Available online at www.sciencedirect.com

SCIENCE DIRECT

Cellular Signalling 17 (2005) 299–310

CELLULAR SIGNALLING

www.elsevier.com/locate/cellsig

Phorbol 12-myristate 13-acetate upregulates cyclooxygenase-2 expression in human pulmonary epithelial cells via Ras, Raf-1, ERK, and NF-κB, but not p38 MAPK, pathways

Ming-Shyan Chang[a], Bing-Chang Chen[b], Ming-Tze Yu[c], Joen-Rong Sheu[d], Tseng-Fu Chen[d], Chien-Huang Lin[a,b,*]

[a]*Graduate Institute of Biomedical Technology, Taipei Medical University, Taipei, Taiwan*
[b]*School of Respiratory Therapy, Taipei Medical University, Taipei, Taiwan*
[c]*Department of Thoracic Medicine, Taipei Medical University-Wan Fang Hospital, Taipei, Taiwan*
[d]*Department of Pharmacology, School of Medicine, Taipei Medical University, Taipei, Taiwan*

Received 12 May 2004; accepted 23 July 2004
Available online 11 September 2004

Abstract

In this study, we investigated the signaling pathway involved in cyclooxygenase-2 (COX-2) expression and prostaglandin E_2 (PGE_2) release by phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator, in human pulmonary epithelial cells (A549). PMA-induced COX-2 expression was attenuated by PKC inhibitors (Go 6976 and Ro 31-8220), a Ras inhibitor (manumycin A), a Raf-1 inhibitor (GW 5074), a MEK inhibitor (PD 098059), and an NF-κB inhibitor (PDTC), but not by a tyrosine kinase inhibitor (genistein) or a p38 MAPK inhibitor (SB 203580). PMA also caused the activation of Ras, Raf-1, and ERK1/2. PMA-induced activation of Ras and Raf-1 was inhibited by Ro 31-8220 and manumycin A. PMA-mediated activation of ERK1/2 was inhibited by Ro 31-8220, manumycin A, GW 5074, and PD 098059. Stimulation of cells with PMA caused IκBα phosphorylation, IκBα degradation, and the formation of a NF-κB-specific DNA–protein complex. The PMA-mediated increase in κB-luciferase activity was inhibited by Ro 31-8220, manumycin A, GW5074, PD 098059, and PDTC. Taken together, these results indicate that PMA might activate PKC to elicit activation of the Ras/Raf-1/ERK1/2 pathway, which in turn initiates NF-κB activation, and finally induces COX-2 expression and PGE_2 release in A549 cells.

Keywords: PMA; Cyclooxygenase-2; PKC; Ras; Raf-1; ERK1/2; NF-κB; A549 cells

1. Introduction

Prostaglandins (PGs) are ubiquitous compounds involved in various homeostatic and inflammatory processes throughout the body [1]. They are formed by the combined action of phospholipase A_2, which liberates arachidonic acid from the *sn*-2 position of cellular membrane phospholipids, and cyclooxygenase (COX), which converts arachidonic acid to the endoperoxide intermediate, PGH_2. PGH_2 is subsequently converted to various PGs by the action of cell-specific synthases [1]. It is now known that at least two distinct isoforms of COX exist [2,3]. COX-1 is generally responsible for the production of PGs under physiological conditions and is known to be constitutively expressed in many cell types

Abbreviations: COX-2; cyclooxygenase-2; DMEM/Ham's F-12; Dulbecco's modified Eagle's medium/Ham's F-12; ECL; enhanced chemiluminescence; EMSA; electrophoretic mobility shift assay; ERK; extracellular signal-regulated kinase; FCS; fetal calf serum; IKK; IκB kinase; LPS; lipopolysaccharide; MEK; MAPK/ERK kinase; MAPK; mitogen-activated protein kinase; MLB; magnesium lysis buffer; NF-κB; nuclear factor-κB; PMA; phorbol-12-myristate-13-acetate; PBS; phosphate-buffered saline; PKC; protein kinase C; PGE_2; prostaglandin E_2; PDTC; pyrrolidine dithiocarbamate; Raf-1 RBD; Ras-binding domain for Raf-1; SDS-PAGE; sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TNF-α; tumor necrosis factor-α.

* Corresponding author. School of Respiratory Therapy, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan. Tel.: +886 2 27361661x3510; fax: +886 2 27397313.
E-mail address: chlin@tmu.edu.tw (C.-H. Lin).

0898-6568/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellsig.2004.07.008

including endothelial cells, platelets, and the gastric mucosa [4]. COX-2 is induced by proinflammatory stimuli, including cytokines [5] and lipopolysaccharide (LPS) [6] in cells in vitro and in inflamed sites in vivo [4]. Furthermore, COX-2 is thought to be the isoform responsible for the production of proinflammatory PGs in various models of inflammation [7].

A549 cells, a human pulmonary epithelial carcinoma cell line with type II alveolar epithelial cell differentiation, show similar features as type II alveolar epithelial cells. Airway epithelial cells have an active role in inflammation by producing various cytokines and eicosanoids [8]. It has been demonstrated that proinflammatory cytokines, such as interleukin 1β (IL-1β), increased COX-2 expression and PGE_2 release in human A549 cells [9]. Phorbol esters such as phorbol 12-myristate 13-acetate (PMA) function as tumor promoters and have been reported to modulate diverse cellular responses such as gene transcription, cellular growth and differentiation, programmed cell death, the immune response, and receptor desensitization through protein kinase C (PKC) signaling pathways. PMA can substitute for diacylglycerol, the endogenous activator of PKC, and it has been used as a model agent to study the mechanisms utilized by growth factors, hormones, and cytokines to regulate the growth and differentiation of cells [10,11]. Phorbol esters, as well as cytokines and LPS, have been shown to upregulate COX-2 expression [12]. We recently reported that PMA upregulates COX-2 expression in human pulmonary epithelial cells (A549) [13]. However, a detailed analysis of the downstream signaling pathways involved in PMA/PKC-induced COX-2 expression has not been done. One well-studied mode of PKC-mediated signaling involves transmission of signals from PKC to mitogen-activated protein kinases (MAPKs). MAPK activation by PMA has been reported to occur via both Ras-dependent and -independent pathways. For example, PC-12 rat adrenal pheochromocytomas [14,15], Jurkat leukemia T cells [16], and primary rat ventricular myocytes [17] exhibit Ras-dependent activation of MAPK by PMA; however, in NIH3T3 mouse myeloma cells [18], COS-1 cells [19], and 293 embryonal kidney cell lines [20], this activation appears to be Ras-independent. Thus, the involvement of Ras in signaling processes initiated by PMA appears to be cell type-dependent and specifically determined by which signaling pathways have been activated and/or the cell's repertoire of kinases.

The classic Ras-mediated pathway involves the binding of Raf-1 and subsequent phosphorylation of Raf-1 at Ser338 by many kinases [21,22], which in turn activate extracellular signal-regulated kinases (ERKs) [23], and consequently phosphorylate many target proteins including transcription factors and protein kinases [24]. Although many cell types have been implicated as having a role with Ras in COX-2 induction [25–27], PMA-induced COX-2 expression has not been investigated in A549 cells. In this study, we show that PMA stimulates COX-2 expression and PGE_2 release in A549 cells through activation of PKC. Such COX-2 upregulation is Ras- and Raf-1-dependent, requires activation of the MAPK/ERK kinase (MEK)/ERK signaling pathway, and ultimately involves activation of a nuclear factor, NF-κB. However, activation of tyrosine kinase and p38 MAPK is not involved in the signaling pathway of PMA-mediated NF-κB activation or COX-2 expression in A549 cells.

2. Materials and methods

2.1. Materials

PMA, actinomycin D, cycloheximide, pyrrolidine dithiocarbamate (PDTC), dithiothreitol (DTT), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), EGTA, EDTA, glycerol, phenylmethylsulphonyl fluoride (PMSF), pepstatin A, leupeptin, sodium dodecyl sulfate (SDS), and Nonidet P-40 (NP-40) were purchased from Sigma (St. Louis, MO). GW 5074 was purchased from Tocris Cookson (Ellisville, MO). Genistein, manumycin A, NS-398, Go 6976, Ro 31-8220, PD 098059, and SB 203580 were purchased from Calbiochem (San Diego, CA). Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12, fetal calf serum (FCS), penicillin/streptomycin, and lipofectamine plus were purchased from Life Technologies (Gaithersburg, MD). A PGE_2 enzyme immunoassay kit was obtained from Cayman (Ann Arbor, MI). Protein A/G beads, antibodies specific for ERK2, ERK phosphorylated at Tyr204, p38 MAPK, IκBα, IκBα phosphorylated at Ser32? Raf-1, and anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody specific for Raf-1 phosphorylated at Ser338 and a Ras activity assay kit were purchased from Upstate (Lake Placid, NY). An antibody specific for p38 MAPK phosphorylated at Thr180/Tyr182 was purchased from Cell Signaling Biotechnology (Beverly, MA). PGL2-ELAM-Luc (which is under the control of one NF-κB binding site) and pBK-CMV-Lac Z were kindly provided by Dr. W.-W. Lin (National Taiwan University, Taipei, Taiwan). [γ-^{32}P]ATP (6,000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Antibodies specific for COX-2 and p65 were purchased from Transduction Laboratories (Lexington, KY). An antibody specific for α-tubulin was purchased from Oncogene Science (Cambridge, UK). Anti-mouse IgG-conjugated alkaline phosphatase was purchased from Jackson Immuno Research Laboratories (West Grove, PA). 4-Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate were purchased from Boehringer Mannheim (Mannheim, Germany). All materials for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad (Hercules, CA).

2.2. Cell culture

A549 cells, a human pulmonary epithelial carcinoma cell line with type II alveolar epithelial cell differentiation, were

obtained from American Type Culture Collection (Livingstone, MT) and grown in DMEM/Ham's F-12 nutrient mixture containing 10% FCS and penicillin/streptomycin (50 U/ml) in a humidified 37 °C incubator. When confluent, cells were disaggregated in a trypsin solution, washed in DMEM/Ham's F-12 supplemented with 10% FCS, centrifuged at 125×*g* for 5 min, then resuspended and subcultured according to standard protocols.

2.3. Measurements of PGE_2 release and COX activity

A549 cells were cultured in 12-well culture plates. For experiments designed to measure the release of PGE_2 due to endogenous arachidonic acid, cells were treated with PMA (10 nM) for indicated time intervals, or pretreated with NS-398 (0.01–1 μM) for 30 min followed by PMA for 12 h, and incubated in a humidified incubator at 37 °C. The medium was then removed and stored at −80 °C until being assayed. PGE_2 was assayed using the PGE_2 enzyme immunoassay kit according to the procedure described by the manufacturer. In separate experiments designed to measure the activity of the COX enzyme, COX activity was quantified by providing cells with exogenous arachidonic acid, the substrate for COX, and measuring its conversion to PGE_2. Briefly, cells were treated with PMA (0.1–100 nM) for 12 h or PMA (10 nM) for indicated time intervals, after which cells were washed with phosphate-buffered saline (PBS, pH 7.4), and fresh medium containing arachidonic acid (30 μM) was added for 30 min at 37 °C; then the medium was collected for the PGE_2 enzyme immunoassay. In some experiments, cells were pretreated with specific inhibitors as indicated followed by PMA (10 nM) and incubated in a humidified incubator at 37 °C for 12 h COX activity was then determined as described above.

2.4. Protein preparation and Western blotting

The expressions of COX-2, α-tubulin, Ras, Raf-1 phosphorylated at Ser338, Raf-1, ERK phosphorylated at Tyr204, ERK2, p38 MAPK phosphorylated at Thr180/Tyr182, and p38 MAPK in A549 cells were evaluated, and the preparation of total proteins and Western blotting were performed as described previously [28]. Briefly, A549 cells were cultured in 6-cm dishes. After reaching confluence, cells were treated with vehicle and PMA (10 nM) or pretreated with specific inhibitors as indicated followed by PMA. After incubation, cells were washed twice in ice-cold PBS and solubilized in extraction buffer containing 10 mM Tris (pH 7.0), 140 mM NaCl, 2 mM PMSF, 5 mM DTT, 0.5% NP-40, 0.05 mM pepstatin A, and 0.2 mM leupeptin. Samples of equal amounts of protein (60 μg) were subjected to SDS-PAGE, then transferred onto a PVDF membrane which was then incubated in TBST buffer (150 mM NaCl, 20 mM Tris–HCl, and 0.02% Tween, pH 7.4) containing 5% non-fat milk. Proteins were visualized by specific primary antibodies and then incubated with horseradish peroxidase- or alkaline phosphatase-conjugated second antibodies. Immunoreactivity was detected using enhanced chemiluminescence (ECL) or NBT/BCIP following the manufacturer's instructions. The quantitative data were obtained using a computing densitometer with Image-Pro plus software (Media Cybernetics, MD).

2.5. Ras activity assay

Ras activity was measured using a Ras activity assay kit. The assay was performed according to the manufacturer's instructions. Briefly, A549 cells were washed twice with ice-cold PBS and lysed in magnesium lysis buffer (MLB) (25 mM HEPES, pH 7.5, 150 mM NaCl, 5% lgepal CA-630, 10 mM $MgCl_2$, 5 mM EDTA, 10% glycerol, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and centrifuged. An equal volume of lysate was incubated with 5 μg of Ras-binding domain for Raf-1 (Raf-1 RBD) at 4 °C overnight, and the beads were washed three times with MLB lysis buffer. Bound Ras proteins were then solubilized in 2× Laemmli sample buffer and quantitatively detected by Western blotting (10% SDS-PAGE) using mouse monoclonal Ras with the ECL system and by densitometry of corresponding bands using scientific imaging systems.

2.6. Preparation of nuclear extracts and the electrophoretic mobility shift assay (EMSA)

A549 cells were cultured in 10-cm Petri dishes. After reaching confluence, cells were treated with vehicle or PMA (10 nM) for 10, 30, 60, and 120 min, then cells were scraped and collected. The cytosolic and nuclear protein fractions were next separated as described previously [29]. Briefly, cells were washed with ice-cold PBS and pelleted. The cell pellet was resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.5 mM DTT, 10 mM aprotinin, 10 mM leupeptin, and 20 mM PMSF) for 15 min on ice, and vortexed for 10 s. Nuclei were pelleted by centrifugation at 15,000×*g* for 1 min. Supernatants containing cytosolic proteins were collected. A pellet containing nuclei was resuspended in hypertonic buffer (20 mM HEPES, pH 7.6, 25% glycerol, 1.5 mM $MgCl_2$, 4 mM EDTA, 0.05 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 20 mM PMSF) for 30 min on ice. Supernatants containing nuclear proteins were collected by centrifugation at 15,000×*g* for 2 min and then stored at −70 °C. Protein levels of phospho-IκBα and IκBα in the cytosolic fraction, and p65 in the cytosolic and nuclear fractions were determined by Western blotting.

A double-stranded oligonucleotide probe containing NF-κB sequences (5′-AGTTGAGGGGACTTTCC-CAGGC-3′; Promega) was purchased and end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The nuclear extract (2.5–5 μg) was incubated with 1 ng of ^{32}P-labeled NF-κB probe (50,000–75,000 cpm) in 10 μl

binding buffer containing 1 μg poly(dI–dc), 15 mM HEPES, pH 7.6, 80 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol at 30 °C for 25 min. DNA/nuclear protein complexes were separated from the DNA probe by electrophoresis on 6% polyacrylamide gels; then the gels were vacuum-dried and subjected to autoradiography with an intensifying screen at −80 °C. Quantitative data were obtained using a computing densitometer with Image-Pro plus software.

2.7. Transfection and κB-luciferase assays

For these assays, 2×10^5 A549 cells were cultured in 12-well plates, and then transfected the following day using Lipofectamine plus (Gibco) with 0.5 μg of pGL2-ELAM-Luc and 1 μg of pBK-CMV-LacZ. After 24 h, the medium was aspirated and replaced with fresh DMEM/Ham's F-12 containing 10% FBS. Cells were treated with vehicle and PMA (10 nM) or pretreated with specific inhibitors as indicated for 30 min before incubation of PMA for 24 h. Luciferase activity was determined with a luciferase assay system (Promega) and was normalized on the basis of LacZ expression. The level of induction of luciferase activity was determined as a ratio in comparison to cells with no stimulation.

2.8. Statistical analysis

Results are shown as the mean±S.E.M. from three to four independent experiments. One-way analysis of variance (ANOVA) followed by, when appropriate, Bonferroni's multiple-range test was used to determine the statistical significance of the difference between means. A p value of less than 0.05 was taken to be statistically significant.

3. Results

3.1. Characterization of COX-2 expression and PGE_2 release induced by PMA

Basal levels of PGE_2 released from A549 cells was low (4.4±0.5 ng/ml, n=4). However, incubation of A549 cells with PMA (10 nM) for various time intervals resulted in a time-dependent increase in PGE_2 release, which reached a maximal level at 12 h (27.1±4.3 ng/ml, n=4) (Fig. 1a). Pretreatment of cells with NS-398 (0.01–1 μM), a selective COX-2 inhibitor [30], for 30 min attenuated PMA-induced PGE_2 release in a concentration-dependent manner. When cells were treated with 1 μM NS-398 completely inhibited PMA-induced PGE_2 release (Fig. 1b). Treatment of A549 cells with PMA (0.01–100 nM, for 12 h) caused concentration-dependent increases in COX activity (measured as described in Materials and methods) and the expression of a 70-kDa COX-2 protein, with a maximal effect caused by 10 nM PMA treatment (Fig. 2a,b). When

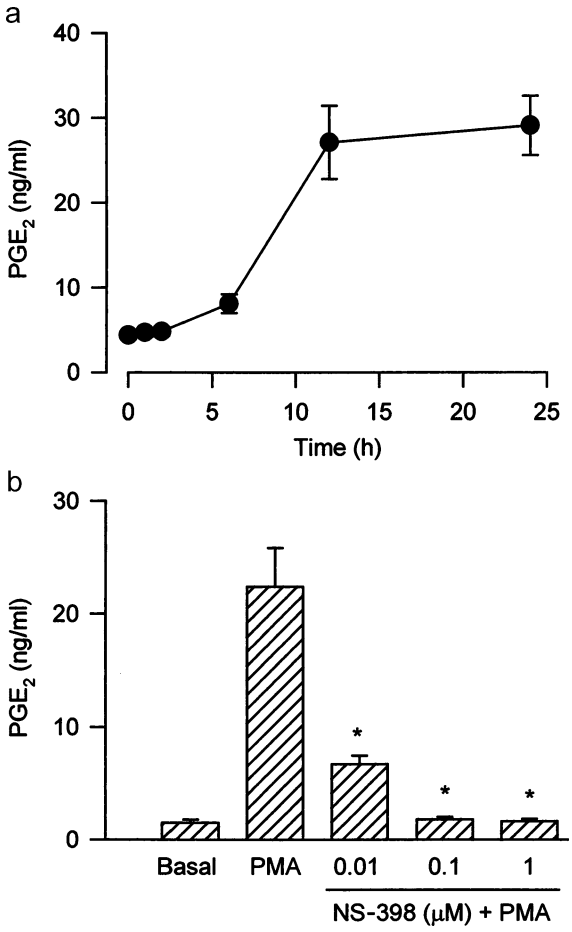


Fig. 1. Time-dependent increase in PGE_2 release caused by PMA and effects of NS398 on PMA-induced PGE_2 release in A549 cells. In (a), cells were incubated with 10 nM PMA for various time intervals, then the medium was collected for PGE_2 measurement. In (b), cells were pretreated with various concentrations of NS398 for 30 min before incubation with 10 nM PMA for 12 h, then the medium was collected for PGE_2 measurement. Results are expressed as the mean±S.E.M. (n=3). *p<0.05 as compared with the PMA-treated group.

cells were treated with 10 nM PMA for various time intervals, COX activity and COX-2 expression increased in time-dependent manners. The maximal effect on the increases of COX activity occurred at 12 h. The earliest induction of COX-2 protein occurred at 2 h, and peaked at 12 h (Fig. 2c,d). In the following experiments, cells were treated with 10 nM PMA for 12 h. Pretreatment of cells with the transcriptional inhibitor, actinomycin D (0.03 and 0.1 μM) [31], or the translational inhibitor, cycloheximide (1 and 3 μM) [32], for 30 min markedly attenuated the PMA-induced increase in COX activity. The PMA-induced increase in COX activity was inhibited by 0.1 μM actinomycin D and 3 μM cycloheximide by 78±3% and 90±2%, respectively (Fig. 3a). Similarly, PMA-induced COX-2 expression was also attenuated by actinomycin D (0.1 μM) or cycloheximide (3 μM) (Fig. 3c). When cells were pretreated for 30 min with Go 6976 (0.1–1 μM), a classical PKC-α,-β, and -γ inhibitor [33], or Ro 31-8220 (0.3–3 μM), an non-selective PKC inhibitor [34], PMA-mediated

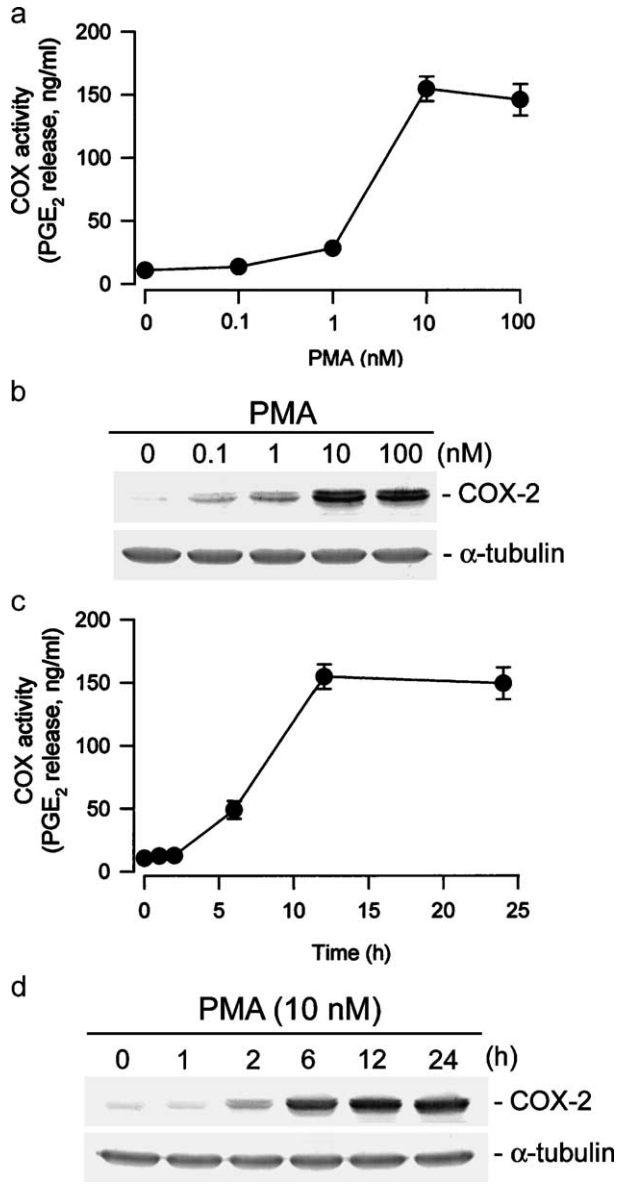


Fig. 2. Concentration- and time-dependent increases in COX activity and COX-2 expression caused by PMA in A549 cells. Cells were incubated with various concentrations of PMA for 12 h (a) or 10 nM PMA for various time intervals (c). The increase in COX activity was measured by examining PGE_2 formation in the presence of 30 μM exogenous arachidonic acid for 30 min. Results are expressed as the mean±S.E.M. (n=4). In (b) and (d), cells were incubated with various concentrations of PMA for 12 h (b) or 10 nM PMA for various time intervals (d), and the extracted proteins were then immunodetected with specific antibodies for COX-2 or α-tubulin as described in Materials and methods. Equal loading in each lane is demonstrated by similar intensities of α-tubulin. Typical traces are representative of three experiments with similar results.

increase in COX activity was inhibited by both Go 6976 and Ro 31-8220 in concentration-dependent manners. Pretreatment of the cells with 1 μM Go 6976 and 3 μM Ro 31-8220 inhibited the PMA response by 87±3% and 84±2%, respectively (Fig. 3b). PMA-induced COX-2 expression was also inhibited by Go 6976 (1 μM) and Ro 31-8220 (3 μM) (Fig. 3d).

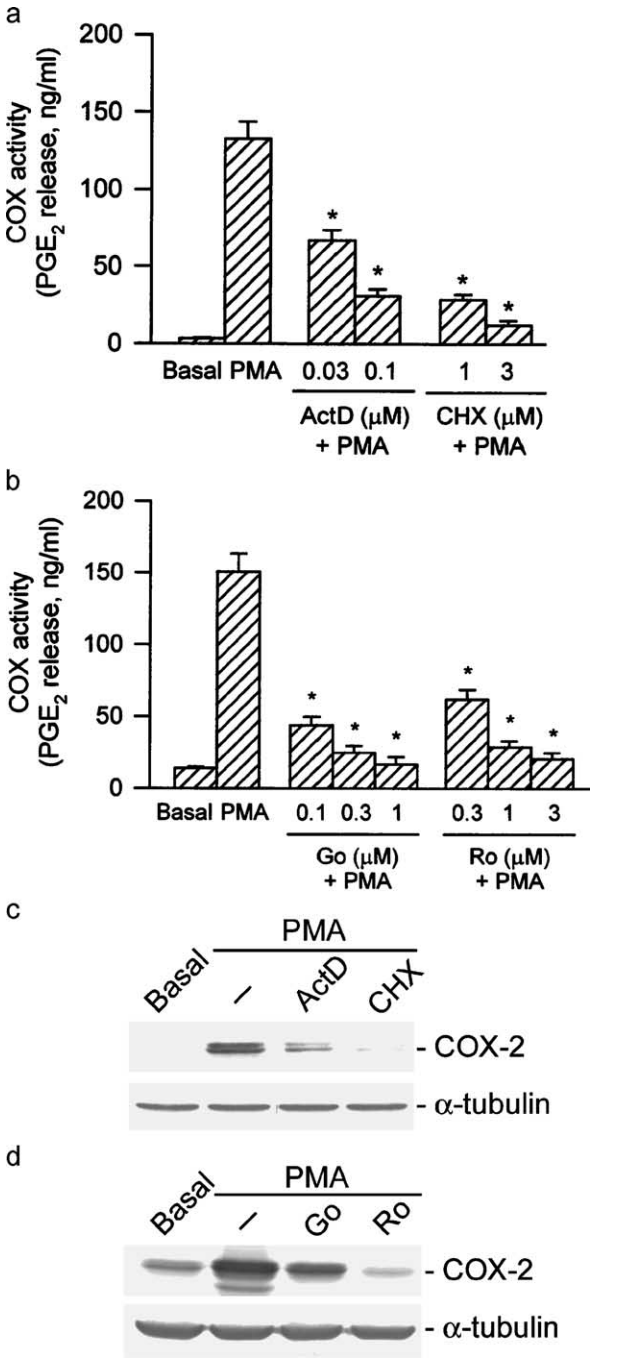


Fig. 3. Effects of actinomycin D, cycloheximide, Go 6976, and Ro 31-8220 on PMA-induced increases in COX activity and COX-2 expression in A549 cells. Cells were pretreated with various concentrations of actinomycin D, cycloheximide (a), Go 6976, or Ro 31-8220 (b) for 30 min before incubation with 10 nM PMA for 12 h. The increase in COX activity was measured by examining PGE_2 formation in the presence of 30 μM exogenous arachidonic acid for 30 min. Results are expressed as the mean±S.E.M. (n=4). *p<0.05 as compared with the PMA-treated group. In (c) and (d), cells were pretreated with 0.1 μM actinomycin D, 3 μM cycloheximide (c), 1 μM Go 6976, or 3 μM Ro 31-8220 (d) for 30 min before incubation with 10 nM PMA for 12 h. Cells were then prepared for immunodetection using a COX-2- or α-tubulin-specific antibody as described in Materials and methods. Equal loading in each lane is demonstrated by similar intensities of α-tubulin. Typical traces are representative of three experiments with similar results. ActD, actinomycin D; CHX, cycloheximide; Go, Go 6976; Ro, Ro 31-8220.

3.2. Involvement of Ras in PMA-induced COX-2 expression

To investigate the involvement of Ras activation in PMA-induced COX-2 expression, A549 cells were treated with manumycin A (a Ras inhibitor) [35]. Pretreatment of the cells for 30 min with manumycin A (1 and 3 μM) attenuated the PMA-induced COX-2 expression by 38±3% and 69±8%, respectively (n=3). However, 3 μM manumycin A had no effect on the basal level of COX-2 expression (Fig. 4a). We further measured the Ras activity in cells

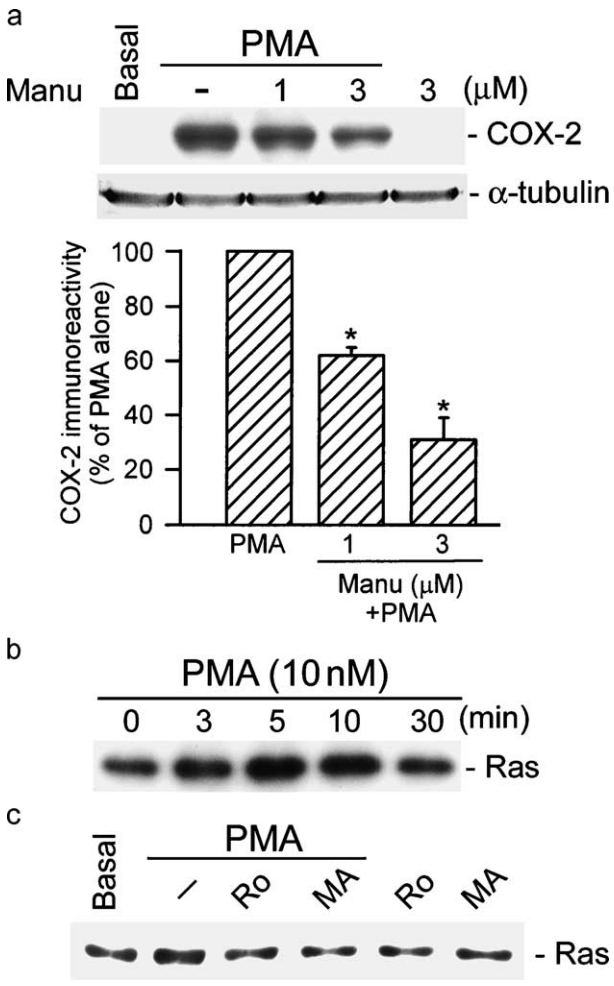


Fig. 4. Effects of manumycin A on PMA-induced COX-2 expression and Ras activation in A549 cells. In (a), cells were pretreated with manumycin A (1 and 3 μM) for 30 min followed by stimulation with 10 nM PMA for another 12 h, and COX-2 expression was determined by immunoblotting with an antibody specific for COX-2. Equal loading in each lane is demonstrated by the similar intensities of α-tubulin. Typical traces are representative of three experiments with similar results, which are presented as the mean±S.E.M. *p<0.05 as compared with the PMA-treated group. In (b), cells were incubated with 10 nM PMA for 0–30 min, and cell lysates were then immunoprecipitated with an antibody specific for Raf-1 RBD. The Ras activity assay is described in Materials and methods. Typical traces represent two experiments with similar results. In (c), cells were pretreated with 1 μM Ro 31-8220 or 3 μM manumycin A for 30 min, and then treated with 10 nM PMA for another 5 min. Cells were then lysed for the Ras activity assay as described above. Typical traces represent two experiments with similar results. Ro, Ro 31-8220; Manu, manumycin A.

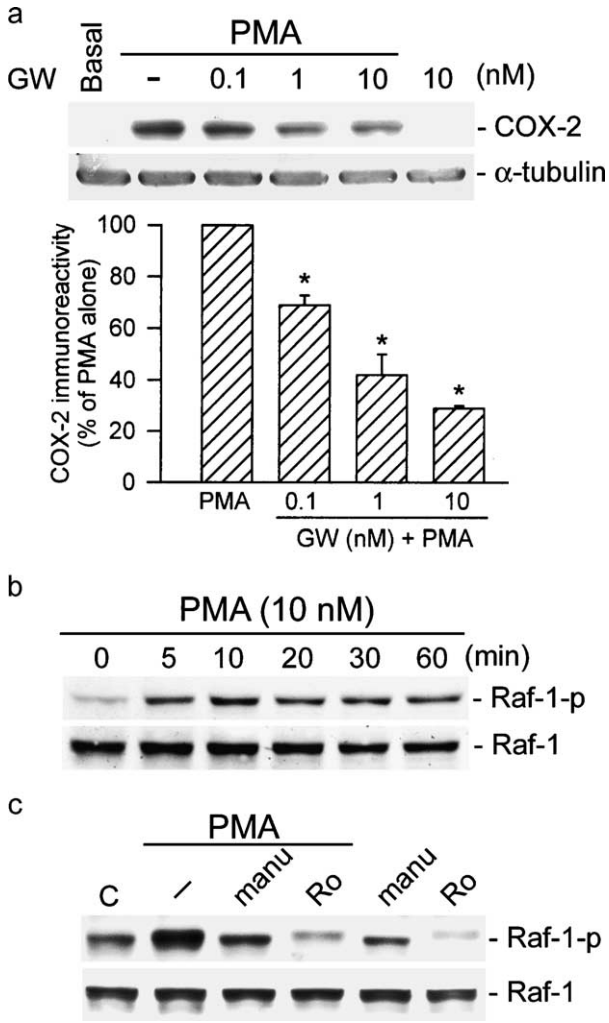


Fig. 5. Effects of GW 5074 on PMA-induced COX-2 expression and Raf-1 phosphorylation in A549 cells. In (a), cells were pretreated with GW 5074 (0.1–10 nM) for 30 min followed by stimulation with 10 nM PMA for another 12 h, and COX-2 expression was determined by immunoblotting with a specific COX-2 antibody. Equal loading in each lane is demonstrated by the similar intensities of α-tubulin. Typical traces represent three experiments with similar results, which are presented as the mean±S.E.M. *p<0.05 as compared with the PMA-treated group. In (b), cells were incubated with 10 nM PMA for 0–30 min, and then Raf-1 phosphorylation (upper panel) and Raf-1 (bottom panel) protein levels were determined. The presence of equal loading in each lane is shown by the similar intensities of Raf-1. Traces represent results from three independent experiments. In (c), cells were pretreated with 3 μM manumycin A or 1 μM Ro 31-8220 for 30 min, and then treated with 10 nM PMA for another 10 min, and then the Raf-1 phosphorylation (upper panel) protein level was determined. Equal loading in each lane is demonstrated by similar intensities of Raf-1 (bottom panel). Typical traces represent three experiments with similar results. Manu, manumycin A; Ro, Ro 31-8220.

treated with PMA. As shown in Fig. 4b, treatment of A549 cells with 10 nM PMA increased the Ras activity in a time-dependent manner. The maximal activation was detected at 5 min after stimulation, and the effect gradually decreased after 10 min of treatment. The PMA-induced increase in Ras activity was markedly inhibited by pretreatment of cells for

30 min with 1 μM Ro 31-8220 and 3 μM manumycin A (Fig. 4c).

3.3. Involvement of Raf-1 in PMA-induced COX-2 expression

To examine whether Raf-1, one of the target proteins for Ras, might play a crucial role in PMA-induced COX-2 expression, the Raf-1 inhibitor, GW 5074 [36], was used. As shown in Fig. 5a, 10 nM GW 5074 alone did not affect the basal COX-2 level, but it inhibited PMA-induced COX-2 expression in a concentration-dependent manner. When cells were treated with 10 nM GW 5074, the PMA-induced COX-2 expression was inhibited by 71±1% (n=3). Raf-1 is associated with Ras-GTP and then by additional modifications such as phosphorylation at Ser338 becomes the active form. The activated Raf-1 then triggers sequential activation of downstream molecules [22]. Thus, phosphorylation of Raf-1 at Ser338 is a critical step in Raf-1 activation. Next, we further examined Raf-1 Ser338 phosphorylation by PMA stimulation in A549 cells; the anti-phospho-Raf-1 antibody at Ser338 was used. When cells were treated with 10 nM PMA for various time intervals, Raf-1 phosphorylation increased at 5 min and peaked at 10 min. After 20 min of treatment, the PMA-induced response declined (Fig. 5b). In addition, PMA-induced Raf-1 phosphorylation was also inhibited by pretreatment with 3 μM manumycin A and 1 μM Ro 31-8220 (Fig. 5c). Results indicated that Raf-1 is a downstream molecule of Ras and is involved in PMA-mediated COX-2 expression.

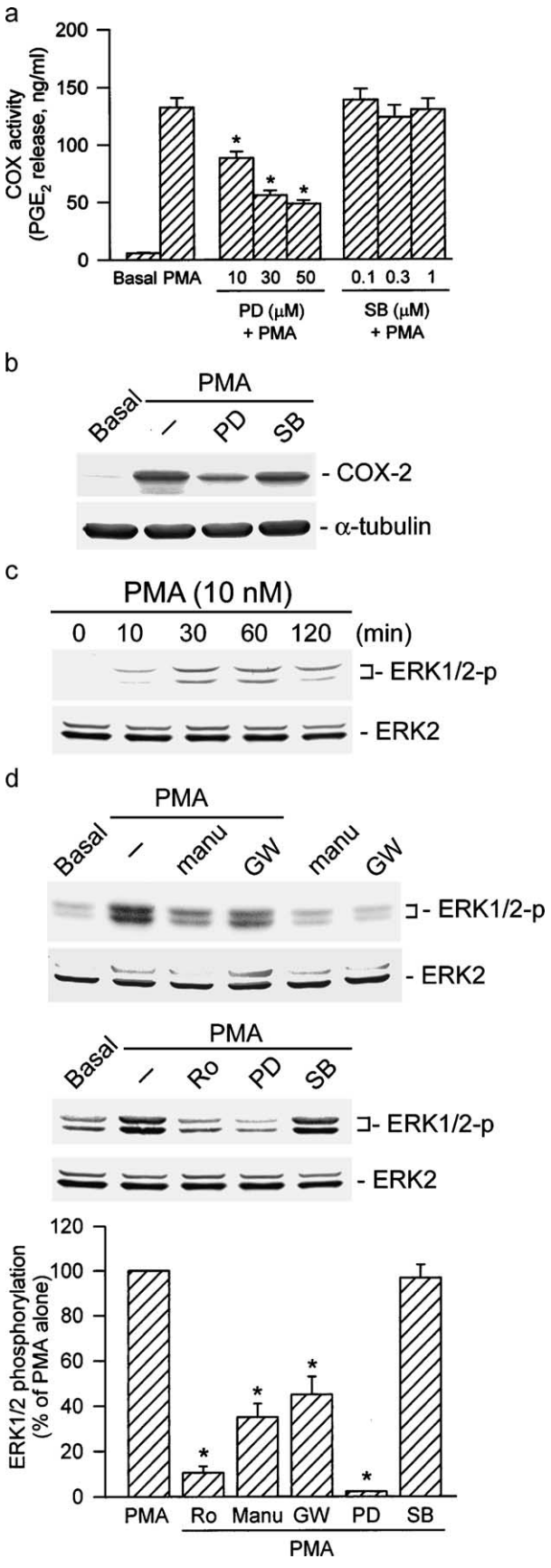


Fig. 6. ERK1/2, but not p38 MAPK, involved in PMA-mediated COX-2 expression in A549 cells. In (a), cells were pretreated with various concentrations of PD 098059 or SB 203580 for 30 min before incubation with 10 nM PMA for 12 h. The increase in COX activity was measured by examining PGE_2 formation in the presence of 30 μM exogenous arachidonic acid for 30 min. Results are expressed as the mean±S.E.M. (n=4). *p<0.05 as compared with the PMA-treated group. In (b), cells were pretreated with 30 μM PD 098059 or 1 μM SB 203580 for 30 min before incubation with 10 nM PMA for 12 h. Cells were then prepared for immunodetection using a COX-2- or α-tubulin-specific antibody as described in Materials and methods. Equal loading in each lane is demonstrated by similar intensities of α-tubulin. Typical traces represent three experiments with similar results. In (c), cells were treated with 10 nM PMA for different time intervals, and ERK1/2 phosphorylation was shown by immunoblotting with an antibody specific for phosphorylated ERK1/2 (ERK1/2-p) (upper panel). Equal loading in each lane is shown by the similar intensities of ERK2 (bottom panel). Traces represent results from three independent experiments. In (d), cells were pretreated with 3 μM manumycin A, 10 nM GW 5074, 1 μM Ro 31-8220, 30 μM PD 098059, or 1 μM SB 203580 for 30 min before incubation with 10 nM PMA for 30 min. Whole-cell lysates were prepared and subjected to Western blot analysis using antibodies specific for phospho-ERK1/2 (ERK1/2-p) or ERK2 as described in Materials and methods. The extent of ERK1/2 activation was quantitated using a densitometer with Image-Pro plus software. Results are expressed as the mean±S.E.M. (n=3). *p<0.05 as compared with the PMA-treated group. Manu, manumycin A; GW, GW 5074; Ro, Ro 31-8220; PD, PD 098059; SB, SB 203580.

3.4. ERK1/2 but not p38 MAPK is involved in PMA-induced COX-2 expression

In order to examine whether ERK1/2 and p38 MAPK activations are involved in the signal transduction pathway leading to COX-2 expression caused by PMA, the MEK inhibitor, PD 098059 [37], and the p38 MAPK inhibitor, SB 203580 [38], were used. Pretreatment of cells for 30 min with PD 098059 (10–50 μM) inhibited the PMA-induced increase in COX activity in a concentration-dependent manner, while SB 203580, at a concentration up to 1 μM, had no effect. PD 098059 (50 μM) inhibited the PMA response by 66±3% (n=3) (Fig. 6a). Pretreatment of the cells with PD 098059 (50 μM) also markedly inhibited PMA-induced COX-2 expression, while SB 203580 (1 μM) had no effect (Fig. 6b). When cells were pretreated for 30 min with the tyrosine kinase inhibitor, genistein (10 and 30 μM) [39], PMA-mediated COX-2 expression was not affected by genistein (data not shown).

Since activation of ERK1/2 and p38 MAPK requires phosphorylation at the threonine and tyrosine residues, immunoblot analysis was performed to examine ERK1/2 and p38 MAPK phosphorylation using specific antibodies for phospho-ERK1/2 or phospho-p38 MAPK. When cells were stimulated with PMA (10 nM) for 10, 30, 60, and 120 min, ERK phosphorylation began at 10 min, peaked at 30–60 min, and then gradually decreased after 120 min of treatment (Fig. 6c). The protein level of ERK2 was not affected by PMA treatment (Fig. 6c). When cells were pretreated for 30 min with Ro 31-8220 (1 μM), manumycin A (3 μM), GW 5074 (10 nM), genistein (30 μM), PD 098059 (30 μM), or SB 203580 (1 μM), PMA-induced ERK1/2 activation was markedly inhibited by Ro 31-8220, manumycin A, GW 5074, and PD 098059, but not by SB 203580 (Fig. 6d) or genistein (data not shown). None of these treatments had any effect on ERK2 expression (Fig. 6d). Treatment of A549 cells with PMA (10 nM) also caused p38 MAPK activation, with a maximum effect at 30 min of treatment. After 60 min of treatment, PMA-induced p38 MAPK activation gradually declined (Fig. 7a). The protein level of p38 MAPK was not affected by PMA treatment (Fig. 7a). PMA-induced p38 MAPK activation was markedly inhibited by pretreatment of cells for 30 min with Ro 31-8220 (1 μM) or SB 203580 (1 μM), but not with PD 098059 (30 μM) (Fig. 7b). None of these treatments had any effect on p38 MAPK expression (Fig. 7b).

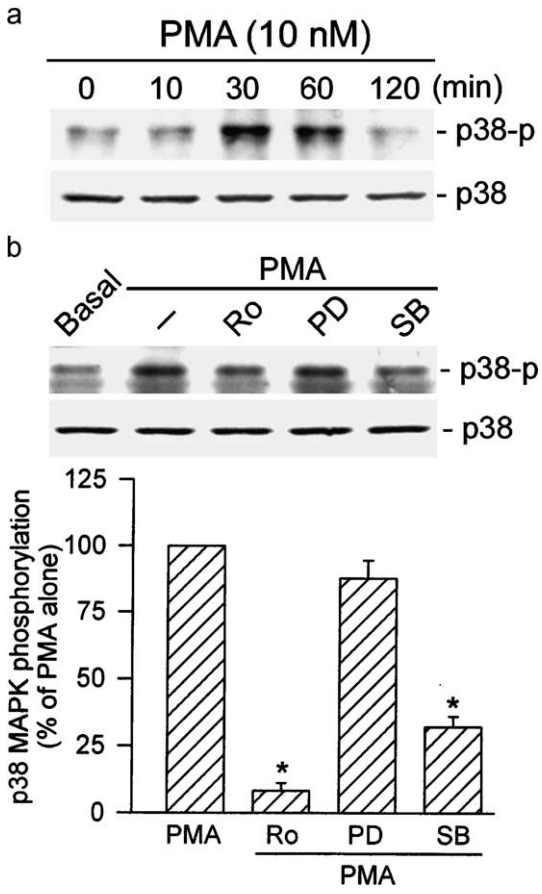
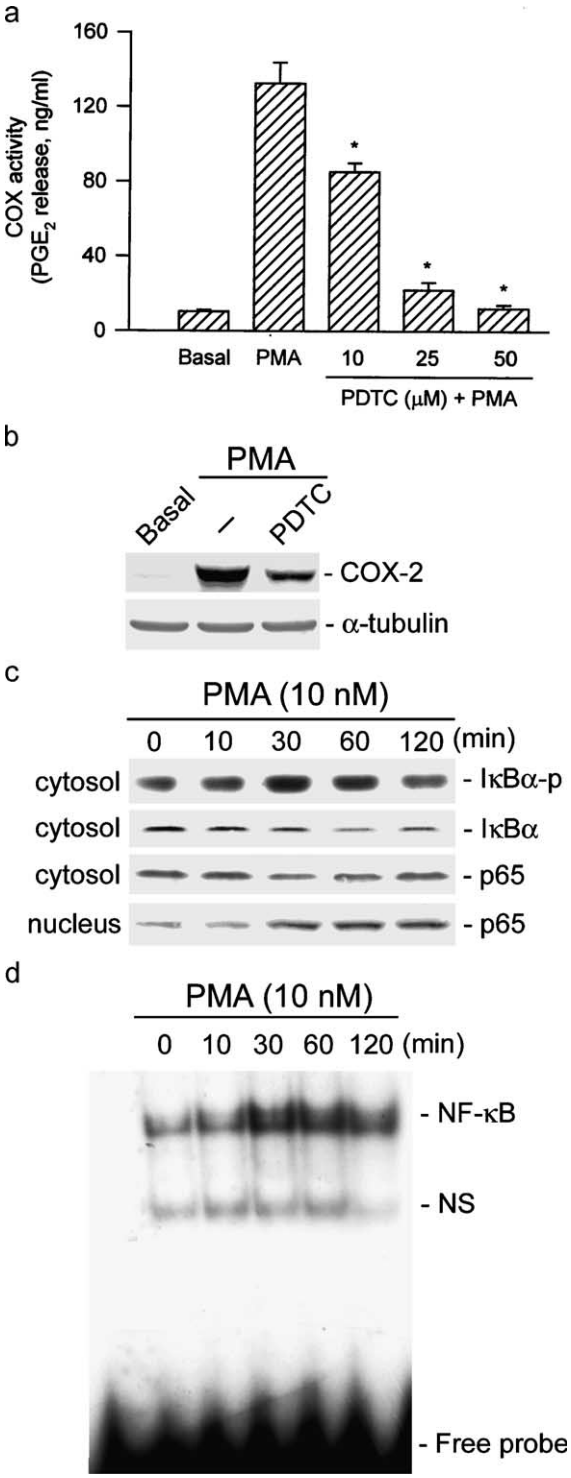


Fig. 7. PMA induction of p38 MAPK activation in A549 cells. In (a), cells were treated with 10 nM PMA for different time intervals, and p38 MAPK phosphorylation was shown by immunoblotting with an antibody specific for phosphorylated p38 MAPK (p-p38) (upper panel). Equal loading in each lane is shown by the similar intensities of p38 MAPK (p38) (bottom panel). Traces represent results from three independent experiments. In (b), cells were pretreated with 1 μM Ro 31-8220, 30 μM PD 098059, or 1 μM SB 203580 for 30 min before incubation with 10 nM PMA for 30 min. Whole-cell lysates were prepared and subjected to Western blot analysis using antibodies specific for p-p38 MAPK (p38-p) or p38 MAPK (p38) as described in Materials and methods. The extent of p38 MAPK activation was quantitated using a densitometer with Image-Pro plus software. Results are expressed as the mean±S.E.M. (n=3). *p<0.05 as compared with the PMA-treated group. Ro, Ro 31-8220; PD, PD 098059; SB, SB 203580.

3.5. Involvement of NF-κB in PMA-induced COX-2 expression

To further study whether NF-κB is involved in the signal transduction pathway leading to COX-2 expression caused by PMA, the specific NF-κB inhibitor, PDTC [40], was used. Pretreatment of cells for 30 min with PDTC (10–50 μM) attenuated the PMA-induced increase in COX activity in a concentration-dependent manner (Fig. 8a). When cells were treated with 50 μM PDTC, the PMA-induced increase in COX activity was inhibited by 90±2% (n=3). Similarly, the PMA-induced COX-2 expression was also attenuated by PDTC (25 μM) (Fig. 8b). Stimulation of cells with PMA (10 nM) results in marked IκBα phosphorylation in the cytosol, with a maximal effect that occurred after 30 min of treatment. Furthermore, PMA also caused marked IκBα degradation after 30–60 min of treatment, and the IκBα protein was resynthesized after 120 min of treatment. In parallel with IκBα phosphorylation and IκBα degradation, PMA resulted in marked p65 translocation from the cytosol to the nucleus which peaked at 30–60 min, and then declined after 120 min of treatment (Fig. 8c). In nuclear extracts of unstimulated cells, a slight intensity of NF-κB-specific DNA–protein complex formation was observed.

Stimulation of cells with PMA (10 nM) for 30–60 min resulted in marked activation of NF-κB-specific DNA–protein complex formation. However, after 120 min of treatment with PMA, the intensities of these DNA–protein complexes decreased (Fig. 8d). Stimulation of cells with PMA (10 nM) for 24 h resulted in a 4.3-fold increase in κB-luciferase activity (Fig. 9). When cells were pretreated for 30 min with Ro 31-8220 (1 μM), manumycin A (3 μM), GW 5074 (10 nM), genistein (30 μM), PD 098059 (50 μM), SB 203580 (1 μM), or PDTC (50 μM), the PMA-induced increase in κB-luciferase activity was markedly inhibited by Ro 31-8220, manumycin A, GW 5074, PD 098059, and PDTC by 93±4%, 58±9%, 62±4%, 87±9%, and 58±5%, respectively, but not by genistein or SB 203580 (Fig. 9).



4. Discussion

The findings of this study showed that PMA induces COX-2 expression and PGE_2 release in human pulmonary epithelial cells (A549) through activation of the PKC signal pathway. Our results suggest that the activations of Ras, Raf-1, MEK, ERK1/2, and NF-κB might be involved in signal transduction leading to the expression of COX-2 caused by PMA/PKC in these cells.

PGs are formed by the combined action of phospholipase A_2, which liberates arachidonic acid from the *sn*-2 position of cellular membrane phospholipids, and COX, which converts arachidonic acid to the endoperoxide intermediate, PGH_2. PGH_2 is subsequently converted to various PGs, such as PGE_2, prostacyclin, and thromboxanes, by the action of cell-specific synthases [1]. In this study, we found that PMA caused concentration- and time-dependent increases in COX activity and COX-2 expression in A549 cells. Furthermore, in the absence of exogenous arachidonic acid, PMA caused a marked increase in PGE_2 release; this effect was inhibited by the selective COX-2 inhibitor, NS398. These findings suggest that PMA might stimulate not only COX-2 expression but also phospholipase A_2 activation in human pulmonary epithelial cells. Previous studies have shown that actinomycin D and cycloheximide are transcriptional and translational inhibitors, respectively [41]. We found that actinomycin D and cycloheximide markedly inhibited the PMA-induced increase in COX

Fig. 8. Involvement of NF-κB in PMA-mediated COX-2 expression in A549 cells. In (a), cells were pretreated with various concentrations of PDTC for 30 min before incubation with 10 nM PMA for 12 h. The increase in COX activity was measured by examining PGE_2 formation in the presence of 30 μM exogenous arachidonic acid for 30 min. Results are expressed as the mean±S.E.M. (n=4). *p<0.05 as compared with the PMA-treated group. In (b), cells were pretreated with 25 μM PDTC for 30 min before incubation with 10 nM PMA for 12 h. Cells were then prepared for immunodetection using COX-2- or α-tubulin-specific antibodies as described in Materials and methods. Equal loading in each lane is demonstrated by similar intensities of α-tubulin. Typical traces represent three experiments with similar results. In (c), cells were treated with 10 nM PMA for various time intervals, and then subcellular (cytosolic and nuclear) fractions were prepared as described in Materials and methods. Levels of phosphorylated IκBα (IκBα-p) and IκBα in the cytosol and p65 in the cytosol and nucleus were immunodetected with phospho-IκBα-, IκBα-, and p65-specific antibodies, respectively. Typical traces represent three experiments with similar results. In (d), NF-κB-specific DNA–protein binding activity in nuclear extracts was determined by the electrophoretic mobility shift assay (EMSA) as described in Materials and methods. The top band represents NF-κB. NS, nonspecific binding. Typical traces represent three experiments with similar results.

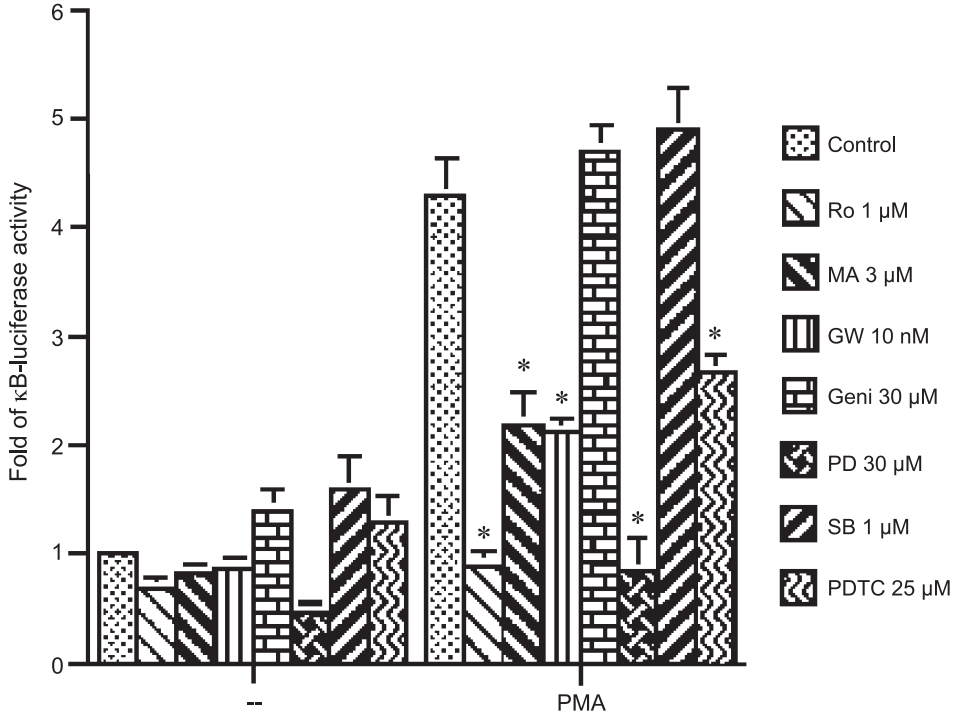


Fig. 9. Involvement of the Ras/Raf-1/ERK signal pathway in PMA-mediated NF-κB activation in A549 cells. Cells were transiently transfected with 0.5 μg of pGL2-ELAM-Luc and 1 μg of pBK-CMV-Lac Z for 24 h. Cells were then pretreated with 1 μM Ro 31-8220, 3 μM manumycin A, 10 nM GW5074, 30 μM genistein, 30 μM PD 098059, 1 μM SB 203580, or 25 μM PDTC for 30 min before being incubated with 10 nM PMA for 24 h. Luciferase activities were determined as described in Materials and methods. Results are expressed as the mean±S.E.M. (n=3). *p<0.05 as compared with the PMA-treated group. Ro, Ro 31-8220; Manu, manumycin A; GW, GW5074; Geni, genistein; PD, PD 098059; SB, SB 203580.

activity and COX-2 expression, suggesting that COX-2 expression is dependent on de novo transcription and translation.

Ras, an oncogenic protein, plays a critical role in inducing COX-2 expression [25–27]. In murine fibroblasts, activation of the Ras/Raf-1/ERK signal pathway is required for COX-2 induction [42]. We found that manumycin A (a Ras inhibitor), GW 5074 (a Raf-1 inhibitor), and PD 098059 (a MEK inhibitor) all inhibited PMA-induced COX-2 expression. Furthermore, treatment of A549 cells with PMA caused the activation of Ras, Raf-1, and ERK. These results suggested that the Ras/Raf-1/ERK signal pathway is very important for COX-2 induction caused by PMA. Furthermore, PMA-induced Ras and Raf-1 activations were inhibited by Ro 31-8220 and manumycin A, indicating that activation of Ras might occur upstream of PMA/PKC-induced Raf-1 activation. We also found that PMA-induced ERK1/2 activation was inhibited by Ro 31-8220, manumycin A, GW 5074, and PD 098059, but not by genistein or SB203580. These results suggest that activations of Ras, Raf-1, and MEK, but not tyrosine kinase or p38 MAPK, occur upstream of PMA/PKC-induced ERK1/2 activation.

It was demonstrated that activation of p38 MAPK is involved in COX-2 expression and PGE_2 release caused by interleukin-1β or lipoteichoic acid [43,44]. A previous report also showed that 12-*O*-tetradecanoylphorbol 13-acetate (TPA), a PKC activator, can induce p38 MAPK activation in RAW 264.7 macrophages [41]. In this study, we found that treatment of A549 cells with PMA resulted in activation of p38 MAPK, and this effect was inhibited by Ro 31-8220 and SB 203580. These results indicate that

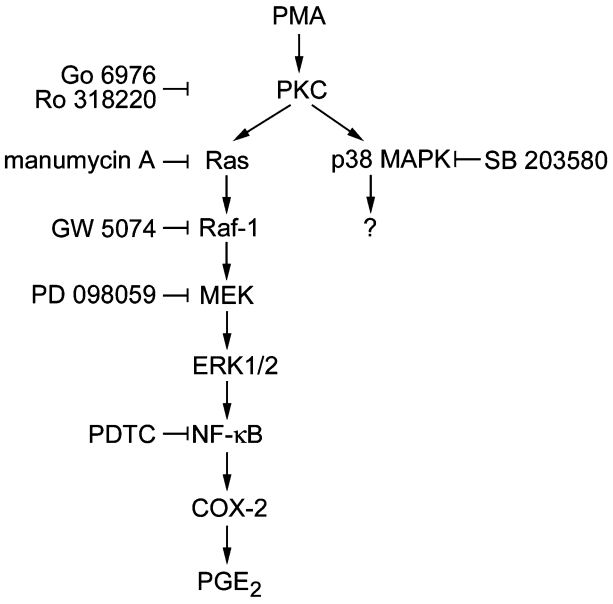


Fig. 10. Schematic summary of signal transduction by PMA which induces COX-2 expression and PGE_2 release in human pulmonary epithelial cells (A549). PMA might activate PKC to elicit Ras, Raf-1, MEK, and ERK1/2 activation, which in turn initiates NF-κB activation, and finally induces COX-2 expression and PGE2 release in A549 cells. However, PMA-mediated p38 MAPK activation is not involved in this event.

PMA-induced p38 MAPK activation is the downstream signal for PKC activation. However, the specific p38 MAPK inhibitor, SB 203580, did not affect PMA-induced COX-2 expression. This result suggests that p38 MAPK activation is not involved in the signaling pathway of PMA/PKC-mediated COX-2 expression, but might be related to the release of immunoregulatory molecules as suggested by Chen and Wang [41]. Activation of tyrosine kinase has also been suggested to be a key event in signal transduction leading to the expression of COX-2 [44,45]. In the present study, we found that the tyrosine kinase inhibitor, genistein, did not affect PMA-induced COX-2 expression, suggesting that tyrosine kinase activation is not involved in PMA/PKC-mediated COX-2 expression.

In mice and humans, the COX-2 promoter has many transcription factors including NF-κB in the 5′ region of the COX-2 gene [12,46]. In addition, NF-κB has been shown to control the induced transcription of the COX-2 gene [47,48]. In this study, we demonstrated that PMA-mediated COX-2 expression was inhibited by the NF-κB inhibitor, PDTC, indicating that activation of NF-κB might be involved in the induction of COX-2 protein caused by PMA. NF-κB is a dimer of transcription factors primarily consisting of p65 or p50. p65 is the transcriptional activation component of the most-common form of the NF-κB heterodimer [49]. In the resting state, this dimer is associated with IκBα/β, which functions to retain NF-κB in the cytosol [50]. With stimulation by cytokines and bacterial products, IκB kinase (IKK) is activated, and then IκBα is directly phosphorylated at Ser32 and Ser36, and IκBβ at Ser19 and Ser23 [51,52]. These phosphorylations lead to ubiquitination of IκBα/β at lysine residues and degradation by the 26S proteasome [53]. This process releases active NF-κB, which is then translocated from the cytosol to the nucleus, binding to specific DNA enhancer sequences (κB-binding sites), and induces gene transcription. Previous studies have shown that TPA can activate the formation of NF-κB-specific DNA–protein complexes in rat astrocytes [54]. In the present study, we found that treatment of A549 cells with PMA resulted in IκBα phosphorylation, IκBα degradation in the cytosol, the translocation of p65 from the cytosol to the nucleus, as well as the formation of NF-κB-specific DNA–protein complexes in the nuclear extracts of A549 cells. Using transient transfection with pGL2-ELAM-κB-luciferase as an indictor of NF-κB activity, we also found that PMA induced an increase in NF-κB activity. A previous report showed that NF-κB activation is mediated via the Ras-dependent signaling pathway, which induces COX-2 transcriptional gene expression in lung epithelial cells [55]. A previous report also showed that in transformed liver epithelial cells, Ras and Raf lead to constitutive activation of NF-κB through the IKKα/β complex [56]. We found that the PMA-mediated increase in κB-luciferase activity was inhibited by Ro 31-8220, manumycin, GW 5074, PD 098059, and PDTC, but not by genistein or SB 203580. These results indicate that PMA/PKC might act through the Ras/Raf-1/ERK1/2 pathway, but not tyrosine kinase or p38 MAPK, to induce NF-κB activation in A549 cells.

In conclusion, PMA might activate PKC to elicit activation of the Ras/Raf-1/MEK/ERK pathway, which in turn initiates NF-κB activation, and finally causes COX-2 expression and PGE_2 release in A549 cells. PMA might also induce activation of p38 MAPK. However, activation of p38 MAPK is not involved in PMA-induced NF-κB activation or COX-2 expression in these cells. A schematic representation of the signaling pathway of PMA-induced COX-2 expression and PGE_2 release in a human pulmonary epithelial cell line (A549) is shown in Fig. 10.

Acknowledgements

This work was supported by a research grant from the National Science Council of the Republic of China (NSC89-2320-B-038-031).

References

[1] J.R. Vane, Y.S. Bakhle, R.M. Botting, Annu. Rev. Pharmacol. Toxicol. 38 (1998) 97–120.
[2] W.L. Xie, J.G. Chipman, D.L. Robertson, R.L. Erikson, D.L. Simmons, Proc. Natl. Acad. Sci. U. S. A. 88 (7) (1991) 2692–2696.
[3] J.A. Mitchell, S. Larkin, T.J. Williams, Biochem. Pharmacol. 50 (10) (1995) 1535–1542.
[4] J.R. Vane, J.A. Mitchell, I. Appleton, A. Tomlinson, D. Bishop-Bailey, J. Croxtall, D.A. Willoughby, Proc. Natl. Acad. Sci. U. S. A. 91 (6) (1994) 2046–2050.
[5] J.A. Maier, T. Hla, T. Maciag, J. Biol. Chem. 265 (19) 10805–10808.
[6] J.A. Mitchell, P. Akarasereenont, C. Thiemermann, R.J. Flower, J.R. Vane, Proc. Natl. Acad. Sci. U. S. A. 90 (24) (1993) 11693–11697.
[7] C.C. Chan, S. Boyce, C. Brideau, A.W. Ford-Hutchinson, R. Gordon, D. Guay, R.G. Hill, C.S. Li, J. Mancini, M. Penneton, et al., J. Pharmacol. Exp. Ther. 274 (3) (1995) 1531–1537.
[8] J.L. Devalia, R.J. Davies, Respir. Med. 87 (6) (1993) 405–408.
[9] J.A. Mitchell, M.G. Belvisi, P. Akarasereenont, R.A. Robbins, O.J. Kwon, J. Croxtall, P.J. Barnes, J.R. Vane, Br. J. Pharmacol. 113 (3) (1994) 1008–1014.
[10] Y. Nishizuka, FASEB J. 9 (7) (1995) 484–496.
[11] D.B. Parekh, W. Ziegler, P.J. Parker, EMBO J. 19 (4) (2000) 496–503.
[12] H. Inoue, C. Yokoyama, S. Hara, Y. Tone, T. Tanabe, J. Biol. Chem. 270 (42) (1995) 24965–24971.
[13] M.S. Chang, W.S. Lee, C.M. Teng, H.M. Lee, J.R. Sheu, G. Hsiao, C.H. Lin, Br. J. Pharmacol. 136 (4) (2002) 558–567.
[14] S.M. Thomas, M. DeMarco, G. D'Arcangelo, S. Halegoua, J.S. Brugge, Cell 68 (6) (1992) 1031–1040.
[15] K.W. Wood, C. Sarnecki, T.M. Roberts, J. Blenis, Cell 68 (6) (1992) 1041–1050.
[16] Y.Q. Li, C.S. Hii, M. Costabile, D. Goh, C.J. Der, A. Ferrante, J. Immunol. 162 (6) (1999) 3316–3320.
[17] A. Chiloeches, H.F. Paterson, R. Marais, A. Clerk, C.J. Marshall, P.H. Sugden, J. Biol. Chem. 274 (28) (1999) 19762–19770.
[18] A.M. de Vries-Smits, B.M. Burgering, S.J. Leevers, C.J. Marshall, J.L. Bos, Nature 357 (6379) (1992) 602–604.
[19] X.F. Ming, B.M. Burgering, S. Wennstrom, L. Claesson-Welsh, C.H. Heldin, J.L. Bos, S.C. Kozma, G. Thomas, Nature 371 (6496) (1994) 426–429.
[20] J.R. Gum, J.W. Hicks, Y.S. Kim, Biochem. J. 325 (Pt 1) (1997) 259–267.

[21] C.J. Marshall, Curr. Opin. Cell Biol. 8 (2) (1996) 197–204.
[22] M. Zang, C. Hayne, Z. Luo, J. Biol. Chem. 277 (6) (2002) 4395–4405.
[23] X.F. Zhang, J. Settleman, J.M. Kyriakis, E. Takeuchi-Suzuki, S.J. Elledge, M.S. Marshall, J.T. Bruder, U.R. Rapp, J. Avruch, Nature 364 (6435) (1993) 308–313.
[24] A.J. Whitmarsh, R.J. Davis, Trends Biochem. Sci. 23 (12) (1998) 481–485.
[25] S.T. Reddy, D.J. Wadleigh, H.R. Herschman, J. Biol. Chem. 275 (5) (2000) 3107–3113.
[26] S.A. Blaine, M. Wick, C. Dessev, R.A. Nemenoff, J. Biol. Chem. 276 (46) (2001) 42737–42743.
[27] K. Subbaramaiah, L. Norton, W. Gerald, A.J. Dannenberg, J. Biol. Chem. 277 (21) (2002) 18649–18657.
[28] C.H. Lin, S.Y. Sheu, H.M. Lee, Y.S. Ho, W.S. Lee, W.C. Ko, J.R. Sheu, Mol. Pharmacol. 57 (1) (2000) 36–43.
[29] C.H. Lin, I.H. Kuan, H.M. Lee, W.S. Lee, J.R. Sheu, Y.S. Ho, C.H. Wang, H.P. Kuo, Br. J. Pharmacol. 134 (3) (2001) 543–552.
[30] M. Ouellet, M.D. Percival, Biochem. J. 306 (Pt 1) (1995) 247–251.
[31] S.J. Martin, S.V. Lennon, A.M. Bonham, T.G. Cotter, J. Immunol. 145 (6) (1990) 1859–1867.
[32] S.C. Chow, I. Peters, S. Orrenius, Exp. Cell Res. 216 (1) (1995) 149–159.
[33] G. Martiny-Baron, M.G. Kazanietz, H. Mischak, P.M. Blumberg, G. Kochs, H. Hug, D. Marme, C. Schachtele, J. Biol. Chem. 268 (13) (1993) 9194–9197.
[34] J. Beltman, F. McCormick, S.J. Cook, J. Biol. Chem. 271 (43) (1996) 27018–27024.
[35] M. Hara, K. Akasaka, S. Akinaga, M. Okabe, H. Nakano, R. Gomez, D. Wood, M. Uh, F. Tamanoi, Proc. Natl. Acad. Sci. U. S. A. 90 (6) (1993) 2281–2285.
[36] E.V. Varga, M. Rubenzik, V. Grife, M. Sugiyama, D. Stropova, W.R. Roeske, H.I. Yamamura, Eur. J. Pharmacol. 451 (1) (2002) 101–102.
[37] L. Pang, T. Sawada, S.J. Decker, A.R. Saltiel, J. Biol. Chem. 270 (23) (1995) 13585–13588.
[38] P.V. LoGrasso, B. Frantz, A.M. Rolando, S.J. O'Keefe, J.D. Hermes, E.A. O'Neill, Biochemistry 36 (34) (1997) 10422–10427.
[39] K. Migita, K. Eguchi, Y. Kawabe, A. Mizokami, T. Tsukada, S. Nagataki, J. Immunol. 153 (8) (1994) 3457–3465.
[40] Z. Pan, V.V. Kravchenko, R.D. Ye, J. Biol. Chem. 270 (14) (1995) 7787–7790.
[41] C.C. Chen, J.K. Wang, Mol. Pharmacol. 55 (3) (1999) 481–488.
[42] W. Xie, B.S. Fletcher, R.D. Andersen, H.R. Herschman, Mol. Cell. Biol. 14 (10) (1994) 6531–6539.
[43] Z. Guan, S.Y. Buckman, B.W. Miller, L.D. Springer, A.R. Morrison, J. Biol. Chem. 273 (44) (1998) 28670–28676.
[44] C.H. Lin, I.H. Kuan, C.H. Wang, H.M. Lee, W.S. Lee, J.R. Sheu, G. Hsiao, C.H. Wu, H.P. Kuo, Eur. J. Pharmacol. 450 (1) (2002) 1–9.
[45] P. Akarasereenont, C. Thiemermann, Biochem. Biophys. Res. Commun. 220 (1) (1996) 181–185.
[46] Y. Kim, S.M. Fischer, J. Biol. Chem. 273 (42) (1998) 27686–27694.
[47] J.F. Schmedtje Jr., Y.S. Ji, W.L. Liu, R.N. DuBois, M.S. Runge, J. Biol. Chem. 272 (1) (1997) 601–608.
[48] C. Jobin, O. Morteau, D.S. Han, R. Balfour Sartor, Immunology 95 (4) (1998) 537–543.
[49] A.S. Baldwin Jr., Annu. Rev. Immunol. 14 (1996) 649–683.
[50] J.E. Thompson, R.J. Phillips, H. Erdjument-Bromage, P. Tempst, S. Ghosh, Cell 80 (4) (1995) 573–582.
[51] J.A. DiDonato, M. Hayakawa, D.M. Rothwarf, E. Zandi, M. Karin, Nature 388 (6642) (1997) 548–554.
[52] T. Maniatis, Science 278 (5339) (1997) 818–819.
[53] Z. Chen, J. Hagler, V.J. Palombella, F. Melandri, D. Scherer, D. Ballard, T. Maniatis, Genes Dev. 9 (13) (1995) 1586–1597.
[54] C.C. Chen, J.K. Wang, W.C. Chen, S.B. Lin, J. Biol. Chem. 273 (31) (1998) 19424–19430.
[55] J.D. Li, W. Feng, M. Gallup, J.H. Kim, J. Gum, Y. Kim, C. Basbaum, Proc. Natl. Acad. Sci. U. S. A. 95 (10) (1998) 5718–5723.
[56] M. Arsura, F. Mercurio, A.L. Oliver, S.S. Thorgeirsson, G.E. Sonenshein, Mol. Cell. Biol. 20 (15) (2000) 5381–5391.