Journal of Cellular Biochemistry 100:1010–1021 (2007)

Role of ROS and MAPK in TPA-Induced ICAM-1 Expression in the Myeloid ML-1 Cell Line

Kassim Traore,[1]* Rajni B. Sharma,[2] C. Lynne Burek,[2] and Michael A. Trush[1]

[1]Department of Environmental Health Sciences, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland 21205
[2]Department of Pathology, School of Medicine, Johns Hopkins University, Baltimore, Maryland 21205

Abstract Intercellular adhesion molecule 1 (ICAM-1) has been implicated in playing a key role in the mechanism of inflammatory process initiated in response to environmental agents, and during normal hematopoietic cell differentiation. Though induction of ICAM-1 by 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) in myeloid cells has been reported, the molecular mechanism by which TPA upregulates ICAM-1 expression remains unclear. In the present study, we investigated the signaling mechanism associated with TPA-induced ICAM-1 expression in ML-1 cells. Herein, our microarray, flow cytometry, and Western blot analysis indicated that ICAM-1 was constitutively expressed at a low level in ML-1 cells, but its expression was further upregulated at both the mRNA and protein levels in response to TPA. ICAM-1 expression in response to TPA was inhibited by pretreatment with GF109203X [a specific inhibitor of protein kinase C (PKC)], or with PD98059 and U0126 (specific inhibitors of MEK), suggesting the importance of PKC, and Erk1/2 signaling cascades in this response. Interestingly, ICAM-1 expression in response to TPA-induced PKC activation was linked to the generation of reactive oxygen species (ROS), as pretreatment with NAC (an ROS scavenger) blocked both ErK1/2 activation and ICAM-1 expression induced by TPA. In addition, TPA-induced ICAM-1 expression was blocked by inhibition of nuclear factor-κB (NF-κB) activation following pretreatment with BAY11-7085 (a specific inhibitor of NF-κB activation). TPA-induced NF-κB activation was shown by increased degradation of IkB (NF-κB specific inhibitory protein). Together, these observations demonstrated that TPA, a potent activator of PKC, induces ICAM-1 expression via a ROS- and ERK1/2-dependent signaling mechanism in ML-1 cells. J. Cell. Biochem. 100: 1010–1021, 2007.

Key words: phorbol 12-myristate 13-acetate; reactive oxygen species; protein kinase C; intercellular adhesion molecule; extracellular signal-regulated kinase; ML-1 cells

A key feature of effective host defense is the ability of leukocytes to leave the bloodstream and enter tissues in response to immune or inflammatory stimuli, which elicits leukocyte extravasation from blood into the various tissues. The principle point of regulation of this event is at the level of adhesive interactions between circulating leukocytes and vascular endothelial cells, a process that is in part regulated by specific endothelial leukocyte adhesion molecules [Springer, 1990; Mustjoki et al., 2001; Pelletier et al., 2004; Savino et al., 2004]. Intracellular adhesion molecule-1 (ICAM-1 or CD54) is an inducible cell surface glycoprotein belonging to the immunoglobulin gene superfamily. ICAM-1, along with its principle target proteins, leukocyte function-associated antigen (LFA-1), integrins and CD44 have been commonly implicated in the adhesion interactions of leukocytes during transendothelial migration and cell adhesive interactions with the extracellular matrix [Dustin and Springer, 1988; Bendall et al., 1993; Mustjoki et al., 2001; Pelletier et al., 2004; Blois et al., 2005; Kumagai et al., 2005]. ICAM-1 expression

Abbreviations used: TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; ICAM-1, intercellular adhesion molecule 1; ROS, reactive oxygen species; ERK, extracellular signal-regulated kinase.

Grant sponsor: National Institute of Environmental Health Sciences (NIEHS); Grant number: T32 ES 07141; Grant sponsor: The Johns Hopkins Center in Urban Environmental Health; Grant number: P30 ES 03819.

*Correspondence to: Kassim Traore, PhD, Department of Environmental Health Sciences, Bloomberg School of Public Health, Johns Hopkins University, 615 North Wolfe Street, Baltimore, MD 21205. E-mail: ktraore@jhsph.edu

Received 8 March 2006; Accepted 12 July 2006

DOI 10.1002/jcb.21101

can be induced by diverse stimuli, including bacterial lipopolysaccharide (LPS) [Kumagai et al., 2005], inflammatory cytokines, such as tumor necrosis factor α (TNF-α) [Huang et al., 2003], interferon-γ (INF-γ) [Chang et al., 2002], and phorbol esters [Wertheimer et al., 1992; Chang et al., 2002]. The expression of ICAM requires de novo synthesis at both the mRNA and protein levels [Braley-Mullen et al., 2001; Chang et al., 2002] suggesting regulation at the transcriptional level. The promoter region of ICAM-1 gene has been identified and characterized, and has shown to contain recognition sequences including two TATA boxes, two nuclear factor-κB (NF-κB) sites, two AP1 sites, two AP2, one INF-γ sites, and two glucocorticoid receptor element sites [Chang et al., 2002]. Of these, the NF-κB sites and binding proteins (transcription factors) appear to be essential for the enhanced ICAM-1 gene expression in response to various stimuli including cytokines [Wertheimer et al., 1992; Braley-Mullen et al., 2001; Chang et al., 2002; Pazdrak et al., 2004; Lin et al., 2005a; Shi and Sarna, 2005].

12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), a potent activator of protein kinase C (PKC) induces the differentiation of myeloid leukemia cells to macrophages [Shimizu et al., 2002]. An early event of this differentiation processes includes expression of different cell surface proteins including ICAM-1 [Goebeler et al., 1993; Bernatchez et al., 1997]. Although, ICAM-1 expression has previously been shown to occur in leukemic cells in response to TPA, the intracellular signaling mechanism by which TPA induces ICAM-1 expression in these cells remains unclear. Recently, we reported that incubation of leukemic cells, THP-1 and ML-1 cells with TPA result in an increased activation of extracellular regulated kinase (ERK1/2) signaling cascade [He et al., 1999; Traore et al., 2005]. In the present study, we examined the signaling pathway involved in TPA-induced ICAM-1 expression in ML-1 cells. Our biochemical and molecular biology analysis show that TPA treatment results in the activation of PKC to signal ICAM-1 expression via an NF-κB-dependent mechanism in ML-1 cells. Moreover the results establish for the first time that the signal transduction cascade used by TPA to induced ICAM-1 gene expression required reactive oxygen species (ROS) generation to initiate the activation of the ERK1/2 signaling cascade.

MATERIALS AND METHODS

Reagents and Antibodies

Selective pharmacologic inhibitors for MEK (PD98059), (U0126), and NF-κB (BAY 11-7085) were purchased from Sigma (St. Louis, MO). The selective inhibitor for PKC (GF109203X) was obtained from ALEXIS Biochemicals (San Diego, CA). The anti-human phospho-Erk1/2 antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). The anti-human ICAM-1 antibodies were purchased from BD Pharmingen (San Diego, CA).

Cell Culture

Human myeloid leukemia, ML-1 cells were obtained from Dr. Ruth Craig, Dartmouth School of Medicine. 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 cm^2 tissue culture flasks, and maintained at 37°C in a humidified 5% CO_2 atmosphere as we previously described [Traore et al., 2005]. Experiments were routinely carried out using cells in the log phase of growth. Phorbol ester (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 at a final concentration of 5 nM for ML-1 cells for various times. 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 and treated with 5 nM TPA for 6 h. Total RNA was extracted using Trizol reagent and purify 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.

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 ß-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 13,000*g* 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 goat anti-human ICAM-1 diluted 1:1,000. After washing the blots, horseradish-conjugated anti-goat IgG, diluted 1:2,000, was added to the blots. Immunoreactivity was detected by chemiluminescence (PerkinElmer, Life Sciences, MA).

FACS Analysis

The levels of ICAM-1 (CD54), phospho-ErK1/2 and phospho-IkB were determined using FACS-Scan analysis. Cells (1×10^6) resuspended in PBS containing 0.1% sodium azide and 5% FBS were incubated on ice for 30 min with 1 μg allophycocyanin (APC)-conjugated anti-ICAM-1 or permeabilized with ice cold methanol then incubated with (Alexa-fluor 488)-conjugated anti-phospho-ErK1/2 or anti-phospho-IkB antibodies for 1 h. The cells were washed twice, fixed in 2% formaldehyde in PBS, and analyzed by FACS-Scan analysis. Negative controls were stained with isotype-matched APC or (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

The generation of cellular ROS, in particular H_2O_2, was assessed using luminol-dependent chemiluminescence as previously described [Traore et al., 2005]. One million cells were preincubated with luminol (10 μM) and horseradish peroxidase (10 μg/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 chemiluminescence was measured continuously in a Berthold LB9505 (Pforzheim, Germany) luminometer at 37°C for 50 min.

RESULTS

TPA Induces ICAM-1 Gene Expression in ML-1 Cells

Following the addition of a differentiating concentration of TPA, ML-1 cells stop dividing and attach to the culture dish. Using anti-ICAM-1 antibodies, we examined the role of ICAM-1 in TPA-induced ML-1 cell adhesion. The lattices formed from strong cell–cell interactions and adhesions were disrupted in the presence of anti-ICAM-1 treatment (Fig. 1A). This observation indicated that ICAM-1, at least in part, plays a role in ML-1 cell attachment. The inspection of microarray data showed that the TPA treatment resulted in an increase of ICAM-1, LFA, CD44, and integrin beta-1 mRNA levels in ML-1 cells within 6 h of treatment (Fig. 1B). Maximum ICAM-1 mRNA

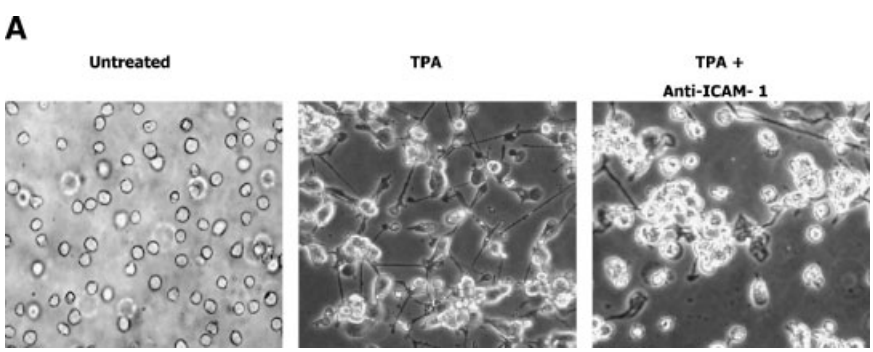


Fig. 1. Role of ICAM-1 in TPA-induced ML-1 cell adhesion. **A**: ML-1 cells were incubated in the presence or in the absence of TPA, or in the presence of anti-ICAM-1 and of TPA for 24 h. Cell attachment was examined by microscopy. To determine the levels of ICAM-1 mRNA, ML-1 cells were treated with TPA for the indicated times. Total RNA was extracted, purified, normalized, and analyzed by microarray analysis using a Human Genome U133 2.0 Plus Array Chip. **B**: The levels of ICAM-1, LFA-1, CD44, and beta-integrins mRNA were assessed after 6 h of treatment with TPA. **C**: The levels of ICAM-1, mRNA were assessed after 6 h, 3 or 6 days of treatment with TPA.

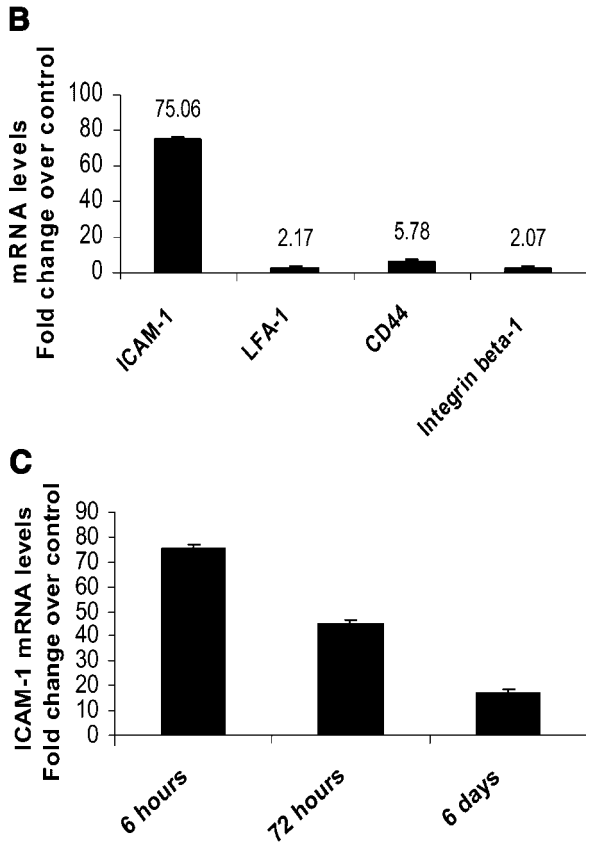


Fig. 1. (*Continued*)

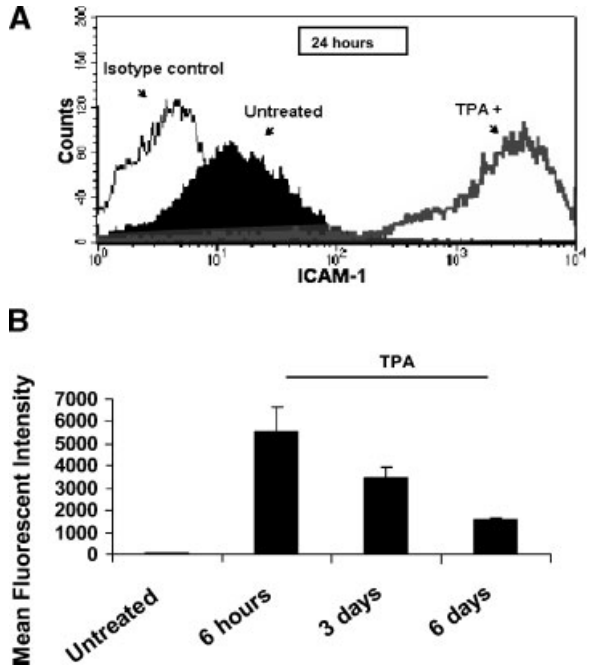


Fig. 2. To determine the levels of ICAM-1 protein expression on the cell surface in response to TPA, ML-1 cells were incubated in the absence or in the presence of TPA (**A**) for 24 h or (**B**) for 1, 3 or 6 days. Cells were harvested, washed, and stained with APC-labeled anti-human ICAM-1, then analyzed by flow cytometry. Data were analyzed by ModFILT statistical software. The figure depicts representative results from one of three replicate experiments.

levels occurred within 6 h of TPA treatment followed by a time-dependent decrease (Fig. 1C). Flow cytometry analysis indicated that ICAM-1 was constitutively expressed at a low level on the ML-1 cell surface, which was significantly upregulated in response to TPA (Fig. 2A). The temporal studies indicated that ICAM-1 protein level was maximal within 24 h of TPA treatment, and decreased progressively in a time-dependent manner (Fig. 2B). Together these observations demonstrated for the first time that TPA induces ICAM-1 expression at the transcription level in ML-1 cells.

Role of PKC in TPA-Induced ICAM-1 Expression in ML-1 Cells

Since TPA is a potent activator of PKC [Traore et al., 2005]. This led us to examine whether the TPA-induced ICAM-1 expression may be mediated via a PKC signaling cascade. Using GF109203X, a specific inhibitor of PKC, we examined the involvement of PKC activation in TPA-induced ICAM-1 expression by Western blot analysis, flow cytometry analysis, and immunocytochemistry analysis. Data shown, respectively, in Figure 3A–C, indicated that pretreatment with GF109203X blocked ICAM-1 protein expression and cell surface suppression in ML-1 cells. Interestingly, GF109203X also blocked ML-1 cell differentiation induced by TPA (data not shown). These results strongly suggest that TPA-induced ICAM-1 expression required activation of PKC in ML-1 cells.

Role of Extracellular Regulated Kinase (ERK) Signaling Cascade in TPA-Induced ICAM-1 Expression in Ml-1 Cells

The ERK signaling cascade has been implicated in regulating ICAM-1 expression induced by various extracellular stimuli [Lin et al., 2005a]. Since we previously have shown that TPA induces ErK1/2 activation in THP-1 cells [Traore et al., 2005] and ML-1 cells [He et al., 1999], this led us to hypothesize that ERK signaling cascade may be involved in signaling TPA-induced ICAM-1 expression in ML-1 cells. Using PD98059 and U0126, both inhibitors of MEK, we assessed the role of MAPK in

signaling TPA-induced ICAM-1 expression in ML-1 cells. The Western blot, flow cytometry, and immunohistochemistry analysis data shown, respectively, in Figure 3A–C, indicated that pretreatment with U01265 (5 μM) effectively and PD98059 (25 μM) partially suppressed ICAM-1 expression induced by TPA in ML-1 cells. We also compared the inhibitory effect of U0126 and PD98059 on ErK1/2 activation in ML-1 cells. Pretreatment with 5 μM U0126 effectively and 25 μM PD98059 again partially suppressed ErK1/2 phosphorylation induced by TPA (Fig. 4). Interestingly, the increased concentration of PD98059 (40 μM) is as efficient as 5 μM of U0126 in inhibiting both ErK1/2 activation and ICAM-1 expression in ML-1 cells, but results in toxicity to the ML-1 cells (data not shown). These observations support the effectiveness of U0126 as a MEK inhibitor in blocking both ErK1/2 activation and ICAM-1 expression. Since PKC is known to mediate TPA activity, using GF109203X we also examined the role of PKC signaling cascade in TPA-induced ErK1/2 phosphorylation in ML-1 cells. Pretreatment with GF109203 also significantly blocked ErK1/2 phosphorylation (Fig. 4). These observations strongly suggested that activation of the ERK signaling cascade, at least in part, is required for TPA-induced ICAM-1 expression in ML-1 cells. The data also demonstrate that the effectiveness of U0126 as an inhibitor of ICAM-1 expression results from its strong inhibitory capacity of ErK1/2 activation.

Role of ROS in TPA-Induced ICAM-1 Gene Expression

ROS has been implicated in regulating ICAM-1 expression induced by many extracellular stimuli including cytokines [Hubbard and Rothlein, 2000; Ichikawa et al., 2004; Li et al., 2005]. Previous studies, including our own have demonstrated that ROS generation is induced in leukemic cell lines THP-1 [Traore et al., 2005] and U-937 [Datta et al., 2000] in response to TPA treatment. To test whether ROS is involved in TPA-induced ICAM-1 expression in ML-1 cells, we first measured ROS generation in ML-1 cells in response to TPA. Here, the generation of ROS was assessed by luminol-dependent

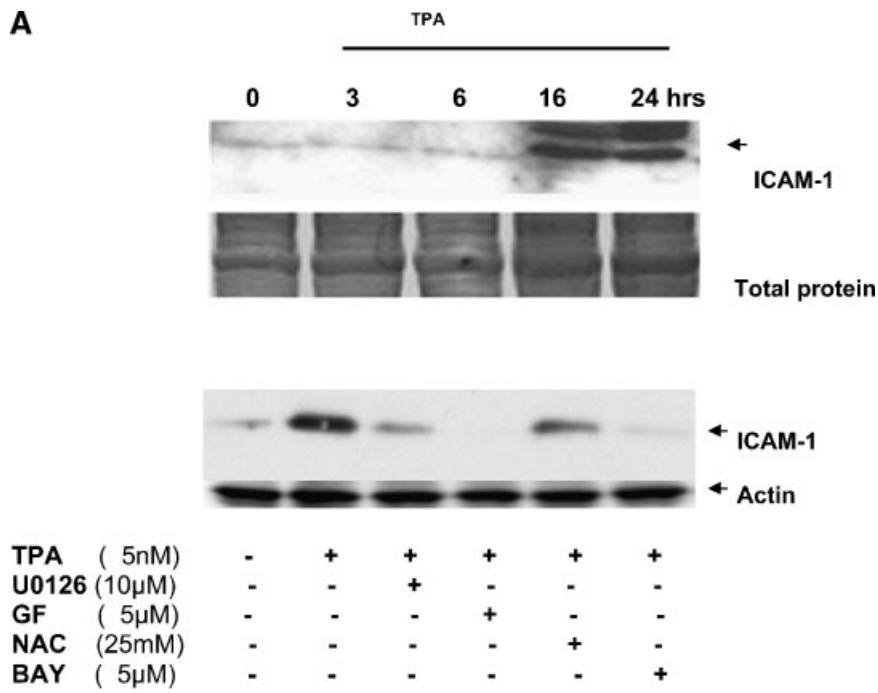
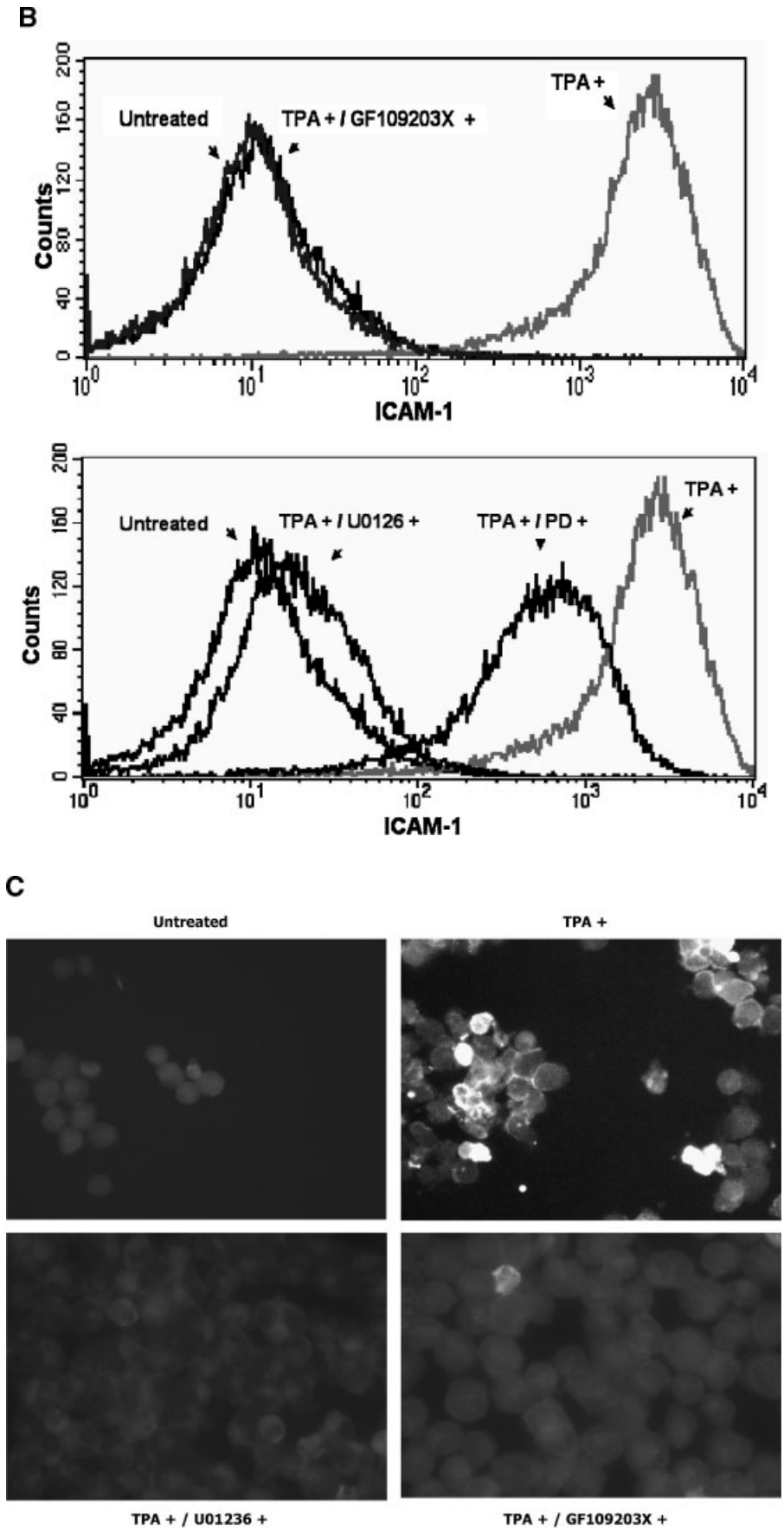


Fig. 3. Effects of PKC, MEK inhibitors, and ROS scavenger on TPA-induced ICAM-1 expression. ML-1 cells were incubated for 20 min in media containing MEK inhibitors (U0126, PD98059), PKC inhibitor GF109203X or ROS scavenger NAC, followed by incubation with 5 nM TPA for 24 h. Cells were harvested and washed with PBS. **A**: Total protein were extracted and analyzed by Western blot analysis using anti-ICAM-1 antibodies. **B**: Cells were stained using APC-labeled anti-human ICAM-1, then analyzed by flow cytometry. **C**: Cells were analyzed by immunocytochemistry using anti-ICAM-1. The figure depicts representative results from one of three replicates experiments.



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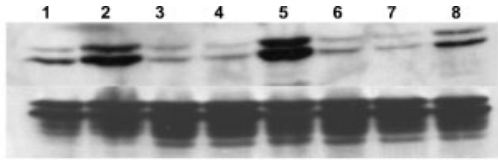

Lane 1: lysate from untreated ML-1 cells
Lane 2, 3 and 4: lysates from cells TPA-treated cells for 1, 3 and 6 hrs respectively
Lane 5: lysates from cells treated with TPA for 1 hr only
Lane 6: lysate from cells pretreated with U0126 (10μM) then treated with TPA for 1hr
Lane 7: lysate from cells pretreated with GF109203X (5μM) then treated with TPA for 1hr
Lane 8: lysate from cells pretreated with PD9059 (25μM) then treated with TPA for 1hr

Fig. 4. Effects of GF109203X, U0126, and PD98059 on TPA-induced Erk1/2 phosphorylation and ICAM-1 expression. ML-1 cells were incubated for 20 min in media containing U0126, PD98059 or GF109203X at indicated concentration followed by TPA treatment for indicated times. Cells were harvested, washed with PBS. Total proteins were extracted and analyzed by Western blot analysis, using rabbit anti-human phospho-p44/p42 (p-Erk1/2) antibodies. The figure depicts representative results from one of three replicates experiments.

chemiluminescence, which in this case monitors H_2O_2 outside the cell. Treatment with TPA resulted in a marked increase in ROS generation, which occurred maximally at 30 min in ML-1 cells (Fig. 5A). Using GF109203X, we then studied the role of PKC in the generation ROS in ML-1 cells in response to TPA. Pretreatment with GF109203X effectively suppressed ROS generation induced by TPA in ML-1 cells (Fig. 5B). In addition, N-acetylcysteine (NAC), a ROS scavenger [Zafarullah et al., 2003], also inhibited TPA-induced ROS generation in ML-1 cells, assuring the involvement of PKC and the effectiveness of NAC as scavenger of ROS generated in response to TPA. Moreover, TPA induced ICAM-1 expression was effectively downregulated by pretreatment with NAC (Figs. 3A and 6B). These results suggest the involvement of ROS in signaling ICAM-1 expression in ML-1 cells in response to TPA. In our recently published article, we proposed that TPA-induced ROS generation is required for ErK1/2 kinase activation in THP-1 cells [Traore et al., 2005]. This led us to investigate the role of ROS in signaling ErK1/2 activation in ML-1 cells. Pretreatment with NAC significantly inhibited Erk1/2 activation induced by TPA in ML-1 (Fig. 6B). These results suggest that ROS generation is a required event during both TPA-induced ErK1/2 activation and ICAM-1 expression in ML-1 cells.

Role of NF-κB in TPA-Induced ICAM-1 Expression

NF-κB has been shown to regulate ICAM-1 expression induced by diverse extracellular stimuli including TNF-α and TPA in many cell lines [Wertheimer et al., 1992; Braley-Mullen et al., 2001; Chang et al., 2002; Pazdrak et al., 2004; Lin et al., 2005b; Shi and Sarna, 2005]. We examined whether NF-κB mediates TPA-induced ICAM-1 expression in ML-1 cells. Using BAY11-7085, a specific inhibitor of NF-κB, we assessed the role of NF-κB in TPA-induced ICAM-1 expression. Pretreatment with BAY11-7085 effectively suppressed TPA-induced ICAM-1 expression in ML-1 cells (Figs. 3A and 7). We also assessed the level of NF-κB activation by determining the level of IkB (NF-κB inhibitory protein) phosphorylation and degradation. Flow cytometry analysis data indicated that the high-level phospho-IkB protein observed in ML-1 cells decreased in a time-dependent fashion in response to TPA treatment (Fig. 8). The progressive decrease in the level of phospho-IkB is probably due to its increased degradation in response to TPA treatment. Together these results suggest that NF-κB, at least in part, is involved in TPA-induced ICAM-1 expression, seemingly at the level of transcription.

Effects of PD98059, U0126, GF109203X, BAY11-7085, and NAC on TPA-Induced ML-1 Adhesion

Herein, we examined the effects of PD98059, U0126, GF109203X, BAY11-7085, and NAC on TPA-induced ML-1 cell adhesion. Pretreatment of ML-1 cells with the indicated pharmacological agents resulted in a marked inhibition of ML-1 cells adhesion induced by TPA treatment (Fig. 9).

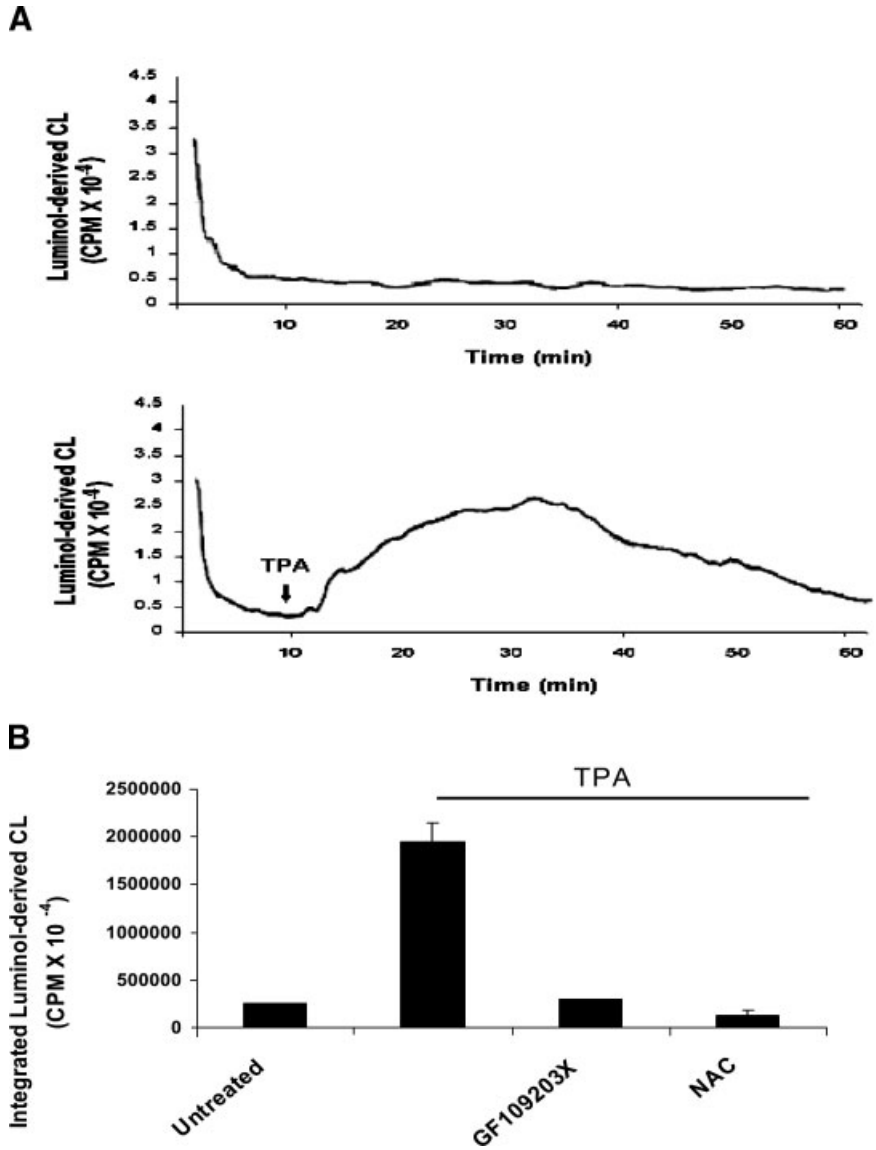

Fig. 5. The generation of ROS in ML-1 cells in response to TPA was assessed by luminol-dependent chemiluminescence. **A**: Cells were incubated in the presence or in the absence of TPA or **B**: Cells were preincubated with GF109203X or NAC for 10 min followed by TPA treatment for 50 min. Luminol-dependent luminescence of 1 million cells was measured in Berthold luminometer for 1 h. The chemiluminesence in (A) are cpm $\times 10^{-4}$ over the indicate time period. The data in (B) are the chemiluminescence from three cell samples integrated over 50 min. The luminol-dependent chemiluminescence of untreated cells is at the level of the instrument background.

DISCUSSION

Intercellular adhesion molecule (ICAM-1) is a cell surface glycoprotein that has been implicated in playing critical roles during different biological processes including hematopoietic cell development in response to various cytokines [Springer, 1990; Mustjoki et al., 2001; Pelletier et al., 2004; Savino et al., 2004]. Previously, we have reported that TPA, a well-known activator of PKC, induces human myeloid cell line ML-1 cell growth arrest and differentiation to macrophages [He et al., 1999]. In the present study, we have investigated the signaling mechanism associated with ICAM-1 expression in ML-1 cells in response to TPA treatment. PKC has been implicated to mediate TPA-induced activities in many cell lines. Wertheimer et al. [1992] have indicated that TPA-induced ICAM-1 expression was mediated via a PKC-dependent fashion in human endothelial cells. In agreement with this finding, we have found that

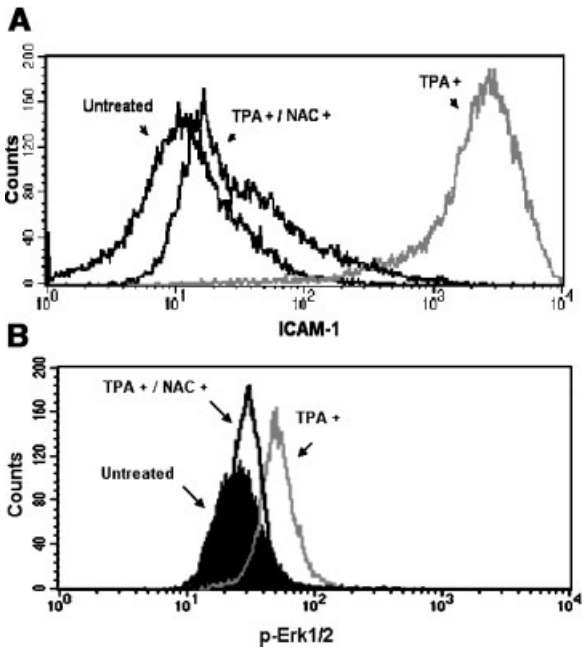

Fig. 6. Effects of ROS scavenger NAC on TPA-induced ICAM-1 expression in ML-1 cells. **A**: Cells were incubated in media containing NAC, a known ROS scavenger for 20 min followed by incubation with media containing TPA for 24 h. Cells were harvested, washed, and stained using anti-human ICAM-1 antibodies and analyzed by flow cytometry. The figure depicts representative results from one of three replicates experiments. **B**: Cells were incubated in media containing NAC for 20 min followed by TPA treatment for 1 h. Cells were harvested, washed, fixed, permeabilized, and stained using (Alexa-Fluor 488) labeled anti-phospho-Erk1/2 and analyzed by flow cytometry. The figure depicts representative results from one of three replicate experiments.

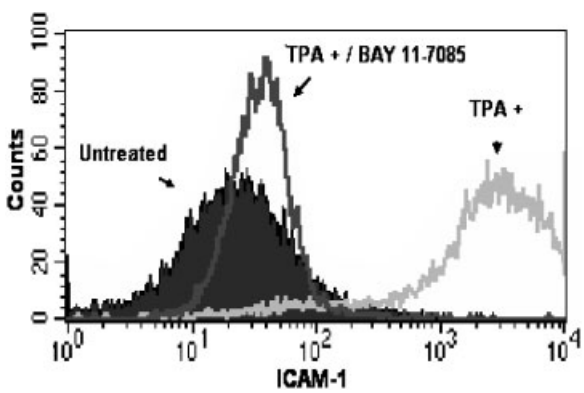

Fig. 7. The effect of BAY11-7085 on TPA-induced expression of CAM-1 was assessed by flow cytometry. ML-1 cells were preincubated with BAY11-7085 for 20 min followed by TPA treatment for 24 h. Cells were harvested, washed, and stained using anti-human ICAM-1, then analyzed by flow cytometry. The figure depicts representative results from one of three replicate experiments.

TPA-induced ICAM-1 protein expression and ML-1 cells adhesion were dependent on PKC activation since pretreatment with GF109203X completely inhibited ICAM-1 expression in ML-1 cells. In addition, pretreatment with GF109203X also completely blocked ML-1 cell adhesion induced by TPA. These observations strongly suggested that PKC activation is crucial in TPA-induced ICAM-1 expression and adhesion of ML-1 cell. Several recent studies [Datta et al., 2000; Traore et al., 2005] have shown that TPA treatment is associated with increased generation of ROS in different cell lines. In the present study, we have demonstrated that ROS are generated in ML-1 cells in response to TPA. An interesting finding in this study is that pretreatment with NAC or GF109203X not only inhibits ROS generation but also ICAM-1 expression in TPA-treated ML-1 cells. These observations strongly suggested not only that PKC activation is required for TPA-induced ROS generation and ICAM-1 expression, but also that the ROS generated in response to PKC activation is indispensable for ICAM-1 expression in ML-1 cells.

MAPKs have been implicated in the expression of ICAM-1 in response to various stimuli including interleukin-1β [Lin et al., 2005b] and TNF-α [Li et al., 2005]. It has been demonstrated that TPA induces ErK1/2 activation via PKC activation [Kaneki et al., 1999; Jang et al., 2005]. In the present study, we have demonstrated that the incubation of ML-1 cells with TPA results in a marked increase in the levels of the phosphorylated form of ErK1/2 in ML-1 cells. ErK1/2 activation was maximal at 1 h and decreased in a time-dependent fashion to baseline, 3–6 h after treatment with TPA. Interestingly, pretreatment with U0126, PD98059, and GF109203X inhibited ErK1/2 activation. However, U0126 and GF109203X not only were effective in inhibiting ErK1/2 activation but also ICAM-1 protein expression; while, PD98059 was only partially effective. U0126 was more potent in inhibiting ICAM-1 expression seemingly because of its strongest inhibitory effects on ErK1/2 activation. Jang et al. [2005] have demonstrated that TPA treatment results in the increased activation of ErK1/2 and is mainly dependent on PKC signaling pathway in U937 cells. In agreement with this observation, we have demonstrated that TPA-induced ErK1/2 phosphorylation in ML-1 cells is also dependent on PKC activation, as

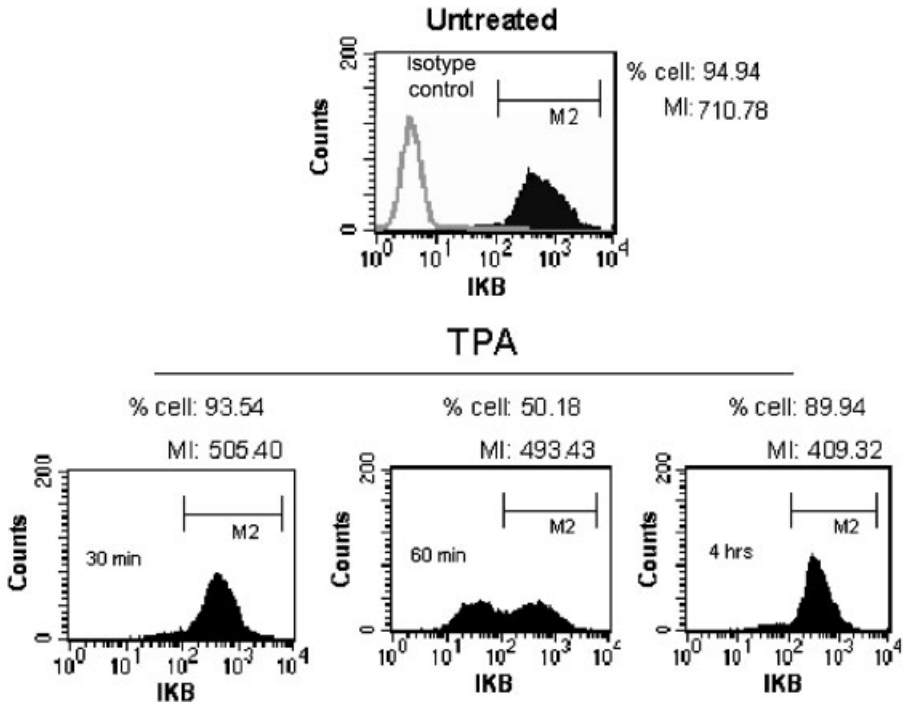

Fig. 8. NF-κB activation was assessed by examining IkB degradation induced by TPA in Ml-1 cells. Five millions cells were treated with TPA for various times, harvested, and stained using anti-phospho-IkB antibodies and analyzed by flow cytometry. Data were analyzed by ModFILT statistical software. The mean fluorescence intensity (MI) for each sample was determined. **Upper panel**: untreated cells. **Lower panel**: cells treated with TPA for various time (30 min, 60 min, and 4 h). The figure depicts representative results from one of three replicate experiments.

GF109203X effectively blocked TPA-induced ErK1/2 activation.

The NF-κB signaling pathway has been implicated in the expression of ICAM-1 induced in response to various stimuli [Wertheimer et al., 1992; Braley-Mullen et al., 2001; Chang et al., 2002; Pazdrak et al., 2004; Lin et al., 2005a; Shi and Sarna, 2005]. Interestingly, our present data indicated that BAY 11-7085 effectively inhibited TPA-induced ICAM-1 expression in ML-1 cells. Jang et al. [2005] have also shown that TPA induces NF-κB p65 activation in U937 cells. In agreement with this study our data have shown that TPA treatment resulted in an increased degradation of IkB, suggesting NF-κB activation in response to TPA treatment in ML-1 cells.

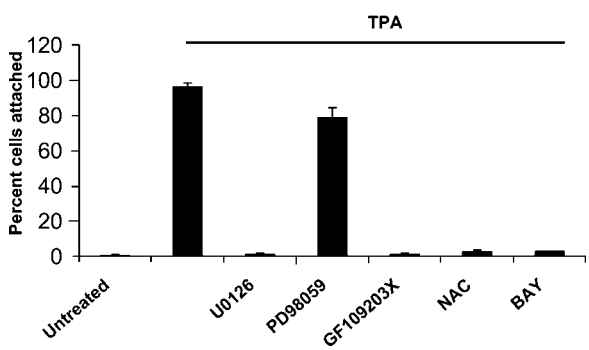

Fig. 9. Effects of U0126, PD98059, GF109203X, NAC, and BAY11-7085 on TPA-induced ML-1 cell adhesion. Three millions cells were incubated for 20 min in media containing 10 μM U0126, 25 μM PD98059, 5 μM GF109203X, 25 mM NAC or 5 μM BAY11-7085 followed by 5 nM TPA for 24 h. Media was collected from each sample. Attached cells were detached by trypsin treatment and collected. The cell counts were determined by hemocytometer and converted into percent cells.

In the present study, we have shown for the first time that ICAM-1 is constitutively expressed at a basal level in proliferating ML-1 cells, an observation which is consistent with a recent report on other leukemic cells [Ichikawa et al., 2004]. In addition, we have presented considerable evidence using pharmacologic agents that TPA a well-known activator of PKC further induces ICAM-1 gene expression to promote ML-1 cell adhesion. Furthermore, we have demonstrated that a ROS-dependent Raf/MEK/ERK pathway plays a crucial role in the regulation of ICAM- 1 expression in myeloid leukemia cells during their differentiation in

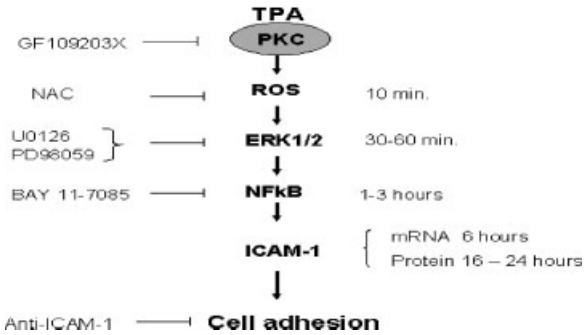

Fig. 10. Proposed signaling mechanism associated with TPA-induced ICAM-1 expression in ML-1 cell.

response to TPA. This is supported by the temporal sequence of events as outlined in Figure 10. Interestingly, Min et al. [2005] have shown that TNF-related activation-induced cytokine (TRANCE) induces ICAM-1 expression in endothelial cells via PKC-, ROS-, and NF-κB-dependent signaling mechanisms. Similarly, Quin et al. [2006] have also demonstrated that ICAM-1 expression is induced in human umbilical vein endothelial cells (HUVEC) via a ROS and NF-κB dependent mechanism, in response to bile acid treatment. However, these studies have omitted Erk1/2 activation as a required event in the signal transduction of ICAM-1 expression. Their conclusions were based on the use of PD98059 to block Erk1/2 activation. Unlike, these studies, our work has demonstrated that Erk1/2 activation is a required event for TPA-induced ICAM-1 expression in ML-1 cells. Furthermore, our data have shown that U0126 is more effective in inhibiting not only Erk1/2 activation but also ICAM-1 expression induced by TPA than is PD98059. Together these observations strongly suggested not only that PKC activation is required for TPA-induced ROS generation and ICAM-1 expression, but also that the ROS generated in response to PKC activation is indispensable for Erk1/2 activation to signal ICAM-1 expression in ML-1 cells. The cellular source of this ROS as well as any additional role of ROS in macrophage differentiation remains to be determined.

ACKNOWLEDGMENTS

We wish to acknowledge the financial support of the National Institute of Environmental Health Sciences (NIEHS) training grant (T32 ES 07141) and a pilot grant from the Johns Hopkins Center in Urban Environmental Health (P30 ES 03819).

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