labeled anti-phospho-Erk1/2 and assessed by flow cytometry. Data were analyzed by ModFILT statistical software. The figure depicts representative results from one of three replicate experiments. **C:** Cells were incubated in media containing TPA or 100 nM H₂O₂ for 1 h. After harvest, cells were lysed in Triton X-100 containing buffer and analyzed for tyrosine phosphatase activity using FDP-derived fluorescence phosphatase assay. The fluorescent intensity were measure at Ex/Em = (485 ± 20)/(528 ± 20). **D:** ML-1 cells were incubated with TPA or DMSO (vehicle) in the presence or absence of 5 mM GF109203X, 10 μM U0126, or 25 mM NAC for 6 h. Total RNA were extract and 2 μg of total RNA were analyzed by RT-PCR as described in materials and methods sections. Results are expressed as means ± SD (n = 3).

Fig. 11.
Proposed mechanism of TPA-induced Erk1/2 regulation during early events of ML-1 cell differentiation to macrophages.

TABLE 1

mRNA Levels of Genes Involved in Cell Cycle Control

Negative regulators	Fold change	Positive regulators	Fold change
CDKN1A (p21 ^{WAF1})	3.78	ANAPC5	-2.62
CYLD	3.76	ANAPC7	-2.16
		BRCA2	-2.20
		E2F2	-2.17
		MCM2	-3.89
		MCM5	-2.30
		MCM6	-3.14
		MYC	-6.36
		WEE1	-2.10
		WT1	-5.54

ML-1 cells were incubated in the growth medium in the presence or absence of TPA for 6 h. Total RNA was extracted purified, normalized, and analyzed by microarray analysis using a human genome U133 2.0 Plus Array Chip.