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Tetradecanoyl phorbol acetate induces expression of Toll-like receptor 2 in U937 cells: involvement of PKC, ERK, and NF-κB

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Abstract

Toll-like receptors (TLRs) have been identified recently as crucial signaling receptors mediating the innate immune recognition. Though induction of TLR2 or TLR4 by 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) in leukemia cells has been reported, however, the mechanism by which TPA up-regulates TLR2 or TLR4 remains poorly understood. In this study, we investigated the effect of TPA on induction of TLR2 in U937 cells. TPA markedly induced TLR2 mRNA and protein expressions. TLR2 expression in response to TPA was attenuated by pretreatments with GF109203X and Go6976 (inhibitors of protein kinase C (PKC)) and PD98059 (an inhibitor of extracellular signal-regulated kinases (ERKs)), but not SB203580 (an inhibitor of p38s) and SP600125 (an inhibitor of c-Jun N-terminal kinases), suggesting involvement of PKC and ERKs in this response. Moreover, TPA-induced PKC activation was linked to generation of reactive oxygen species, which were dispensable for TLR2 expression in U937 cells. Pretreatments with GF109203X blocked TPA-induced phosphorylation of ERKs, suggesting activation of ERKs by PKC. In addition, TPA induced nuclear factor-κB (NF-κB) activation, which was shown by increased nuclear translocation of p65 NF-κB and degradation of IκB-α, a NF-κB inhibitory protein. Importantly, TPA-induced TLR2 expression was inhibited by blockage of NF-κB activation using NF-κB inhibitors, including MG132 and BAY11-7085. Specifically, TPA-induced nuclear translocation of NF-κB was effectively attenuated by GF109203X and PD98059, suggesting PKC and ERK regulation of NF-κB nuclear localization in response to TPA. Together, these results suggest that TPA-induced TLR2 expression in U937 cells may be at least in part mediated through activation of PKC and ERKs as well as NF-κB transcription factor, and that cross-talk between PKC or ERKs and NF-κB may exist.

Keywords: TPA; TLR2; PKC; ERKs; NF-κB

The Toll protein was originally discovered as a modulator of embryonic development in *Drosophila* [1]. Recently, several mammalian Toll homologues have been identified and designated as Toll-like receptors (TLRs). At least 10 human TLRs have been cloned and some shown to mediate cellular response to specific patterns of microbial components that are conserved motifs in pathogens termed pathogen-associated molecular patterns [2,3]. TLRs have essential roles in the innate recognition of pathogen-associated molecular patterns and in triggering acquired immunity [3,4]. Among TLRs, the best characterized TLRs are TLR2 and TLR4. TLR2 is predominantly distributed in monocytes/macrophages and polymorphonuclear cells (PMNs), and is involved in response to Gram-positive bacteria [5,6]. On the other

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hand, TLR4 is required for response to lipopolysaccharide (LPS) derived from Gram-negative bacteria and is predominantly present in monocytes/macrophages, PMNs, intestinal epithelial cells, and umbilical vein endothelial cells [5–7].

Macrophages are effector cells of the innate immune system and play an important role in initiating and maintaining the immune response. Thus, the elevated expression of TLR2 and/or TLR4 may contribute to the resensitization of macrophages to invasive pathogens, but the regulation of expression of TLRs is poorly understood. Accordingly, expression of TLR2 and/or TLR4 has been shown to be induced by LPS, interferon-γ, inflammatory cytokines, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), or retinoic acid in various cell types [8–11]. Human promonocytic leukemia U937 cells differentiate into monocyte/macrophage-like cells when exposed to various agents, such as TPA, 1,25-dihydroxyvitamin D3, and retinoic acid [12]. In this process, TPA activates protein kinase C (PKC), a family of serine/threonine-specific kinases, which is involved in a pleiotypic set of processes including cell differentiation and growth [13].

In the present study, we investigated the effect of TPA on expression of TLR2 and TLR4 in U937 cells, and the signaling mechanism associated. We found that exposure of TPA into U937 cells induced increase in expression of TLR2, but not TLR4. Although some of the molecular events associated with TPA-associated differentiation are known in U937 cells, the molecular signaling mechanism responsible for TPA-induced TLR2 expression is not fully known. Interestingly, our data suggest that the increased expression of TLR2 by TPA is at least in part mediated through activation of PKC, extracellular signal-regulated kinases (ERKs), and nuclear factor-κB (NF-κB) transcriptional factor.

Materials and methods

Cell culture. U937 promonocytic leukemia cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Typically, 3×10^5 cells/ml were seeded in T-25 flasks as 4 ml cultures, and maintained in the tissue culture incubator for 12–16 h before the addition of TPA or other reagents.

Drugs and materials. Antibodies against phospho-ERKs (p-ERKs), ERKs, phospho-p38s (p-p38s), p38s, phospho-c-Jun N-terminal kinases (p-JNKs), and JNKs were purchased from Cell Signaling (Beverly, MA). Antibodies against IκB-α, p65 NF-κB, β-actin, β-tubulin, and mouse anti-goat FITC-conjugated IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-human TLR2.1 antibody was purchased from eBioscience (San Diego, CA). PD98059, GF109203X, and Go6976 were purchased from Biomol (Plymouth, PA). MG132, BAY11-7085, and SP600125 were obtained from Calbiochem (La Jolla, CA). Cycloheximide, TPA, vitamin E, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), and 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) were purchased from Sigma–Aldrich (St. Louis, MO).

Reverse transcription-polymerase chain reaction. The expression values of TLR2, TLR4, and CD14 were quantified by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard. Total cellular RNA was isolated from cells and reverse-transcribed using a random hexadeoxynucleotide primer (Perkin–Elmer, Branchburg, NJ), and reverse transcriptase (Perkin–Elmer, Branchburg, NJ) in 20 μl volume. PCR amplification was performed by using 0.25 μl AmpliTaq DNA polymerase (Perkin–Elmer, Branchburg, NJ), 1 μl of each 10 mM deoxyribonucleotide triphosphosphate, 6.25 μl GeneAmp 10× PCR buffer II (Perkin–Elmer, Branchburg, NJ), 4 μl of 25 mM $MgCl_2$, 1 μl of each specific sense and anti-sense primer at 25 μM, and water with the hot-start method to enhance the sensitivity and specificity of amplification. The PCR products were analyzed on 1.5% agarose gel. The primer sequences and product sizes were as follows: TRL2 forward 5′-GCC AAA GTC TTG ATT GAT TGG-3′, reverse 5′-TTG AAG TTC TCC AGC TCC TG-3′, 394 base pair (bp); TLR4 forward 5′-TGG ATA CGT TTC CTT ATA AG-3′, reverse 5′-GAA ATG GAG GCA CCC CTT TC-3′, 506 bp; CD14 forward 5′-ACT CCC TCA ATC TGT CGT TCG CTG-3′, reverse 5′-CTG AAG CCA AGG CAG TTT GAG TCC-3′, 339 bp; and GAPDH forward 5′-CGT CTT CAC CAC CAT GGA GA-3′, reverse 5′-CGG CCA TCA CGC CAC AGT TT-3′, 300 bp.

Measurement of reactive oxygen species generation. DCFH-DA was used to detect intracellular generation of reactive oxygen species (ROS) by TPA. DCFH-DA is a stable, nonfluorescent, and nonpolar compound that can diffuse through cell membranes. Once inside the cell, the acetyl groups are primarily cleaved by cytosolic enzymes to form the polar nonfluorescent dichlorofluorescein (DCFH), which is then rapidly oxidized to highly fluorescent dichlorofluorescein (DCF) in the presence of ROS. Cells were harvested, washed twice with phosphate-buffered saline (PBS), and suspended in PBS (1×10^6 cells/ml). Cell suspension (500 μl) was placed in a tube, loaded with DCFH-DA to a final concentration of 20 μM, and incubated at 37 °C for 20 min. After the addition of TPA, cells were incubated at 37 °C for various times. Then ROS generation was measured by the fluorescence intensity (FL-1, 530 nm) of 10,000 cells with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Mean fluorescence intensity was obtained by histogram statistics using the CellQuest program.

Western blot analysis. Whole cell extracts were prepared in the lysis buffer as described previously [14]. For phospho-protein detection, cells were washed with ice-cold PBS containing 1 mM Na_3VO_4 and 1 mM NaF, and lysed in a buffer (20 mM Tris–Cl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM NaF, 2 mM EDTA, 200 nM aprotinin, 20 μM leupeptin, 50 μM phenanthroline, and 280 μM benzamidine–HCl). To isolate cytosolic and nuclear proteins, cells were homogenized in ice-cold hypotonic buffer (10 mM Hepes, 10 mM KCl, 3 mM $MgCl_2$, 0.5% NP-40, 2 mM PMSF, 1 mM DTT, and 200 nM aprotinin) for 20 min and centrifuged 12,000 rpm for 10 min. The supernatant was saved as a cytosolic fraction. The pellets were homogenized in ice-cold nuclear extract buffer [10 mM Tris–Cl (pH 7.5), 0.5 M NaCl, 2.5% glycerol, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 2 mM PMSF, and 200 nM aprotinin] for 20 min and centrifuged 12,000 rpm for 10 min. The supernatant was saved as a nuclear fraction. The protein concentration of extracts was estimated with Bradford reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. Equal amounts of protein (40 μg/lane) were resolved by 10–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was then washed with Tris-buffered saline (10 mM Tris, 150 mM NaCl) containing 0.05% Tween 20 (TBST) and blocked in TBST containing 5% non-fat dried milk. The membrane was further incubated with respective specific antibodies such as p-ERKs (1:2000), ERKs (1:2000), p-JNKs (1:1000), JNKs (1:2000), p-p38s (1:1000), p38s (1:2000), p65 NF-κB (1:2000), IκB-α (1:2000), β-actin (1:10000), and β-tubulin (1:5000). The membrane was continuously incubated with appropriate secondary

antibodies coupled to horseradish peroxidase, and developed in the ECL Western detection reagents (Amersham–Pharmacia Biotech, Piscataway, NJ, USA).

Analysis of expression of surface TLR2. TLR2 flow cytometry was performed using monoclonal anti-human TLR2.1 antibody (eBioscience, San Diego, CA) by the manufacturer's instruction. Briefly, 1×10^6 cells were washed in PBS containing bovine serum albumin and sodium azide, and then incubated with TLR2.1 antibody at a 1/100 dilution for 30 min at 4 °C. After washing, FITC-conjugated goat anti-mouse antibody was added and suspension was incubated at 4 °C for 30 min. Cells were resuspended in 400 μl PBS and analyzed by BD FACSVantage flow cytometer (Becton–Dickinson Biosciences, San Jose, CA). The data from flow cytometry were analyzed by WinMDI 2.8 software. Negative control was prepared by incubating with an isotype-matched control antibody (IgG2a).

Results

TPA induces TLR2 mRNA expression in U937 cells

First of all, endogenous expression level of TLR2, TLR4, or CD14 was analyzed in human U937, HL60, and K562 cells by RT-PCR using respective primers. THP-1 cells served as positive sources for human TLR2 and TLR4 mRNA expressions. As shown in Fig. 1A, HL60 cells expressed both TLR2 and TLR4 mRNAs. On the other hand, U937 cells expressed only TLR4. K562 cells also expressed very low level of TLR4 mRNA. Expression of CD14 was detected in HL60 and THP-1 cells. TPA has been known to induce differentiation of these cell lines into monocytic- and macrophage-like cells and to induce expression of TLR2 or TLR4 mRNA in HL60 and U937 cells [8,15].

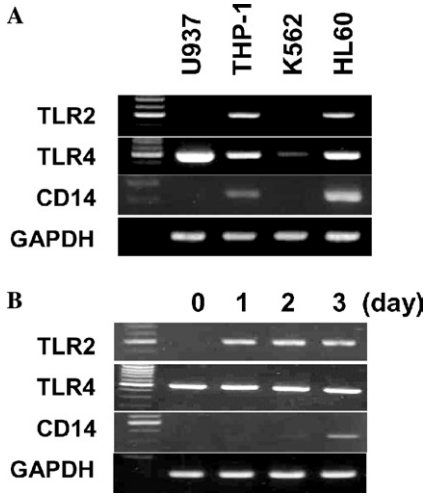

Fig. 1. Expressions of TLR2, TLR4, and CD14 in human leukemic cells. (A) Total RNAs were extracted from U937, THP-1, K562, and HL60 cells, and analyzed by RT-PCR with specific primers for TLR2, TLR4, and CD14. GAPDH was analyzed to verify similar cDNA loading. (B) Up-regulation of TLR2 mRNA expression in response to TPA in U937 cells. U937 cells were treated with TPA for the indicated days. Expressions of TLR2, TLR4, and CD14 were analyzed by RT-PCR with specific primers for TLR2, TLR4, CD14, and GAPDH.

To analyze whether TPA could induce expression of TLR2 or TLR4 mRNA in U937 and K562 cells, TPA was exposed to these cell lines for 3 days. As shown in Fig. 1B, TPA treatment in U937 cells induced a significant increase of expression of TLR2, which was sustained up to 3 days, but little change in TLR4 mRNA expression. In K562 cells, there was no expression of TLR2 or 4 by TPA treatment (data not shown). Kinetic data additionally demonstrated that the significant induction was detected at 4 h at the mRNA level (Fig. 2A) and 16 h on the cell surface (Fig. 2B) in TPA-induced U937 cells. In analysis of expression of the cell surface TLR2, negative control was prepared by incubating with an isotype-matched control antibody (data not shown). To determine whether increase in TLR2 expression induced by TPA in U937 cells was dependent on new protein synthesis, U937 cells were preincubated with cycloheximide, a protein synthesis inhibitor, for 1 h before TPA treatment. Interestingly, pretreatment with cycloheximide attenuated TPA-induced TLR2 mRNA up-regulation, suggesting that TLR2 expression by TPA in U937 cells required de novo protein synthesis (Fig. 2C).

Effects of PKC inhibitors on TPA-induced TLR2 expression

Because TPA is a well-known PKC activator, we hypothesized that TPA-induced TLR2 expression might be associated with activation of PKC. Interestingly, as shown in Fig. 3, pretreatments with GF109203X and Go6976, inhibitors of PKC, significantly blocked not only differentiation of U937 cells (data not shown) but also expression of TLR2 mRNA (Fig. 3A) and protein (Fig. 3B) induced by TPA. MTT assay confirmed that these inhibitors at concentrations applied had no cytotoxic effects (data not shown). These results strongly suggest that TPA-induced TLR2 expression was correlated with activation of PKC in U937 cells.

Role of ROS in TPA-induced TLR2 expression

It has been shown that TPA treatment of myeloid leukemia cell such as U937 cells induces generation of ROS [16]. This promptly led us to investigate whether TPA induces TLR2 expression via ROS production in U937 cells. Data of flow cytometer confirmed ROS generation in U937 cells at 10 min TPA treatment (Fig. 4A, upper panel), which was evaluated by ROS-mediated oxidation of DCFH-DA that preloaded for 20 min into the cells before TPA stimulation. The role of ROS in the TPA-induced TLR2 mRNA and protein expression in U937 cells was next assessed. Pretreatments with vitamin E and GF109203X effectively inhibited the increase of DCF fluorescence by TPA (Fig. 4A, lower panel). However, pretreatments with well-known antioxidants

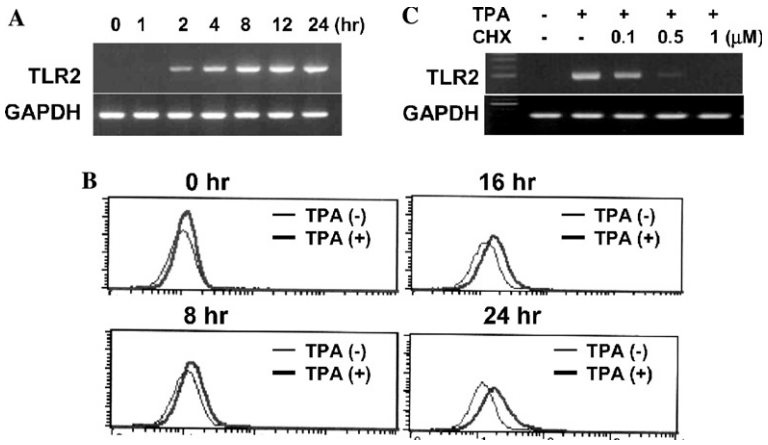

Fig. 2. Expression of TLR2 mRNA and protein in U937 cells treated with TPA. (A) U937 cells were treated with TPA for the indicated times. Total RNA was extracted and analyzed by RT-PCR with specific primers for TLR2 and GAPDH. (B) Cell surface expression of TLR2 in U937 cells treated with TPA for the indicated times. After treatment of U937 cells with TPA, cells were stained with goat anti-human TLR2 monoclonal antibody and FITC-conjugated mouse anti-goat antibody, and then analyzed by a flow cytometer. Representative data from three independent experiments are shown. (C) The effects of cycloheximide (CHX) on TPA-induced TLR2 mRNA expression. U937 cells were pretreated with the indicated concentrations of CHX for 1 h and then stimulated with TPA for 24 h. Total RNA was extracted and analyzed by RT-PCR with specific primers for TLR2 and GAPDH.

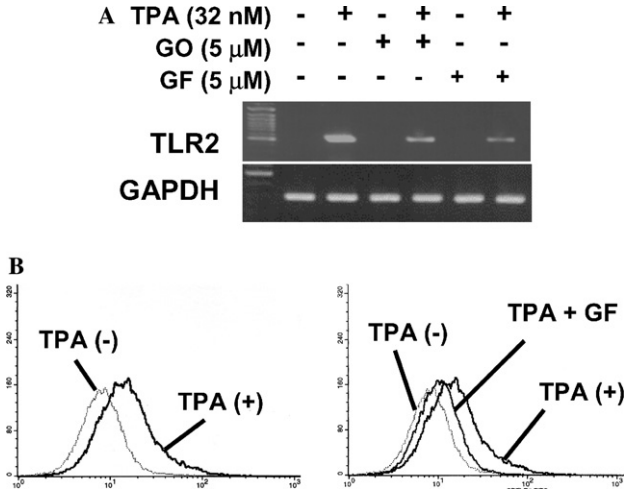

Fig. 3. Activation of PKC is critical for TPA-induced TLR2 expression in U937 cells. (A) U937 cells were pretreated for 1 h with the indicated concentrations of Go6976 (GO) and GF109203X (GF), and then cells were treated with TPA for 24 h. Total RNA was prepared and used for TLR2 or GAPDH by RT-PCR. (B) U937 cells were pretreated for 1 h with GF (5 μM), and then cells were treated with TPA for 24 h. Cells were harvested and assayed for cell surface expression of TLR2 as described in Materials and methods. Representative data from three independent experiments are shown.

such as vitamin E and Trolox had no effect on TPA-induced TLR2 mRNA and surface protein expressions (Figs. 4B and C). ROS generation by PKC such as PKC-βII has been postulated [16]. Therefore, we investigated the effect of PKC inhibition on ROS production by TPA in U937 cells. Interestingly, pretreatment with GF109203X that inhibited TPA-induced expression of TLR2 mRNA (Fig. 3A) and surface protein (Fig. 3B) blocked the increase of DCF fluorescence induced by TPA in U937 cells (Fig. 4A). These results strongly suggest that PKC mediates TPA-induced ROS generation, but ROS production by TPA is dispensable for TLR2 mRNA and surface protein expression in U937 cells.

Role of mitogen activated protein kinases in TPA-induced TLR2 expression

Since TPA is known to have pleiotypic effects on cell function [13], we measured the effects of TPA on activation of JNKs, ERKs, and p38s using respective antibodies that specifically recognize the active phosphorylated forms. As shown in Fig. 5A, treatment with TPA induced activation of ERKs and JNKs, but not p38s. Stripping and subsequent reprobing the same blot with antibodies against total JNKs, ERKs, and p38s demonstrated no change of total proteins by TPA treatment, indicating that TPA led to activation of pre-existing ERKs and JNKs. Using PD98059, an inhibitor of ERKs, or SP600125, an inhibitor of JNKs, we further investigated whether or not TPA-induced TLR2 expression is dependent on activation of ERKs or JNKs. Pretreatment with PD98059 significantly inhibited the morphological changes associated with differentiation (data not shown), and TLR2 mRNA and protein expressions by TPA. However, pretreatment with SP600125 had no effect on TPA-induced morphological changes associated with differentiation, and TLR2 mRNA and protein expressions (Figs. 5B and C). These results suggest that ERKs play an important role in TPA-induced differentiation and TLR2 expression. PKC activation is connected to activation of Raf-1 and mitogen activated protein kinase (MAPK) kinases (MEKs), well-known upstream activators of ERKs or

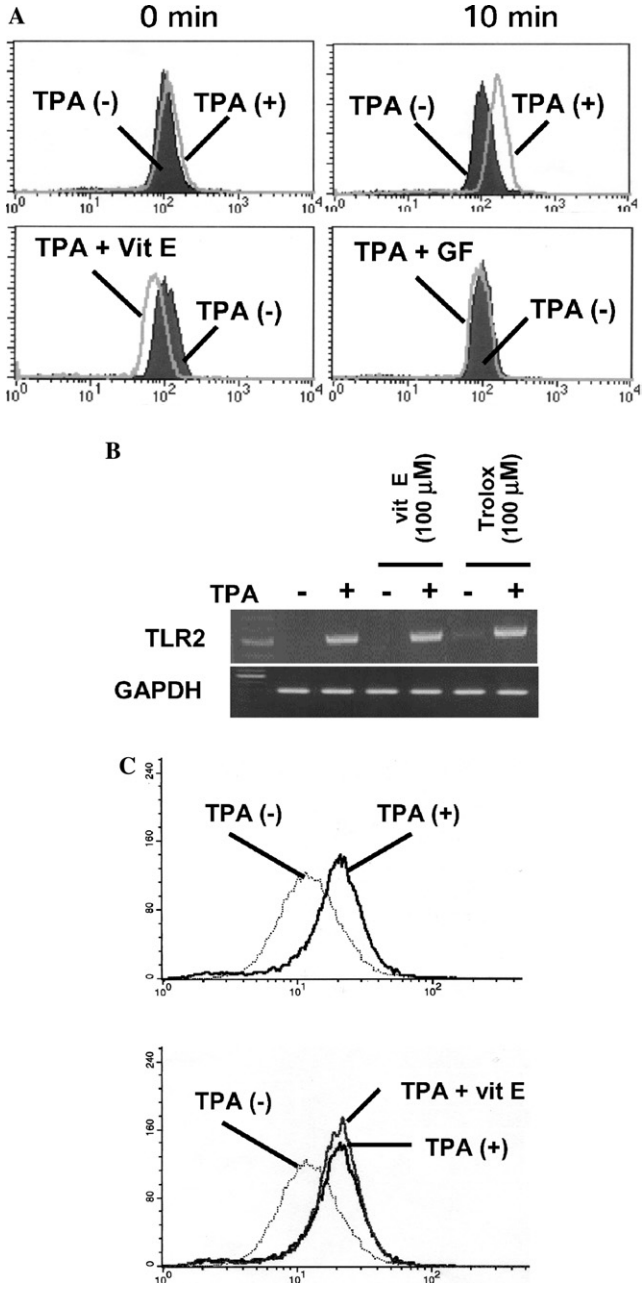

Fig. 4. PKC mediates TPA-induced ROS generation, but ROS production by TPA is dispensable for TLR2 mRNA and protein expression in U937 cells. (A) U937 cells were preloaded with 20 μM DCFH-DA for 20 min at 37 °C, and then TPA was added into the cells and incubated for the indicated times at 37 °C. DCF fluorescence was measured using a flow cytometer (upper panel) and expressed as histograms. Cells were pretreated with 100 μM vitamin E (vit E) or 5 μM GF109203X (GF) for 1 h, then preloaded with DCFH-DA, and treated with TPA for 10 min. DCF fluorescence was analyzed with a flow cytometer and expressed as histograms. Representative data from three independent experiments are shown. (B) U937 cells were pretreated with the indicated concentrations of vit E or Trolox for 1 h and then treated with TPA for additional 24 h. Total RNA was prepared and used for TLR2 or GAPDH by RT-PCR. (C) U937 cells were pretreated for 1 h with vit E (100 μM), and then cells were treated with TPA for 24 h. Cells were harvested and assayed for cell surface expression of TLR2 as described in Materials and methods. Representative data from three independent experiments are shown.

other MAPKs. Indeed, the data of inhibition of TPA-induced ERKs activation by pretreatment with GF109203X strongly suggest that TPA-induced activation of ERKs is mediated by PKC activation (Fig. 5D).

Role of NF-κB in TPA-induced TLR2 expression

NF-κB has been shown to regulate TLR2 expression by extracellular stimuli [9,10,17,18]. To test whether NF-κB mediates TPA-induced TLR2 mRNA expression in U937 cells, we measured the effect of TPA on NF-κB activation in U937 cells. Here the activation of NF-κB was assessed by the degree of nuclear translocation of p65 NF-κB and by the degradation of IκB-α, a NF-κB inhibitory protein, triggered by TPA treatment. Treatment with TPA in U937 cells resulted in the marked nuclear translocation of p65 NF-κB and concomitantly the strong degradation of IκB-α in the cytosol, which maximally occurred at 2–6 h TPA treatment (Fig. 6A). Using MG132 and BAY11-7085, inhibitors of NF-κB, we then studied the role of NF-κB in TPA-induced TLR2 mRNA expression in U937 cells. Pretreatment of MG132 or BAY11-7085 effectively suppressed TPA-induced nuclear translocation of p65 NF-κB in U937 cells (Fig. 6B), assuring the effectiveness of MG132 and BAY11-7085 as NF-κB inhibitors. Importantly, TPA-induced TLR2 mRNA expression was effectively inhibited by pretreatment with MG132 and BAY11-7085 (Fig. 6C). These results strongly suggest that NF-κB in part involves in TPA-induced TLR2 expression probably at the level of TLR2 transcription. Since regulation of NF-κB activation by PKC and ERKs has been reported [19–22], and TPA-induced TLR2 mRNA and protein expression was mainly influenced by PKC and ERKs in this study, we therefore investigated whether TPA-induced activation of NF-κB is mediated through PKC or ERKs pathway. Notably, pretreatment with GF109203X or PD98059 effectively suppressed nuclear translocation of p65 NF-κB in response to TPA treatment (Fig 6D). In contrast, pretreatment with SB203580, an inhibitor of p38s, or SP600125, an inhibitor of JNKs, had no effect on nuclear translocation of p65 NF-κB by TPA treatment (data not shown). These results may suggest that PKC and ERKs, but not p38s or JNKs, appear to be upstream of NF-κB activation in U937 cells in response to TPA treatment.

Discussion

Macrophages are patrolling cells of the innate immune system and express TLRs. TLRs are a family of pattern recognition receptors recently identified as crucial signaling receptors mediating the innate immune recognition [3,4]. Though TLRs may contribute to the

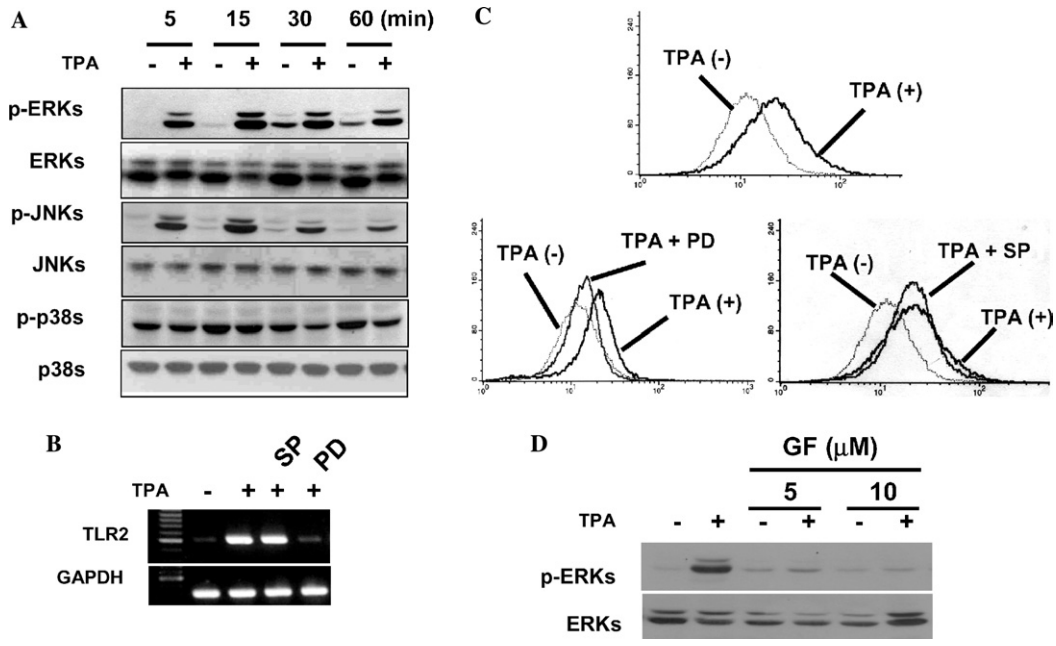

Fig. 5. Role of mitogen activated protein kinases on TPA-induced TLR2 expression. (A) U937 cells were treated with TPA for the indicated times. At each time, whole cell lysates were prepared and used for p-ERKs, ERKs, p-p38s, p38s, p-JNKs, or JNKs Western with respective antibodies. (B) U937 cells were pretreated with 25 μM PD98059 (PD) and 25 μM SP600125 (SP) for 1 h and then treated with TPA for additional 24 h. Total RNA was prepared and used for TLR2 or GAPDH by RT-PCR. (C) U937 cells were pretreated for 1 h with 25 μM PD and 25 μM SP, and then cells were treated with TPA for 24 h. Cells were harvested and assayed for cell surface expression of TLR2 as described in Materials and methods. Representative data from three independent experiments are shown. (D) U937 cells were pretreated with the indicated concentrations of GF109203X (GF) for 1 h and then treated with TPA for additional 15 min. Whole cell lysates were prepared and used for p-ERKs and ERKs Western with respective antibodies.

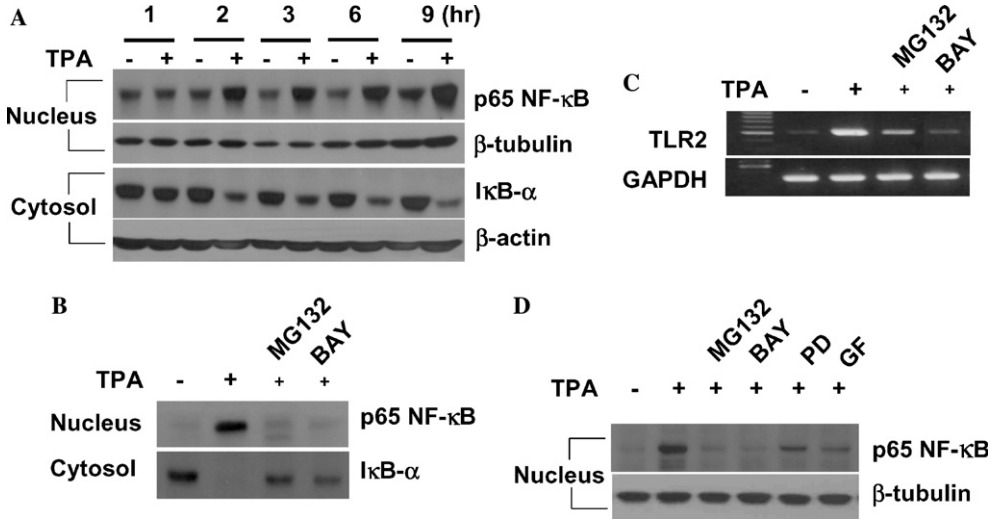

Fig. 6. TPA induces activation of NF-κB, which is important for TLR2 mRNA expression. (A) U937 cells were treated without or with TPA for the indicated times. At each time, nuclear and cytosolic fractions were prepared and used for p65 NF-κB, IκB-α, β-tubulin, or β-actin Western. (B) U937 cells were pretreated with 5 μM MG132 or 5 μM BAY11-7085 (BAY) and then treated with TPA for additional 3 h. Nuclear and cytosolic fractions were prepared and used for p65 NF-κB Western. (C) U937 cells were pretreated with MG132 (5 μM) or BAY (5 μM) and then treated with TPA for additional 24 h. Total RNA was prepared and used for TLR2 or GAPDH RT-PCR. (D) U937 cells were pretreated with 5 μM MG132, 5 μM BAY, 25 μM PD98059 (PD), and 5 μM GF109203X (GF), and then treated with TPA for additional 3 h. Nuclear fraction was prepared and used for p65 NF-κB or β-tubulin Western.

resensitization of macrophages to invasive pathogens, however, the mechanism by which TLR expression is regulated in cells is largely unknown. In this study, we have investigated the molecular signaling mechanisms of TLR2 expression induced by TPA in a human promonocytic leukemia cell line U937.

U937 cells have been recognized to be a useful cell culture model in the study of differentiation [12]. U937 cells respond to TPA that causes activation of PKC with growth arrest and induction of monocyte/macrophage phenotype. Li et al. [8] have shown that treatments of TPA and retinoic acid can induce TLR2 expression in HL-60 cell in a PKC-dependent manner. In agreement with this, we have found that TPA-induced TLR2 mRNA and surface protein expressions in U937 cells are dependent on PKC because pretreatments with PKC inhibitors such as GF109203X and Go6976 effectively inhibited TPA-induced TLR2 mRNA and protein expressions. It has been demonstrated that TPA treatment of U937 cells is associated with the generation of ROS [16]. In the present study, we have shown that ROS are generated in cells treated with TPA, and pretreatment with vitamin E effectively suppresses ROS production by TPA. However, we have observed that TPA-induced TLR2 surface protein expression is not affected by pretreatment with vitamin E. An interesting finding of this study is that blockage of PKC by GF109203X inhibits not only ROS production but also TLR2 expression (mRNA and protein) in TPA-treated U937 cells. These results strongly suggest that PKC activation is required for TPA-induced ROS generation and TLR2 expression in U937 cells, but ROS production by PKC is dispensable for TPA-induced TLR2 mRNA and protein expression in U937 cells.

MAPKs are linked to the expression of TLR2 by various stimuli including interleukin-1β [23–25]. It has been shown that TPA-induced activations of JNKs and ERKs, members of MAPKs, are mediated by PKC activation [26,27]. In the present study, we have demonstrated that TPA treatment causes a marked increase in the level of phosphorylated ERKs and JNKs in U937 cells. Notably, though phosphorylation of ERKs and JNKs is induced by TPA, only PD98059 pretreatment (but not SP600125) attenuates TPA-induced TLR2 mRNA and surface protein expressions, suggesting an important role of ERKs in TPA-induced TLR2 expression in U937 cells. Sozeri et al. [28] have shown that TPA treatment leads to hyperphosphorylation of Raf, suggesting c-Raf to be a downstream effector of PKC in TPA-triggered protein kinase cascade that may eventually link to transcriptional activation of TPA-inducible genes. Blumer and Johnson [27] have shown that Raf-1 phosphorylates and activates MEK-1/2 which in turn activates ERK-1/2. In agreement with these, our data suggest that TPA-induced ERKs activation in U937 cells is largely dependent on the PKC pathway because pretreatment with GF109203X effectively inhibits TPA-induced activation of ERKs.

NF-κB signaling pathway has been implicated in the expression of TLR2 induced by various stimuli [9,10,17,18,29]. In this study, we have demonstrated that TPA treatment markedly induces nuclear translocation of p65 NF-κB and concomitantly strong degradation of IκB-α, suggesting NF-κB activation by TPA in U937 cells. Interestingly, the present data showing that pretreatment of MG132 or BAY11-7085 effectively suppresses TPA-induced nuclear translocation of p65 NF-κB as well as TLR2 mRNA expression support the notion that NF-κB in part mediates TPA-induced TLR2 expression probably by up-regulating TLR2 transcription in U937 cells. Since TPA-induced TLR2 mRNA and protein expression in U937 cells is required for activation of PKC, ERKs, and NF-κB, therefore, we speculated that TPA-induced NF-κB activation might be correlated with activation of PKC and ERKs. We have found that pretreatments with GF109203X and PD98059, respectively, attenuate nuclear translocation of p65 NF-κB by TPA. These results may, therefore, suggest possible cross-talk between PKC or ERKs and NF-κB in mediating TPA-induced TLR2 expression in U937 cells.

In conclusion, findings of the present study demonstrate that TPA induces TLR2 mRNA and protein expressions in U937 cells via activation of PKC, ERKs as well as NF-κB. Activation of PKC or ERKs by TPA may involve in TPA-induced TLR2 expression in part by cross-talk with NF-κB. Thus, the inducibility of TLR2 expression by TPA in U937 cells appears to be determined by multiple signaling molecules. Further efforts to investigate other signaling pathways associated with TPA-induced TLR2 expression in U937 cells are warranted.

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