

# Prostaglandin E<sub>2</sub> Blocks Menadione-Induced Apoptosis through the Ras/Raf/Erk Signaling Pathway in Promonocytic Leukemia Cell Lines

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Altered oxidative stress has long been observed in cancer cells, and this biochemical property of cancer cells represents a specific vulnerability that can be exploited for therapeutic benefit. The major role of an elevated oxidative stress for the efficacy of molecular targeted drugs is under investigation. Menadione is considered an attractive model for the study of oxidative stress, which can induce apoptosis in human leukemia HL-60 cell lines. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) via its receptors not only promotes cell survival but also reverses apoptosis and promotes cancer progression. Here, we present evidence for the biological role of PGE<sub>2</sub> as a protective agent of oxidative stress-induced apoptosis in monocytic cells. Pretreatment of HL-60 cells with PGE<sub>2</sub> markedly ameliorated the menadione-induced apoptosis and inhibited the degradation of PARP and lamin B. The EP<sub>2</sub> receptor antagonist AH6809 abrogated the inhibitory effect of PGE<sub>2</sub>, suggesting the role of the EP<sub>2</sub>/cAMP system. The PKA inhibitor H89 also reversed apoptosis and decreased the PKA activity that was elevated 10-fold by PGE<sub>2</sub>. The treatment of HL-60 cells with NAC or zinc chloride showed a similar protective effect as with PGE<sub>2</sub> on menadione-treated cells. Furthermore, PGE<sub>2</sub> activated the Ras/Raf/MEK pathway, which in turn initiated ERK activation, and ultimately protected menadione-induced apoptosis. These results imply that PGE<sub>2</sub> via cell survival pathways may protect oxidative stress-induced apoptosis in monocytic cells. This study warrants further pre-clinical investigation as well as application towards leukemia clinics.

## INTRODUCTION

Oxidative stress is like two sides of a coin and used with caution can destroy with cancer cells, since cancer cells exhibit increased level of reactive oxygen species (ROS) compared to normal cells (Trachootham et al., 2009). Mounting evidence suggest that excessive ROS generation occurs in hypoxic environments and it is possible to enhance the ROS level to such extent that it can damage proteins, lipids, DNA, and ultimately lead to cell death (Coffey et al., 2000). Hence, glutathione

(GSH) depletion along with ROS generation can effectively be used to induce cell death in cancer cells irrespective of their sensitivity toward anticancer drugs. Among the anti-cancer agents, menadione is a polycyclic aromatic ketone (2-methyl-1,4-naphthoquinone), which has the ability to generate intracellular ROS at multiple cellular sites through redox cycling reactions (Brière et al., 2004). Menadione induced apoptosis through the downregulation of ERK and JNK, followed by the activation of caspase 3 and poly ADP-ribose polymerase (PARP) cleavage (Osada et al., 2008).

Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is generated by the enzymes phospholipase A<sub>2</sub> (PLA<sub>2</sub>), cyclooxygenase (COX), and prostaglandin synthase (PGES), which stimulates key downstream signaling cascades and have roles in the maintenance of normal physiology as well as in the various pathological conditions including different cancers (Smith et al., 2000). The PGE<sub>2</sub> differentially mediates its physiological role via receptors, due to the binding affinity of these receptors with PGE<sub>2</sub> (Sampey et al., 2005; Smith et al., 2000). It has been reported that prostaglandin E receptors 1 and 3 (EP<sub>1</sub> and EP<sub>3</sub>) mingle with G<sub>αq</sub> to activate Ca<sup>2+</sup> signaling and with G<sub>αi</sub> to inhibit adenylyl cyclase, respectively. The other two receptors EP<sub>2</sub> and EP<sub>4</sub> couple to G<sub>αs</sub> and in turn stimulate adenylyl cyclase (Chun et al., 2010). Ras is a small GTP-binding protein, which is the common upstream molecule of several signaling pathways including Raf-MEK-ERK. PGE<sub>2</sub> activates a Ras-Raf-ERK pathway through the EP<sub>2</sub> receptor, which in turn upregulates COX-2 expression and stimulates cancer cell proliferation (Sung et al., 2006). In addition, EP<sub>2</sub> contributed to tumor formation through the activation of protein kinase A (PKA), epidermal growth factor receptor (EGFR), and several effectors such as cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), H-Ras, Src, protein kinase B (AKT) and ERK (Chun et al., 2009).

Leukemia is cancer of the blood or bone marrow, which is characterized by unusual proliferation of blood cells. The human promyelocytic leukemia cell line (HL-60) is considered a useful model to study various genetic and biochemical factors regulating the development of the myeloid feature (Fontana et al., 1981). Therefore, the aim of this study was to elucidate the mechanisms of cell death induced by menadione as well as the

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anti-apoptotic role of PGE<sub>2</sub>. Menadione induced apoptotic cell death through oxidative stress, which is inhibited by PGE<sub>2</sub> possibly through the scavenging of ROS and EP<sub>2</sub> receptor in HL-60 cells. Furthermore, oxidative stress-induced signaling pathways were investigated by examining the role of PGE<sub>2</sub> with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), taxol, etoposide, and menadione. It was observed that PGE<sub>2</sub> reverses menadione-induced apoptosis through the modulation of the Ras/Raf/ERK signaling pathway. We hope that the findings of this study will be a potential step forward in the understanding of the PGE<sub>2</sub> role in apoptosis and further insights into the clinical applications of menadione for leukemia.

## MATERIALS AND METHODS

### Reagents and cell culture

Menadione was obtained from the Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO) to produce a 100 mM stock solution. PGE<sub>2</sub>, butaprost, AH6809, and EP<sub>2</sub> antagonist, were purchased from the Cayman Chemical Company. PD98059 was purchased from BioMol Plymouth Meeting. Paclitaxel (Taxol) was purchased from Bristol-Myers Squibb Princeton. HL-60 cells obtained from ATCC, CCL-240™ were cultured at a density of  $5 \times 10^5$  in RPMI-1640 medium powdered with L-glutamine, containing 10% heat inactivated fetal bovine serum (FBS) and supplemented with 1% (v/v) antibiotic-penicillin streptomycin (Gibco, Invitrogen Corporation) at 37°C in a 5% CO<sub>2</sub>-humidified atmosphere. When the cells reached 70% confluence, the growth medium was removed from the cells and the cells were washed with phosphate-buffered saline (PBS) to remove all traces of serum. Trypsin/EDTA solution dispensed into culture dish completely covered the cell layer and incubated the cells for 2 min at 37°C to ensure that the cells completely detached from the base of the dish. Cells were centrifuged at  $1,000 \times g$  for 5 min and after the cell counting through the standard trypan blue method and hemacytometer, the cell pellet was resuspend in fresh growth media with 10% FBS. Then the cells were seeded onto a new plate.

### Cell cycle analysis

Cells were trypsinized and collected by centrifugation at  $1,000 \times g$  for 5 min. The collected cells were washed with PBS and resuspended at  $1 \times 10^6$  cells/ml. For fixation, the cell suspension was kept overnight at 4°C, after the addition of 70% ethanol. Then, the cells were stained with propidium iodide solution (50 µg/ml propidium iodide, 0.1% Triton X-100, 0.1 mM EDTA, and 50 µg/ml RNase) for 20 min at 4°C. Stained DNA was analyzed by fluorescence activated cell sorting (FACS Calibur; Beckton Dickinson). For the detection of propidium iodide, cells were exited at 488 nm and their emissions were determined between 515 and 545 nm (Thayyullathil et al., 2008).

### Determination of apoptosis

Cell viability was determined by a trypan blue exclusion test. A morphological analysis of apoptosis was performed after staining with Hoechst 33342 dye. Cells were fixed in 4% paraformaldehyde and permeabilized with 1X PBS and 0.5% Triton X-100 the nuclei were stained for 20 min with Hoechst dye. The coverslips were then washed, mounted onto slides, and viewed with a fluorescence microscope.

Apoptotic DNA fragments were isolated according to a previously described method and DNA was separated by electrophoresis on 1% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light (Herrmann et al., 1994).

### Cell morphology studies

After treating the cells with 1 µM PGE<sub>2</sub> for 2 h followed by 10 µM of menadione. The HL-60 cells were then incubated at 37°C with 5 µM 2',7'-dichlorofluorescein-diacetate (DCFHDA) (Calbiochem, USA) for 10 min. After indicated time, we gathered the cells and wash with 1× PBS for 3 times. The cell morphology was then observed by a fluorescence microscope (Axiovert 40 CFL, Carl Zeiss, USA) and the camera used was the Axiocam MRC 5 (Park et al., 2002).

### Measurement of ROS production

Interacellular ROS concentrations were measured with the oxidant-sensitive fluorescent probe DCFHDA with inverted microscopy. Cells were grown to a density of  $1 \times 10^6$  cells per 35 mm culture dish and maintained in the growth medium for 24 h. The cells were exposed to 5 µM DCFHDA for 30 min, and treated with 10 µM menadione for 12 h. The cells were washed with PBS and cover glass was put on the dish. DCF fluorescence (excitation, 485 nm; emission, 530 nm) was imaged on an inverted microscope (Yu and Kim, 2011).

### Measurement of lipid peroxidation

Lipid peroxidation was estimated with the fluorescent probe diphenyl-1-pyrenylphosphine (DPPP) as previously described (Park et al., 2002). After HL-60 cells were incubated with 5 µM DPPP for 15 min in the dark, the cells were exposed to 10 µM menadione or 1 µM PGE<sub>2</sub> for 1 h. The images of DPPP fluorescence by reactive species were analyzed by the Zeiss Axiovert 200 inverted microscope at the fluorescence DAPI region (excitation, 351 nm; emission, 380 nm).

### Protein kinase assay

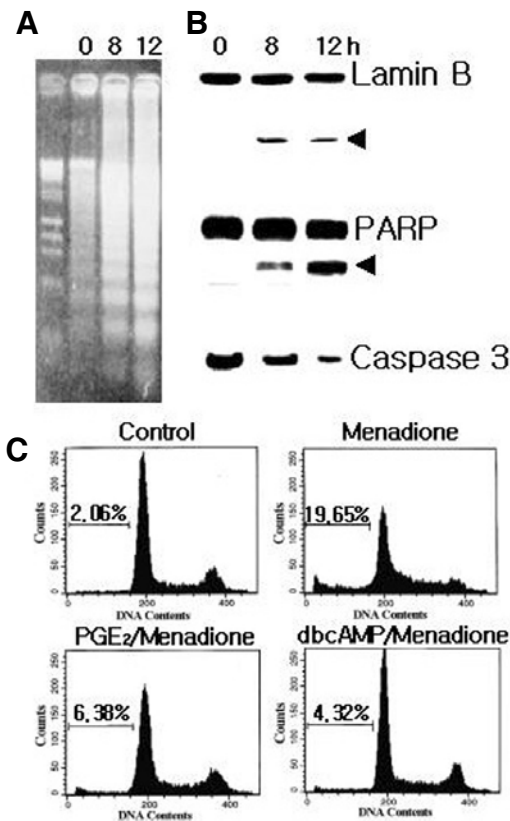
cAMP-dependent protein kinase activity was determined by measuring the transfer <sup>32</sup>P-ATP to a peptide substrate that was then captured on P81 phosphocellulose papers. Aliquots of cell lysates were incubated with 50 mM Tris at pH 7.5, 200 µM kemptide, 40 mM MgCl<sub>2</sub>, and 400 µM ATP. After 5 min at 30°C, the reactions were terminated by adding EDTA to a final concentration of 20 mM. Relative PKA activity was quantified with a liquid scintillation counter as described previously (Blum et al., 1999).

### Western blotting

Gathered cells were washed with 1× PBS and lysed in a RIPA lysis buffer, which contain 50 mM Tris HCl (pH 7.4), 1% NP-40, 40 mM sodium fluoride (NaF), 10 mM sodium chloride (NaCl), 10 mM sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM dithiothreitol (DTT), and protease inhibitor. The cell lysates were centrifuged at  $12,000 \times g$  for 15 min and protein was quantified through the Bio-Rad protein assay, was mixed with 4X loading buffer and boiled for 5 min. Samples were then resolved by SDS-PAGE and the separated proteins were transferred onto nitrocellulose membranes by the transfer method using transfer buffer (CAPS and methanol). The blots were blocked with 5% non-fat milk in Tris buffer saline containing 0.1% Tween 20 (1x TBST) and then incubated with primary antibodies followed by secondary antibodies. Western blot data presented here are representative of three independent experiments. The signals were detected by ECL Western blotting detection kit from Amersham Biosciences (USA) by using chemiluminescence system (Thayyullathil et al., 2008).

### Ras activity assay

Ras activity was measured with a Ras activity assay kit (Upstate). The assay was performed according to the manufac-



**Fig. 1.** Menadione induced apoptosis of HL-60 cells. Cells ( $1 \times 10^6$  cells/ml) were cultured in the absence or presence of  $10 \mu\text{M}$  menadione. (A) Analysis of menadione-induced DNA fragmentation of cells by agarose gel electrophoresis. (B) Lamin B (upper panel) and PARP (middle panel) are cleaved as a result of caspase-3 activation (lower panel) in apoptotic cells after treatment with menadione. At the indicated times, cell lysates were separated by SDS-PAGE and proteins were detected by immunoblotting. (C) Inhibition of menadione-induced apoptosis. HL-60 cells were pretreated with  $1 \mu\text{M}$  PGE<sub>2</sub> or  $100 \mu\text{M}$  dibutyryl cAMP (dbcAMP). After 15 min, cells were incubated in the absence (control) or presence of menadione for 12 h. Cells were harvested and stained with propidium iodide, and their DNA content was analyzed by flow cytometry. Apoptotic cells are shown by the accumulation of cells with a DNA content of below  $2n$ . Very similar results were obtained from three independent experiments.

turer's instructions. Briefly, cells were washed twice with ice-cold PBS, lysed in lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 25 mM NaF, 10% glycerol, 10  $\mu\text{g}/\text{ml}$  aprotinin, and 10  $\mu\text{g}/\text{ml}$  leupeptin) and centrifuged. An equal volume of lysate was incubated with 7  $\mu\text{g}$  of the Ras-binding domain for Raf-1 at  $4^\circ\text{C}$  for 2 h, and the beads were washed three times with lysis buffer. Bound Ras proteins were then detected by Western blotting using mouse monoclonal Ras antibody with the ECL system.

#### Statistical analysis

All the data presented as the mean values  $\pm$  SD (standard deviation) were from 3 separate experiments. The data were evaluated by SPSS software with the dependent student *t*-test and where applicable (multiple group comparisons), the data were subjected to one-way analysis of variance (ANOVA). The

**Table 1.** Anti-apoptotic action of prostaglandin E<sub>2</sub> in human leukemia cell lines

Cell type	Treatment	% Apoptosis	Apoptosis inhibition
HL-60	Menadione	$20.57 \pm 3.55$	-
	+ PGE <sub>2</sub>	$9.47 \pm 2.41$	$54.05 \pm 7.60$
	+ dibutyryl cAMP	$12.16 \pm 3.48$	$41.51 \pm 6.66$
	+ N-acetylcysteine	$15.24 \pm 2.31$	$30.65 \pm 4.65$
	+ Zinc chloride	$12.19 \pm 1.15$	$40.42 \pm 1.04$
	Hydrogen peroxide	$35.15 \pm 2.39$	-
	+ PGE <sub>2</sub>	$22.10 \pm 4.75$	$37.45 \pm 9.79$
	+ dibutyryl cAMP	$24.77 \pm 4.85$	$30.95 \pm 1.74$
	+ N-acetylcysteine	$24.03 \pm 6.05$	$33.01 \pm 7.60$
	+ Zinc chloride	$17.66 \pm 4.96$	$51.42 \pm 6.27$
U937	Etoposide	$21.02 \pm 4.84$	-
	+ PGE <sub>2</sub>	$12.17 \pm 1.59$	$40.87 \pm 8.65$
	Taxol	$18.75 \pm 3.12$	-
	+ PGE <sub>2</sub>	$13.01 \pm 2.85$	$30.74 \pm 8.58$
	Menadione	$27.03 \pm 6.85$	-
	+ PGE <sub>2</sub>	$13.83 \pm 1.75$	$47.98 \pm 6.70$
	Etoposide	$28.55 \pm 8.05$	-
	+ PGE <sub>2</sub>	$21.65 \pm 6.60$	$24.57 \pm 2.87$

Cells were pretreated with PGE<sub>2</sub> ( $1 \mu\text{M}$ ), dibutyryl cAMP ( $100 \mu\text{M}$ ), N-acetylcysteine ( $15 \text{ mM}$ ), or zinc chloride ( $45 \mu\text{M}$ ) for 10 min and then incubated with menadione, H<sub>2</sub>O<sub>2</sub> ( $100 \mu\text{M}$ ), etoposide ( $8.5 \mu\text{M}$ ), or taxol ( $200 \text{ nM}$ ) for 12 h. Apoptotic cells were determined by flow cytometry. The treatment of HL-60 cells with NAC or zinc chloride and U937 cells with etoposide showed a similar protective effect as with PGE<sub>2</sub> on menadione-treated cells. All results are the means  $\pm$  SD of at least three independent experiments.

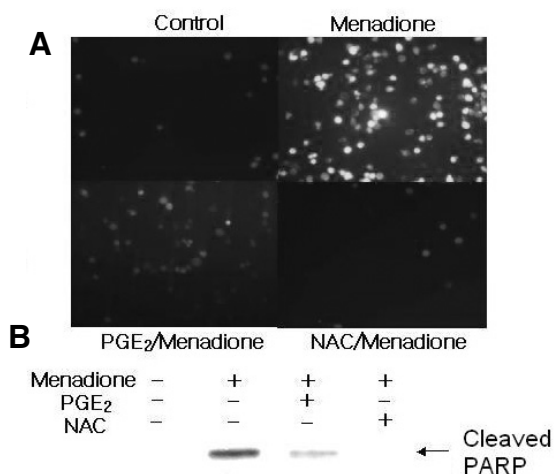
significance of the differences between the mean was determined by the Tukey range test. Statistical significance was at  $P \leq 0.05$ .

## RESULTS

### PGE<sub>2</sub> prevents oxidative- and menadione-induced apoptosis in HL-60 cells

Menadione has been shown to stimulate ROS generation through activation of NADPH/quinone oxidase in various cells. As shown by the gel electrophoresis (Fig. 1A), menadione induced DNA fragmentation with rapid kinetics, which are considered the hallmark of apoptosis. Lamin B and PARP were cleaved with similar kinetics as the DNA fragmentation, yielding cleavage bands (Fig. 1B) that were detected after 4 h of menadione treatment. Since PARP and lamin B have been shown to be cleaved by caspase 3, therefore the menadione treated whole cell lysates were analyzed by Western blot with anti-caspase 3. The disappearance of a 32 kDa caspase zymogen was used to show the activation of caspase 3 (Fig. 1B). These data suggest that the caspase 3 dependent pathway might be responsible for the menadione-induced apoptosis.

To investigate the underlying mechanism of cAMP-dependent inhibition of apoptosis, we first examined the effect of cell-permeable cAMP analog dibutyryl cAMP on the menadione-induced apoptosis in HL-60 cells. To quantify the degree of apoptosis, the amount of sub-G1 DNA from the fixed nuclei



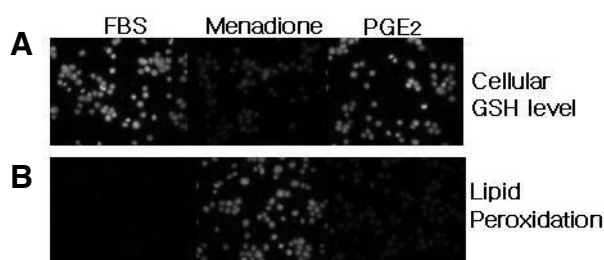
**Fig. 2.** Menadione-induced apoptosis is mediated by ROS production. (A) After cells were pretreated with 20 mM N-acetylcysteine (NAC) for 1 h, they were exposed to menadione. The cells were further incubated with DCFHDA, a cell-permeable dichlorofluorescein that can be used as an intracellular probe for oxidative stress for 30 min. Accumulation of the probe in the cells was measured by an increase at 530 nm when the sample was excited at 485 nm. Fluorescence images of DCF-loaded cells were obtained under a microscope. (B) PARP cleavage product was analyzed by Western blotting with anti-PARP antibody.

was analyzed by flow cytometry (Fig. 1C). HL-60 cells were exposed to menadione, which resulted in the increased accumulation of the sub-G1 phase. The addition of dibutyl cAMP blocked menadione-induced apoptotic cell death. As expected, treatment of HL-60 cells with 1  $\mu$ M PGE<sub>2</sub> also effectively suppressed menadione-induced apoptosis.

To determine whether cAMP and PGE<sub>2</sub> could also inhibit the induction of apoptosis by agents other than menadione, HL-60 cells were treated with H<sub>2</sub>O<sub>2</sub>, etoposide, and taxol, and then allowed to recover in either the absence or presence of dibutyl cAMP or PGE<sub>2</sub>. The flow cytometry results showed that PGE<sub>2</sub> and dibutyl cAMP also attenuated the generation of apoptosis through these cytotoxic oxidative agents (Table 1). The anti-apoptotic actions of PGE<sub>2</sub> on drug-induced apoptosis were more effective than dibutyl cAMP. To confirm this scenario, the anti-apoptotic effect of PGE<sub>2</sub> was corroborated in U937 myeloid cells, indicating that it is not a cell line-specific phenomenon. The treatment of HL-60 cells with N-acetylcysteine (NAC) or zinc chloride showed a similar protective effect on menadione-treated cells (Table 1).

#### PGE<sub>2</sub> may act as a scavenger of ROS

Menadione is known to catalyze the formation of H<sub>2</sub>O<sub>2</sub> and hydroxyl radical (OH<sup>•</sup>), and these ROS are thought to mediate, at least in part, cellular toxicity. To confirm whether menadione-induced apoptosis in HL-60 cells is associated with the generation of cellular oxidative stress, the levels of intracellular peroxides were evaluated with DCFHDA. It is assumed that menadione-induced apoptosis in HL-60 cells occur through increases in DCF-detectable ROS (Fig. 2). Exposure of the HL-60 cells to radical scavenger such as PGE<sub>2</sub> or NAC caused a clear decrease in the DCF fluorescence, consistent with the level of cleaved PARP (Fig. 2). These data strengthen the conclusion that PGE<sub>2</sub> provides protection from the apoptotic actions of menadione by decreasing the steady-state level of



**Fig. 3.** Measurement of cellular GSH and lipid peroxidation in menadione and PGE<sub>2</sub> treated cells. (A) HL-60 cells ( $1 \times 10^6$  cells/ml) were stained with 5  $\mu$ M CMAC for 15 min in the dark and then exposed to 10  $\mu$ M or 1  $\mu$ M of PGE<sub>2</sub>. Intracellular GSH levels were expressed relative to the FBS treated cells. Menadione treatment decreased cellular GSH levels, which was reversed by pretreatment of PGE<sub>2</sub>. (B) DPPP fluorescent intensity (lipid peroxidation) was decreased markedly in PGE<sub>2</sub>-treated cells, whereas menadione treatment increased the lipid peroxidation evident fluorescent intensity. Fluorescence images were obtained under microscopy.

intracellular oxidants.

Glutathione (GSH) is one of the most abundant intracellular antioxidants, and measuring the changes in its concentration provides an alternative method to monitor oxidative stress in the cells. GSH-sensitive fluorescent dye t-butoxycarbonyl-Leu-Met-7-amino-4-chloromethylcoumarin (CMAC) can be used as a useful probe to evaluate the level of intracellular GSH. Cellular GSH levels were decreased in menadione-treated HL-60 cells (Fig. 3A). However, the depletion of GSH was significantly inhibited by the pretreatment with 1  $\mu$ M PGE<sub>2</sub>. To establish the extent to which oxidation of phospholipids was associated with the execution of the apoptotic program induced by menadione, DPPP was used as a probe to assess lipid peroxidation in monocytes. The DPPP fluorescent intensity was decreased markedly in PGE<sub>2</sub>-treated cells, whereas it increased in menadione-treated cells (Fig. 3B).

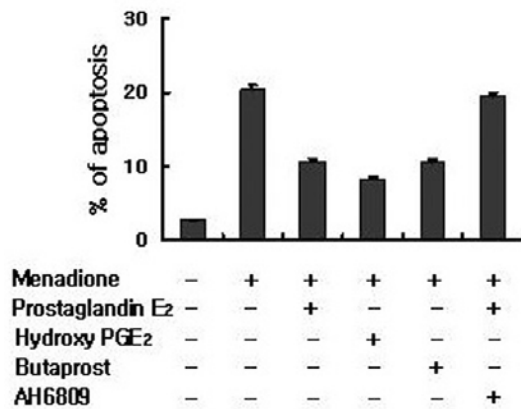
#### PGE<sub>2</sub>-induced anti-apoptotic actions is mediated through EP<sub>2</sub> receptor

Once the role of PGE<sub>2</sub> in menadione-induced apoptosis in HL-60 cells was established, we next evaluated its functional relevance in anti-apoptotic actions. Above our results suggest that PGE<sub>2</sub>-induced anti-apoptotic effects might be mediated through a mechanism involving the cAMP system. Therefore, it seems that the activation of adenylate cyclase coupled with the activation of EP<sub>2</sub> and EP<sub>4</sub> receptors, plays an important role in the signal transduction mechanism associated with the PGE<sub>2</sub>-mediated protection from menadione-induced apoptosis.

To further support our conclusion for the role of PGE<sub>2</sub> receptors, we compare their expression in HL-60 cells with the specific agonist for each receptor in order to identify EP receptors involved in the PGE<sub>2</sub>-mediated protection from menadione-induced apoptosis. Stimulation of the EP<sub>2</sub> receptor with butaprost inhibited apoptotic cell death in HL-60 cells, whereas stimulation of EP<sub>3</sub> or EP<sub>4</sub> with sulprostone or PGE<sub>1</sub> alcohol had no significant effect on cell survival (Fig. 4). Additionally, the EP<sub>2</sub> antagonist AH-6809 abrogated the inhibitory effects of PGE<sub>2</sub> on menadione-induced apoptosis (Fig. 4). Therefore, the EP<sub>2</sub> receptor appeared to play a major role in mediating the PGE<sub>2</sub>-stimulated anti-apoptosis in HL-60 cells.

#### EP<sub>2</sub> receptor activates PKA signaling pathway

To elucidate further the mechanism by which PGE<sub>2</sub> regulated

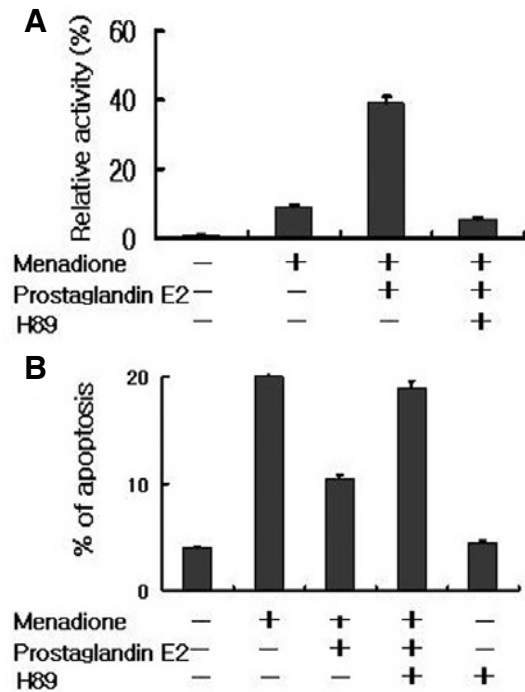


**Fig. 4.** PGE<sub>2</sub> protects apoptosis through EP<sub>2</sub>. HL-60 cells were incubated with butaprost, AH6809 and hydroxy PGE<sub>2</sub>. After treatment with PGE<sub>2</sub> or menadione, cells were stained with propidium iodide and their DNA content was analyzed by flow cytometry. Stimulation of the EP<sub>2</sub> receptor with butaprost or antagonist AH6809 inhibited apoptotic cell death in HL-60 cells, whereas stimulation of EP<sub>3</sub> or EP<sub>4</sub> with sulprostone or PGE<sub>1</sub> alcohol had no significant effect on cell survival.

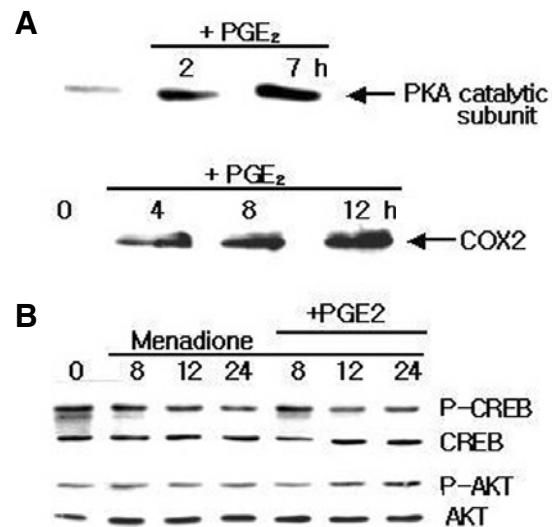
HL-60 apoptosis, we examined its downstream signaling pathways. It has been proposed that PGE<sub>2</sub> stimulation of the EP<sub>2</sub> receptor leads to the activation of PKA through the elevation of intracellular cAMP levels. To characterize the EP<sub>2</sub> receptor mediated signaling pathways involved in the anti-apoptotic activity of PGE<sub>2</sub>, the effects of PGE<sub>2</sub> on PKA activity were determined. PKA was measured in HL-60 cells with a radioisotope assay that utilizes the phosphorylation of the synthetic substrate kemptide (Fig. 5A). The PKA activity increased more than four-fold after treatment with PGE<sub>2</sub>. The ability of PGE<sub>2</sub> to activate PKA was completely blocked by the addition of H-89, a specific PKA inhibitor. Prior to the incubation of HL-60 cells with H-89 for 15 min also suppressed anti-apoptotic activity of PGE<sub>2</sub> by 85%, compared to the single treated PGE<sub>2</sub> cells (Fig. 5B). These results indicate that PGE<sub>2</sub>-mediated cell survival in response to oxidative stress requires PKA activity.

Studies have shown that PKA is a cytosolic, tetrameric holoenzymes that consists of two regulatory subunits associated with two catalytic subunits. The dissociated, active catalytic subunits can then affect the cell physiology through the phosphorylation of a wide variety of protein substrates in the cytoplasm and nucleus. cAMP-mediated transcriptional responses involve the ability of PKA to phosphorylate cAMP response element binding protein (CREB). To explore the functional significance of PKA activation by PGE<sub>2</sub>, we first tested translocation PKA catalytic subunits to nucleus in PGE<sub>2</sub>-challenged HL-60 cells was first tested. As shown in Fig. 6A, PGE<sub>2</sub> treatment induced translocation of PKA catalytic subunits to the nucleus. However, in PGE<sub>2</sub> stimulated cells the activated PKA did not phosphorylate cAMP response element-binding (CREB) (Fig. 6B) and other cAMP-responsive transcription factors such as activating transcription factors (ATF-1 or ATF-2) (data not shown).

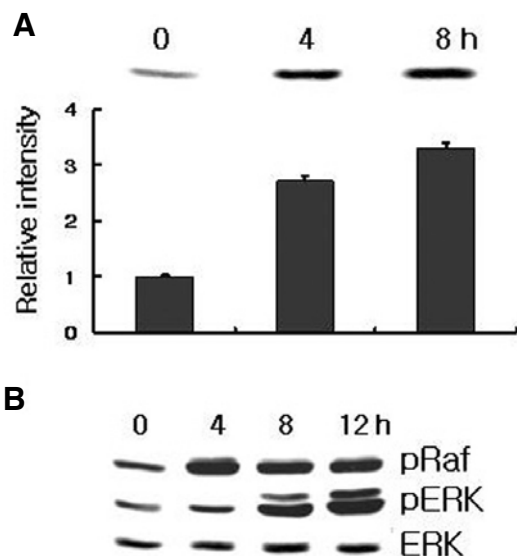
Another possible signaling pathway for the effects of PGE<sub>2</sub> on apoptosis is the phosphorylation of Akt. Since phosphorylation of Akt at Ser<sup>473</sup> is required for its full activation, the phosphorylation of endogenous AKT was examined with an antibody that specifically recognizes Akt phosphorylation at the Serine residue. We did not observe any activation of endogenous Akt



**Fig. 5.** PGE<sub>2</sub> activate protein kinase A. (A) HL-60 cells were pre-treated with vehicle alone, PGE<sub>2</sub>, or H89 for 15 min prior to being exposed to menadione. Cells were harvested at 2 h and measured PKA activity by *in vitro* kinase assay detailed in the "Materials and Methods". (B) The cells were incubated with H89, PGE<sub>2</sub> and then treated with menadione. After, stained with propidium iodide and their DNA content was analyzed by flow cytometry.



**Fig. 6.** Effect of PGE<sub>2</sub> on the expression of PKA, COX<sub>2</sub>, and transcription factors. (A) HL-60 cells were treated with 1  $\mu$ M PGE<sub>2</sub> and PKA catalytic subunit and COX<sub>2</sub> expression was detected after indicated time. Immunoblotting analysis revealed that PGE<sub>2</sub> enhanced the expression of PKA and COX<sub>2</sub>. (B) Cells were pre-treated with or without PGE<sub>2</sub>, and incubated with 10  $\mu$ M of menadione for 8, 12 and 24 h. PGE<sub>2</sub> pretreatment inhibited the menadione-induced apoptosis via CREB and AKT.

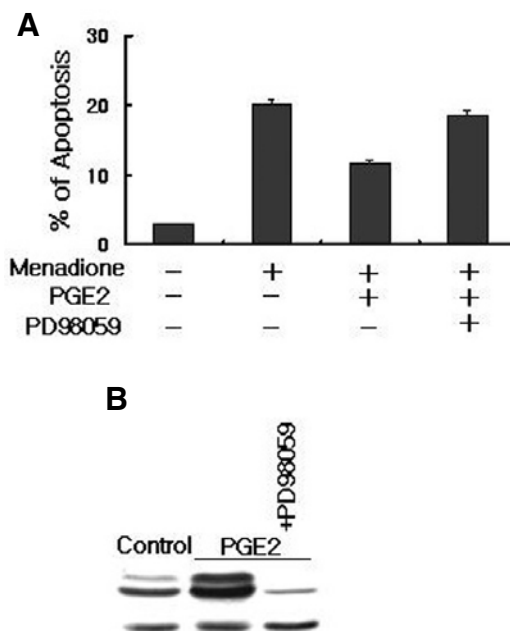


**Fig. 7.** PGE<sub>2</sub> induce Raf and ERK phosphorylation. (A) Ras activity was assessed by immunoblotting samples for Ras immunoprecipitated from lysates using Raf-1 RBD. Densitometer measurements of Ras activity (units as pixels) are shown below the blot. (B) Phosphorylated Raf-1 and Erk were detected by immunoblotting analysis with antibody specific for phosphorylated Raf-1 and Erk (pRaf and pErk, respectively). Protein loading was determined by stripping and reprobing the same nitrocellulose membrane for total Raf-1 and Erk protein level. Results are representative of three independent experiments.

when the cells were treated with PGE<sub>2</sub> (Fig. 6B). Thus, it did not seem likely that CREB and Akt could be involved in anti-apoptosis mediated by PGE<sub>2</sub>. Taken together, these results demonstrate that i) activation of PKA plays an important role in the regulation of PGE<sub>2</sub>-mediated anti-apoptotic effects, and ii) activation of CREB and Akt is not required for the protection of apoptosis in the monocytic HL-60 cells.

#### Activation of ERK is a critical event in PGE<sub>2</sub>-induced anti-apoptosis in HL-60 cells

Since Ras-Raf is implicated in the triggering of several different signal transduction pathways, we have verified that the integrity of the downstream EP<sub>2</sub> receptor-targeted pathway was indeed required for the anti-apoptotic effects of PGE<sub>2</sub>. We directly measured the Raf activity in response to PGE<sub>2</sub>. Figure 7 shows that treatment of HL-60 cells with PGE<sub>2</sub> induced an increase in Raf activity, which was assessed by immunoblotting samples of Raf immunoprecipitated from lysates using Raf-1-RBD. It seems that anti-apoptotic effect of EP<sub>2</sub> leads to reinforcement of the activated Ras signal pathway due to the regulation of the cAMP levels in HL-60 cells simulated with PGE<sub>2</sub>. Western blot analysis also revealed that PGE<sub>2</sub> treatment increased the levels of phosphorylated Raf and phosphorylated ERK in HL-60 cells (Fig. 7). To further insights into the signaling pathway leading to the protection of apoptosis in PGE<sub>2</sub>-induced HL-60 cells, a specific MEK1 inhibitor, PD98059, was used (Fig. 8). Pretreatment of HL-60 cells with PD98059 inhibited the PGE<sub>2</sub> induced stimulation of Raf and ERK and reversed the anti-apoptotic effect in menadione treated HL-60 cells. Inhibition of the MEK pathway with PD98059 abolished the protective effect upon HL-60 cells, suggesting the role of the Ras-Raf-MEK-ERK pathway on this effect (Fig. 8). Taken together, these findings



**Fig. 8.** The PGE<sub>2</sub> effect is mediated by the ERK signaling pathway. (A) HL-60 cells were pretreated with specific MEK inhibitor, PD98059 for 1 h before treatment with PGE<sub>2</sub> and menadione. Cells were harvested and stained with propidium iodide, and their DNA content was analyzed by flow cytometry. PD98059 abolished the protective effects of PGE<sub>2</sub> on menadione-induced apoptosis. Apoptotic cell death is shown by the accumulation of cells with a DNA content of below 2n. Results were obtained from three independent experiments. (B) Cells were treated with PGE<sub>2</sub> or PD98059 and ERK was detected by immunoblotting analysis with antibody specific for phosphorylated ERK (upper panel). PD98059 inhibited PGE<sub>2</sub>-induced phosphorylation of ERK in HL-60 cells. Actin was used as a control (lower panel).

suggest that the activation of ERK is a critical event in PGE<sub>2</sub>-induced anti-apoptosis in HL-60 cells.

#### DISCUSSION

Despite the recent advances in the understanding of signaling pathways, the role of PGE<sub>2</sub> in mediating the effects of drugs has not been well publicized, and many questions have yet to be answered. PGE<sub>2</sub> facilitates tumor progression by binding to its cognate receptors EP<sub>1-4</sub> with the subsequent enhancement of cellular proliferation and angiogenesis, inhibiting apoptosis, enhancing cellular invasiveness, and modulating of immunosuppression (Narumiya et al., 1999). Our results demonstrate that PGE<sub>2</sub> has a major role in promoting the survival of monocytes under oxidative-stress, and this effect might be mediated through its transmembrane receptor EP<sub>2</sub>. It was observed that treatment of HL-60 cells with menadione leads to the DNA fragmentation and PARP and lamin B cleavage (Caricchio et al., 1999; Suzuki and Ono, 1999). PGE<sub>2</sub> markedly increased the intracellular concentration of cAMP and reversed the menadione-induced apoptosis through the decreased degradation of PARP and lamin B (Figs. 1B and 1C). Several reports have demonstrated that the second messenger cAMP has anti-apoptotic actions in monocytic cell lines (García-Bermejo et al., 1998; Jun et al., 1998). cAMP also prevented apoptosis in a variety of other cell types including human neutrophil (Rossi et

al., 1995), but the mechanism by which cAMP influences apoptosis is still unknown. The underlying mechanism was confirmed by the addition of cell-permeable cAMP analog dibutyryl and by the quantified amount of sub-G1 DNA, which showed that the addition of dibutyryl cAMP blocked menadione induced apoptotic cell death. The flow cytometry analysis revealed that PGE<sub>2</sub> and dibutyryl cAMP inhibited the apoptosis induced by oxidative agents such as H<sub>2</sub>O<sub>2</sub>, etoposide and taxol in HL-60 cells and also attenuated the generation of apoptosis in U937 cells (Table 1).

Additionally, apoptotic cell death induced by menadione is thought to be mediated by intracellular ROS generation as shown previously (Criddle et al., 2006). PGE<sub>2</sub> inhibited the menadione-induced apoptosis through the scavenging of ROS, observed by the decrease in the DCF fluorescence (Fig. 2). In accordance with this assumption, the GSH cellular level was significantly decreased in menadione treated cells (Checker et al., 2011). As shown in Fig. 3B, the fluorescent intensity of CMAC was decreased markedly in PGE<sub>2</sub>-treated cells, whereas it was increased in menadione-treated cells.

PGE<sub>2</sub> is generated from arachidonic acid by cyclooxygenases, which binds to G protein-coupled receptors and increases the intracellular cAMP level (Coleman et al., 1994). It has been shown that HL-60 cells expressed three subtypes of PGE receptors including EP<sub>2</sub>, EP<sub>3</sub>, and EP<sub>4</sub> (Ishiguro et al., 1998). Different studies have demonstrated the role of EP<sub>2</sub> receptor in the enhanced effect of PGE<sub>2</sub> on cellular and physiological processes (Ishiguro et al., 1998). Using pharmacological agonists for all four EP receptors, we noticed a significant increase in cAMP only with the EP<sub>2</sub> specific agonist butaprost, suggesting that the other receptors do not mediate cell survival role of PGE<sub>2</sub>. Additionally, the EP<sub>2</sub> antagonist AH-6809 abrogated the inhibitory effects of PGE<sub>2</sub> on menadione-induced apoptosis (Fig. 4). These results are consistent with the previously published lines regarding the functional role of EP<sub>2</sub>-PGE<sub>2</sub> in enhancing tumor promotion or preventing apoptosis (Aronoff et al., 2004; Kuo et al., 2009). Furthermore, Fig. 5A very clearly shows that the EP<sub>2</sub> receptor activated PKA through an elevation of the intracellular cAMP level. PKA activity increased four-fold in PGE<sub>2</sub> treated cells. The specific PKA inhibitor H89, completely prevented the PGE<sub>2</sub> anti-apoptotic activity (85%) as shown in Fig. 5B, which suggest that PKA is an essential component of PGE<sub>2</sub>-mediated cell survival in response to oxidative stress.

To further insight into the survival role of PGE<sub>2</sub>, we explored the efficient mechanism of PKA and AKT activation by PGE<sub>2</sub>. Studies have shown that cAMP-mediated transcriptional responses required PKA to phosphorylate CREB (Seino and Shibasaki, 2005). It was observed that in PGE<sub>2</sub> stimulated cells the translocated active PKA catalytic subunit did not phosphorylate CREB as well as other cAMP-responsive transcription factors such as ATF-1 and ATF-2. On the other hand, signaling through the PGE<sub>2</sub> receptor (EP<sub>2</sub>) was coupled to the activation of Akt. Akt phosphorylation mediates anti-apoptotic and pro-survival events (Chang et al., 2003a). When the cells were treated with PGE<sub>2</sub>, no endogenous Akt was observed, which suggest that activation of Akt and CREB is not required for the protection of apoptosis in the monocytic HL-60 cells. Although, it has been observed that the PGE<sub>2</sub> induced increase of CREB transcriptional activity is PKA dependent, however, this may be a cell specific role of PGE<sub>2</sub> (Bidwell et al., 2010).

PGE<sub>2</sub> exerted the majority of its actions through transmembrane receptors and the physiological role of PGE<sub>2</sub> via EP<sub>2</sub> is diverse. It has been reported that PGE<sub>2</sub> stimulates cell proliferation through the activation of ERK, suggesting the Ras signal-

ing pathway (Yu et al., 2009). Overexpression of the activated Ras/Raf/ERK signaling pathway is associated with cell proliferation and inhibition of apoptosis (Chang et al., 2003b). Since Ras is implicated in the triggering of ERK, we verified that the integrity of the downstream EP<sub>2</sub> receptor-targeted pathway was indeed required for the anti-apoptotic effects of PGE<sub>2</sub>. PGE<sub>2</sub> treatment increased the phosphorylation of Raf and the ERK levels in HL-60 cells (Fig. 7), which suggest that the ERK signaling pathway is involved in the protection of apoptosis in PGE<sub>2</sub>-induced HL-60 cells. The activation of ERK1/2 is critical for PGE<sub>2</sub>-induced cell proliferation and is located upstream of COX-2 gene expression (Shen et al., 2004). PD98059, a specific inhibitor of MEK1, was used to block ERK1/2 activation, and PGE<sub>2</sub>-induced apoptosis was measured. It is noted that PD98059 inhibited the anti-apoptotic effect of PGE<sub>2</sub>, followed by the inhibition of ERK1/2 phosphorylation induced by PGE<sub>2</sub> in HL-60 cells (Fig. 8). The functional outcome of ERK activation in HL-60 cells does not always mean a cell will go into a proliferative response. Rather, in certain cell types and under certain environmental circumstances, inhibition of ERK protects cells from apoptotic cell death (Goulet et al., 2005). However, in the present study, PGE<sub>2</sub> might induce ERK phosphorylation at concentrations that stimulate HL-60 cell proliferation, which can be supported through the previously published work (Yu et al., 2009). Our results show that PGE<sub>2</sub> activates the Ras pathway, which in turn initiates ERK activation, and ultimately protects oxidative-induced apoptosis in HL-60 monocytic cells.

In the current study, we present evidence for the biological role of PGE<sub>2</sub> in promoting the survival of monocytes under oxidative-stress. Pretreatment of HL-60 cells with PGE<sub>2</sub> markedly inhibited the menadione-induced apoptosis through the scavenging of ROS generation followed by an increase in the cellular GSH level and a decrease in lipid peroxidation. Challenging the HL-60 cells with PGE<sub>2</sub> triggers an intracellular signaling pathway that leads to the activation of cAMP transcriptional activity. The selective EP<sub>2</sub> receptor mediates anti-apoptotic and pro-survival events in PGE<sub>2</sub> treated HL-60 cells. Additionally, the specific inhibition of ERK revealed that the anti-apoptotic effects of PGE<sub>2</sub> might be related to the Ras/Raf/ERK signaling pathway. Our preliminary results will shed light on the hypothesis that PGE<sub>2</sub> is directly responsible for the protection of monocytes under oxidative-stress. The importance of non-steroidal anti-inflammatory drugs (NSAIDs) to inhibit PGE<sub>2</sub> remains a clinically relevant agent for the treatment of inflammation. The PGE<sub>2</sub> inhibitors will play a pivotal role in the treatment and prevention not only for leukemia but also for other types of cancers. Further studies are warranted to investigate the modulating effects of PGE<sub>2</sub> on the apoptotic signaling pathways of HL-60 cells as well as other cancer types either *in vitro* or *in vivo*.

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## REFERENCES

- Aronoff, D.M., Canetti, C., and Peters-Golden, M. (2004). Prostaglandin E2 inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-mediated increase in intracellular cyclic AMP. *Immunology* 173, 559-565.
- Bidwell, P., Joh, K., Leaver, H.A., and Rizzo, M.T. (2010). Prostaglandin E2 activates cAMP response element-binding protein in glioma cells via a signaling pathway involving PKA-dependent inhibition of ERK. *Prostaglandins Other Lipid Mediat.* 91, 18-29.
- Blum, S., Moore, A.N., Adams, F., and Dash, P.K. (1999). A mitogen-activated protein kinase cascade in the CA1/CA2 subfield of the dorsal hippocampus is essential for long-term spatial mem-

- ory. *J. Neurosci.* 19, 3535-3544.
- Brière, J.J., Schlemmer, D., Chretien, D., and Rustin, P. (2004). Quinone analogues regulate mitochondrial substrate competitive oxidation. *Biochem. Biophys. Res. Commun.* 316, 1138-1142.
- Caricchio, R., Kovalenko, D., Kaufmann, W.K., and Cohen, P.L. (1999). Apoptosis provoked by the oxidative stress inducer menadione (Vitamin K3) is mediated by the Fas/Fas ligand system. *Clin. Immunol.* 93, 65-74.
- Chang, F., Lee, J.T., Navolanic, P.M., Steelman, L.S., Shelton, J.G., Blalock, W.L., Franklin, R.A., and McCubrey, J.A. (2003a). Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. *Leukemia* 17, 590-603.
- Chang, F., Steelman, L.S., Lee, J.T., Shelton, J.G., Navolanic, P.M., Blalock, W.L., Franklin, R.A., and McCubrey, J.A. (2003b). Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. *Leukemia* 17, 1263-1293.
- Checker, R., Sharma, D., Sandur, S.K., Khan, N.M., Patwardhan, R.S., Kohli, V., and Sainis, K.B. (2011). Vitamin K3 suppressed inflammatory and immune responses in a redox-dependent manner. *Free Radic. Res.* 45, 975-985.
- Chun, K.S., Lao, H.C., Trempus, C.S., Okada, M., and Langenbach, R. (2009). The prostaglandin receptor EP2 activates multiple signaling pathways and beta-arrestin1 complex formation during mouse skin papilloma development. *Carcinogenesis* 30, 1620-1627.
- Chun, K.S., Lao, H.C., and Langenbach, R. (2010). The prostaglandin E2 receptor, EP2, stimulates keratinocyte proliferation in mouse skin by G protein-dependent and  $\beta$ -arrestin1-dependent signaling pathways. *J. Biol. Chem.* 285, 39672-39681.
- Coffey, R.N., Watson, R.W., Hegarty, N.J., O'Neill, A., Gibbons, N., Brady, H.R., and Fitzpatrick, J.M. (2000). Thiol-mediated apoptosis in prostate carcinoma cells. *Cancer* 88, 2092-2104.
- Coleman, R.A., Smith, W.L., and Narumiya, S. (1994). International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol. Rev.* 46, 205-229.
- Criddle, D.N., Gillies, S., Baumgartner-Wilson, H.K., Jaffar, M., Chinje, E.C., Passmore, S., Chvanov, M., Barrow, S., Gerasimenko, O.V., and Tepikin, A.V., et al. (2006). Menadione-induced reactive oxygen species generation via redox cycling promotes apoptosis of murine pancreatic acinar cells. *J. Biol. Chem.* 281, 40485-40492.
- Fontana, J.A., Colbert, D.A., and Deisseroth, A.B. (1981). Identification of a population of bipotent stem cells in the HL-60 human promyelocytic leukemia cell line. *Proc. Natl. Acad. Sci. USA* 78, 3863-3866.
- García-Bermejo, L., Pérez, C., Vilaboa, N.E., de Blas, E., and Aller, P. (1998). cAMP increasing agents attenuate the generation of apoptosis by etoposide in promonocytic leukemia cells. *J. Cell Sci.* 111, 637-644.
- Goulet, A.C., Chigbrow, M., Frisk, P., and Nelson, M.A. (2005). Selenomethionine induces sustained ERK phosphorylation leading to cell-cycle arrest in human colon cancer cells. *Carcinogenesis* 26, 109-117.
- Herrmann, M., Lorenz, H.M., Voll, R., Grunke, M., Woith, W., and Kalden, J.R. (1994). A rapid and simple method for the isolation of apoptotic DNA fragments. *Nucleic Acids Res* 22, 5506-5507.
- Ishiguro, S., Takahashi, N., Nemoto, K., Negishi, M., and Ichikawa, A. (1998). Potentiation of retinoic acid-induced differentiation of HL-60 cells by prostaglandin EP2 receptor. *Prostaglandins Other Lipid Mediat.* 56, 145-153.
- Kuo, K.T., Wang, H.W., Chou, T.Y., Hsu, W.H., Hsu, H.S., Lin, C.H., and Wang, L.S. (2009). Prognostic role of PGE2 receptor EP2 in esophageal squamous cell carcinoma. *Ann. Surg. Oncol.* 16, 352-360.
- Narumiya, S., Sugimoto, Y., and Ushikubi, F. (1999). Prostanoid receptors: structures, properties, and functions. *Physiol. Rev.* 79, 1193-1226.
- Osada, S., Tomita, H., Tanaka, Y., Tokuyama, Y., Tanaka, H., Sakashita, F., and Takahashi, T. (2008). The utility of vitamin K3 (menadione) against pancreatic cancer. *Anticancer Res.* 28, 45-50.
- Park, J.E., Yang, J.H., Yoon, S.J., Lee, J.H., Yang, E.S., and Park, J.W. (2002). Lipid peroxidation-mediated cytotoxicity and DNA damage in U937 cells. *Biochimie* 84, 1199-1205.
- Rossi, A.G., Cousin, J.M., Dransfield, I., Lawson, M.F., Chilvers, E.R., and Haslett, C. (1995). Agents that elevate cAMP inhibit human neutrophil apoptosis. *Biochem. Biophys. Res. Commun.* 217, 892-899.
- Sampey, A.V., Monrad, S., and Crofford, L.J. (2005). Microsomal prostaglandin E synthase-1: the inducible synthase for prostaglandin E2. *Arthritis Res. Ther.* 7, 114-117.
- Seino, S., and Shibasaki, T. (2005). PKA-dependent and PKA-independent pathways for cAMP-regulated exocytosis. *Physiol. Rev.* 85, 1303-1342.
- Shen, S.C., Ko, C.H., Hsu, K.C., and Chen, Y.C. (2004). 3-OH flavone inhibition of epidermal growth factor-induced proliferation through blocking prostaglandin E2 production. *Int. J. Cancer* 108, 502-510.
- Smith, W.L., DeWitt, D.L., and Garavito, R.M. (2000). Cyclooxygenases: structural, cellular, and molecular biology. *Annu. Rev. Biochem.* 69, 145-182.
- Sung, Y.M., He, G., Hwang, D.H., and Fischer, S.M. (2006). Overexpression of the prostaglandin E2 receptor EP2 results in enhanced skin tumor development. *Oncogene* 25, 5507-5516.
- Suzuki, Y., and Ono, Y. (1999). Involvement of reactive oxygen species produced via NADPH oxidase in tyrosine phosphorylation in human B- and T-lineage lymphoid cells. *Biochem. Biophys. Res. Commun.* 255, 262-267.
- Thayyullathil, F., Chathoth, S., Hago, A., Patel, M., and Galadari, S. (2008). Rapid reactive oxygen species (ROS) generation induced by curcumin leads to caspase-dependent and -independent apoptosis in L929 cells. *Free Radic. Biol. Med.* 45, 1403-1412.
- Trachootham, D., Alexandre, J., and Huang, P. (2009). Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nat. Rev. Drug. Discov.* 8, 579-591.
- Yu, J.S., and Kim, A.K. (2011). Wogonin induces apoptosis by activation of ERK and p38 MAPKs signaling pathways and generation of reactive oxygen species in human breast cancer cells. *Mol. Cells* 31, 327-335.
- Yu, L., Wu, W.K., Li, Z.J., Li, H.T., Wu, Y.C., and Cho, C.H. (2009). Prostaglandin E(2) promotes cell proliferation via protein kinase C/extracellular signal regulated kinase pathway-dependent induction of c-Myc expression in human esophageal squamous cell carcinoma cells. *Int. J. Cancer* 125, 2540-2546.