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RESEARCH COMMUNICATION

Phorbol Ester TPA Modulates Chemoresistance in the Drug Sensitive Breast Cancer Cell Line MCF-7 by Inducing Expression of Drug Efflux Transporter ABCG2

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Abstract

Recent studies have indicated a link between levels of cyclooxygenase-2 (COX-2) and development of the multidrug resistance (MDR) phenotype. The ATP-binding cassette sub-family G member 2 (ABCG2) is a major MDR-related transporter protein that is frequently overexpressed in cancer patients. In this study, we aimed to evaluate any positive correlation between COX-2 and ABCG2 gene expression using the COX-2 inducer 12-O-tetradecanoylphorbol-13-acetate (TPA) in human breast cancer cell lines. ABCG2 mRNA and protein expression was studied using real-time RT-PCR and flow cytometry, respectively. A significant increase of COX-2 mRNA expression (up to 11-fold by 4 h) was induced by TPA in MDA-MB-231 cells, this induction effect being lower in MCF-7 cells. TPA caused a considerable increase up to 9-fold in ABCG2 mRNA expression in parental MCF-7 cells, while it caused a small enhancement in ABCG2 expression up to 67 % by 4 h followed by a time-dependent decrease in ABCG2 mRNA expression in MDA-MB-231 cells. TPA treatment resulted in a slight increase of ABCG2 protein expression in MCF-7 cells, while a time-dependent decrease in ABCG2 protein expression was occurred in MDA-MB-231 cells. In conclusion, based on the observed effects of TPA in MDA-Mb-231 cells, it is proposed that TPA up-regulates ABCG2 expression in the drug sensitive MCF-7 breast cancer cell line through COX-2 unrelated pathways.

Keywords: ATP-binding cassette transporter - ABCG2 - breast cancer cell lines - cyclooxygenase-2

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Introduction

In cancer treatment, prolonged chemotherapy could lead to the selective survival of multidrug resistant (MDR) cells that exhibit simultaneous resistance to a wide spectrum of structurally and functionally unrelated chemotherapeutic agents. One of the mechanisms leading to MDR is the over-expression of membrane efflux protein breast cancer resistance protein (BCRP).

The 72 kDa breast cancer resistance protein (BCRP) is the second member of the subfamily G of the human ATP binding cassette (ABC) transporter superfamily, designated as ABCG2 (Mao & Unadkat, 2005). ABCG2 is present in normal tissues, such as placental syncytiotrophoblasts, pancreas, small intestine, colon, hepatocytes, breast and in venous and capillary endothelial cells of almost all tissues (Maliepaard et al., 2001; Doyle & Ross, 2003). The functional characteristics and the distribution of ABCG2 suggest that this transporter protects the organism from many potentially harmful xenobiotics by decreasing intracellular concentration of them (Doyle & Ross, 2003; Huls et al., 2009). Overexpression of ABCG2 is associated with high levels of resistance to a variety of anticancer agents. ABCG2 expression has been detected in a large number of hematological malignancies and solid tumors, indicating that this transporter may play an important role in clinical drug resistance of cancers (Ross et al., 2000; Diestra et al., 2002).

In recent years, there have been increasing interest in examining several factors such as COX-2 (Cyclooxygenase-2) causing multidrug resistance via increased the expression of MDR transporters proteins. Cyclooxygenases, also known as prostaglandin endoperoxide synthases or prostaglandin H synthases, comprises a group of enzymes that participate in the conversion of arachidonic acid to prostaglandins that affect a number of physiological and pathological states (Smith & Dewitt, 1996). The expression of the two COX isoenzymes is differently regulated. Constitutively expressed COX-1 supplies normal tissues with prostaglandins required to maintain physiological organ functions (O'Neill & Ford-Hutchinson, 1993), such as cytoprotection of the gastric mucosa (Chan et al., 1995) and regulation of renal blood flow (Tanioka et al., 2003). On the other hand, COX-2 is

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highly induced by inflammatory signals, growth factors and cytokines at sites of inflammation (Smith & Dewitt, 1996; Dubois et al., 1998). Therefore, it is assumed that COX-2 plays an important role in the prostaglandin E2 (PGE2) production involved in pathophysiological processes (Trebino et al., 2003). COX-2 may be implicated in tumor promotion through modulating cell proliferation, inhibiting apoptosis, control of cell migration, cell adhesion, tumor invasion and suppression of immune response (Cao & Prescott, 2002). In recent years, it has been reported that COX-2 modulates ABC transporter expression and is involved in the development of the MDR phenotype. A correlation between expression of COX-2 and MDR1 was found in breast cancer (Ratnasinghe et al., 2001), hepatocellular carcinoma (Fantappiè & R, 2002), and acute myeloblastic lymphoma (Puhlmann et al., 2005). Previously, we demonstrated that induction of COX-2 expression could increase ABCG2 activity (mitoxantrone efflux) in breast cancer cell lines (Kalalinia et al., 2010).

In the present study, we addressed the question as to whether a regulative link exists between the COX-2 expression and the mRNA and protein expression of ABCG2 transporter in breast cancer cell lines. Our results suggest that COX-2 inducer TPA up-regulate ABCG2 expression which looks like to happen with a COX-2-independent mechanism.

Materials and Methods

Reagents and Antibodies

Antibody to human COX-2 was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). BCRP/ABCG2 antibody (BXP-21) was obtained from Abcam (Cambridge, UK). TPA, bovine serum albumin (BSA), penicillin–streptomycin were purchased from Sigma–Aldrich (Germany). RPMI 1640 with L-glutamine and FBS were purchased from Biosera (UK) and Gibco (USA), respectively.

Cell culture and treatments

We used the human breast carcinoma cell lines. MCF-7 and its ABCG2-overexpressing, mitoxantrone-resistance derivative, MCF7/MX cell lines was generously provided by Dr. Erasmus Schneider (Wadsworth Center, New York State Department of Health, USA) and MDA-MB-231 cells was generously provided by Dr. Mohammad Kazem Koohi (Faculty of Veterinary Medicine, university of Tehran, Iran). MDA-MB-231 cells were chosen as a control cell line because they are known to express COX-2. Cells were cultured in RPMI 1640 supplemented with fetal bovine serum (FBS) 10% (w/v), penicillin (50,000 units/L), and streptomycin (50 mg/L) at 37 °C in the presence of CO_2 5%. MX-resistant cells were grown in the medium containing mitoxantrone at a 10^{-7} M concentration to maintain the multidrug-resistant phenotype. MCF7/MX cells were cultured on MX free medium for at least 7 days prior to experiments. Since FBS has been shown to induce COX-2 (due to the presence of growth factors) (Shen et al., 2004), subconfluent cells were washed and changed to serum-free medium containing BSA 0.1% (w/v) for 24 h before treatment. For real-time PCR studies, the breast cancer cells were incubated for 4-48 h with TPA (0–100 nM). For flow-cytometry analysis, all cell lines were treated with TPA 10 nM for 4, 24 and 48 h. After specific treatments, cells were trypsinized and harvested for isolation of total RNA or used for protein expression studies by flow cytometry.

RNA Extraction and Reverse Transcription-Real-Time Polymerase Chain Reaction (RT-PCR)

In order to evaluate ABCG2 and COX-2 expression in breast cancer cell lines under TPA treatment, real-time RT-PCR analysis was performed. Total RNA was isolated from subconfluent treated cells (with TPA 0-100 nM for 4-48 h) using High Pure RNA Isolation Kit from Roche Applied Science according to the protocol provided by the manufacturer. To prevent DNA contamination, RNA was treated with ribonuclease-free DNase (which supplied with RNA Isolation Kit). The total amount of extracted RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).The purity of the RNA samples were determined by the ratio of absorbance at 260 nm and 280 nm and the ratio of absorbance at 260 nm and 230 nm, acceptable ratios were in the range of 1.8-2.2. One step real-time RT-PCR was performed on the RNA samples using EXPRESS One-Step SYBR® GreenER™ Universal kit (Invitrogen) and Stratagene 3000 p sequence detector system. The sequences of primer pairs (TAG Copenhagen A/S, Denmark) were: ABCG2: 5´-TATCAATGGGATCATGAAACCTGG-3´ (forward) and 5´-GCGGTGCTCCATTTATCAGAAC-3´(reverse); COX-2,5´-AATCATTCACCAGGCAAATTG- 3´(forward) and 5´-TCTGTACTGCGGGTGGAACA- 3´(reverse); β-actin: 5´-TCATGAAGTGTGACGTGGACATC-3´ (forward) and 5´-CAGGAGGAGCAATGATCTTGATCT-3´ (reverse) (Rapisarda et al., 2002; Lin et al., 2003; Buchholz et al., 2006). Reactions were performed with an initial step at 57 °C for 5 min (cDNA synthesis), followed by a denaturation step at 95 °C for 2 min, 40 cycles at 95 °C for 15 s, 60 °C for 1 min. Amplification of a single product for each primer set was confirmed by the dissociation curve analysis. Standard curves were prepared for target (ABCG2 and COX-2) and reference (β-actin) genes. Relative expression levels for each gene of interest were normalized to that of the β-actin by the MxPro-Mx3000P system. The results were expressed as the target/reference ratio of the treated samples divided by the target/reference ratio of the untreated control sample.

Intracellular staining of COX-2

The COX-2 protein expression of the breast cancer cell lines under treatment with TPA 10 nM for 4, 24 and 48 h was measured by flow cytometric assay which was developed for internal detection of COX-2 in fixed and permeabilized cells. Briefly, 1×10^6 cells were washed with BSA 2% (w/v) in PBS. The cells were fixed by adding Leucoperm reagent A (fixation medium) and incubated for 15 min at RT (Room Temperature). Following washing with PBS which contained Tween 0.01% (w/v) (PBST), the cells were permeabilized by resuspending with Leucoperm reagent B (Permeabilization Medium) and

incubated at RT for 15 min. After washing and blocking the cells with BSA 10% (w/v), cells were incubated for 30 min at RT with 50 ng of phycoerythrin (PE) - conjugated anti-COX-2 mAbs. The cells were washed with PBST and resuspended in sheath fluid for immediate analysis or in formaldehyde 0.5% and stored at 2-8°C in the dark for later analysis within 24 hours.

Indirect staining of ABCG2

To study the effect of COX-2 induction by TPA on the expression of ABCG2, Flow cytometry detection of ABCG2 expression in control and treated cells was performed using the method described by Minderman et al. (Minderman et al., 2002) with slight modifications. Cells were washed with PBS and fixed in formaldehyde 3.7% (w/w) for 10 min at room temperature, then in ice-cold methanol 90% (v/v) for 10 min. Thereafter, cells were washed with PBST and blocked with BSA 10% (w/v) for 1 h at room temperature. Cells were then incubated for 60 min on ice with primary monoclonal antibody BXP-21 (1:100 in BSA 2% (w/v)), which recognizes internal epitopes of the ABCG2 protein (Maliepaard et al., 2001). After washing with PBST, Cells were incubated for 20 min on ice and protected from light with a Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody with a final dilution 1:50 in PBST to detect the primary anti-ABCG2 mAb. After the final wash with PBST, cells were resuspended in PBS with BSA 2% (w/v) and stored on ice in the dark prior to analysis.

Flow cytometric detection of COX-2 and ABCG2 expression

Samples were analyzed on a Partec™ cytometer equipped with a standard argon laser 488-nm excitation, and with 530/30 band pass filter for FL1 and a 585/42 band pass filter for FL2 for detection of FITC and PE fluorescence, respectively. To exclude cell debris and clumps, samples were gated on forward scatter versus side scatter. Fluorescence of 10,000 cells was quantified from histogram plots using the mean fluorescence intensity (MFI). Flow cytometry data were processed and analyzed using FloMax version 2.52 and WinMdi version 2.9 software. Fold change was calculated by dividing the MFI of the treated sample (MFI treated) by that of the untreated sample (MFI untreated).

Statistical analysis

All experiments were performed in duplicate or triplicate on three independent occasions. Statistically significant differences between values were determined using multivariate analysis of variance (MANOVA) with Tukey's post-hoc. All the data are expressed as mean±S.D., and P<0.05 was considered to be statistically significant.

Results

The first objective was to confirm the induction of the COX-2 expression in MCF-7, MCF7/MX and MDA-MB-231 cells with TPA. Figure 1 indicates that TPA can stimulate the expression of COX-2 within MDA-MB-231 cells. This induction effect of the TPA on the expression of COX-2 was concentration-dependent and decreased as the incubation time of this cell line increased. The basal levels of COX-2 mRNA expression were increased more than 11-fold in 4 h and it was negligible 48 h after treatment. The most induction effect of TPA on the expression of COX-2 was observed with TPA 10 nM by 12 h and TPA 20 nM by 24 h in MCF-7 and MCF7-MX, respectively.

The differential COX-2 protein expression as a result of COX-2 mRNA overexpression with TPA 10 nM was also evaluated by flow cytometry. As shown in Figure 2, COX-2 protein was up-regulated in MCF-7 (4-48 h) and MDA-MB-231(4 h), as a difference of more than 60% was considered significant in MCF-7 cells. On the other hand, COX-2 protein expression was slightly reduced (about 15 % vs. control) under TPA treatment in MCF7/MX cells.

The relationship between induction of COX-2 expression and expression of ABCG2 was studied using real-time RT-PCR. Breast cancer cells were treated with TPA 0-100 nM for 4-48 h. Figure 3 shows the results of these preliminary experiments. We observed a slight

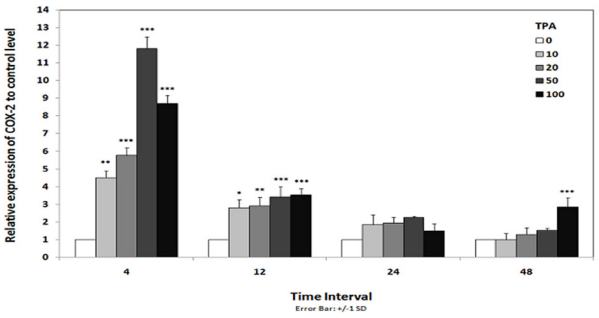

Figure 1. Time Course Study of the Effects of TPA on COX-2 mRNA Expression in MDA-MB-231 Cells. Cells were treated with TPA (0-100 nM for 4-48 h) and real-time RT-PCR analysis was performed on total RNA extracted from control and treated cells. Values were normalized to the β-actin content of samples. The results were expressed as the target/reference ratio of the treated samples divided by the target/reference ratio of the untreated control sample and expressed as mean±SD (n=3); *, p<0.05; **, p<0.01; ***, p<0.001

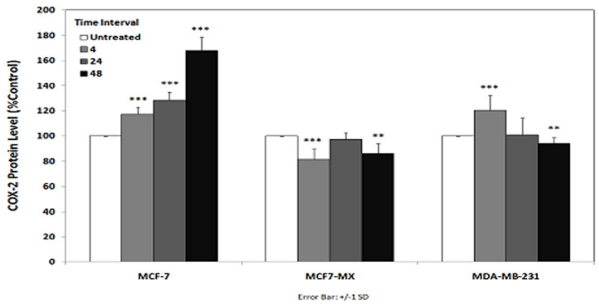

Figure 2. COX-2 Protein Expression in MCF-7, MCF7/MX and MDA-MB-231 Cell Lines. Cells were treated with TPA (10 nM for 4, 24 and 48 h) and COX-2 protein expression was measured by flow cytometric assay which developed for internal detection of COX-2. Briefly, cells were fixed and permeabilized by Leucoperm (fixation and permeabilization medium) and blocked with BSA. Then, they were incubated with PE- conjugated anti-COX-2 mAbs for 30 min at RT. COX-2 protein level expressed as ratio of: $MFI_{treated\ sample}$ /$MFI_{untreated\ sample}$ The values are shown as mean±S.D. (n=3); *, p <0.05; **, p <0.01; ***, p <0.001".

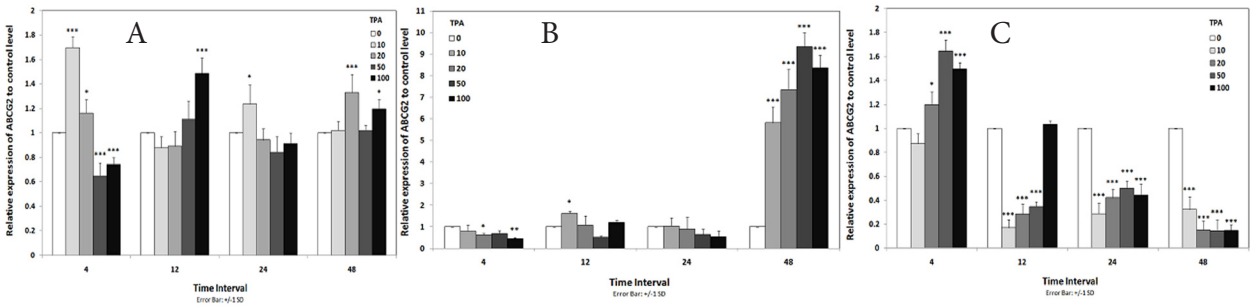

Figure 3. Effects of TPA on the Levels of ABCG2 mRNA in A, MCF-7-MX, B, MCF-7, and C, MDA-MB-231 Cell Lines. Cells were treated with TPA (0-100 nM for 4-48 h) and ABCG2 mRNA expression was measured by real-time RT-PCR using total RNA extracted from control and treated cells. Relative expression levels for each gene were normalized to that of the β-actin. The results were expressed as: (target/reference ratio)$_{\text{treated samples}}$/(target/reference ratio)$_{\text{untreated control sampl}}$. Values were expressed as mean±SD (n=3); *, p<0.05; **, p<0.01; ***, p<0.001".

increase in ABCG2 expression in resistance cell line MCF7/MX (Figure 3A), while a considerable increase up to 9-fold in ABCG2 expression was observed in the parental MCF-7 cells (Figure 3B). On the other hand, interesting results were observed in MDA-MB-231 (Figure 3C), whereas small enhancement in ABCG2 expression up to 67 % was observed in 4 h while increasing the incubation time reverse this process and showed a time-dependent decrease to about 85 % in ABCG2 expression.

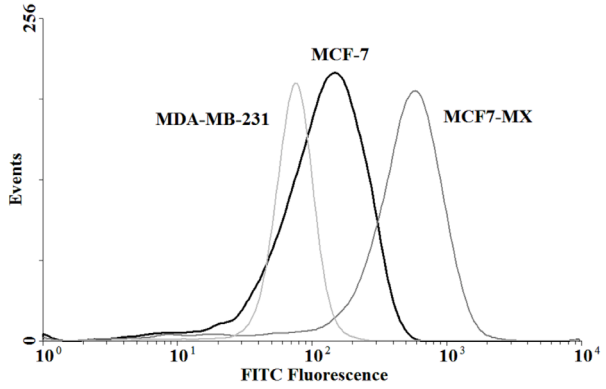

Figure 4. Expression of ABCG2 Protein in Wild Type and Resistant Cell Lines was Studied by Flow Cytometry with the BXP-21 Monoclonal Antibody. Cells were fixed and permeabilized by formaldehyde 3.7% (w/w) and methanol 90% (v/v). Thereafter, cells were blocked with BSA 10% (w/v) and then incubated with primary monoclonal antibody BXP-21. After washing, Cells were incubated with a FITC-conjugated goat anti-mouse antibody. Fluorescence of 10,000 cells was quantified from histogram plots using the FITC fluorescence.

It was necessary to investigate whether the increased gene expression was associated with increased translation of the protein. All cell lines were treated with TPA (10 nM for 4, 24 and 48 h), and ABCG2 protein expression were analyzed by flow cytometry. At basal level (without treatment), higher FITC fluorescence of MCF-7/MX histogram was observed in compared to FITC fluorescence of MCF-7 and MDA-MB231 histogram which confirmed the overexpression of ABCG2 protein in MCF7/MX cells (Figure 4). The results showed a slight increase of ABCG2 expression in MCF-7 with the highest effect at 48 h (Figure 5A), while no significant changes in the level of ABCG2 expression was observed in MCF7/MX (Figure 5B). On the contrary, treatment of MDA-MB-231 cells with TPA resulted in a time-dependent decrease in ABCG2 protein expression wherever the maximum intensity (about 10 %) was seen at 48 hours (Figure 5C). Using flow cytometry analysis we were able to confirm the real-time PCR results.

Discussion

Multidrug resistance may be one of the major reasons for the failure of chemotherapy in human cancers. Energy dependent drug efflux involving ABCG2 is believed to result in reduced drug accumulation and sensitivity to a variety of natural products used in the treatment of cancers. We have previously showed that proinflammatory cytokines IL-1β and TNF-α induced ABCG2 and pregnane X receptor (PXR) expression in breast cancer cell line MCF-7 (Mosaffa et al., 2009; Malekshah et al., 2011). In another study, it has been indicated that dexamethasone (an anti-inflammatory drug) reduced the

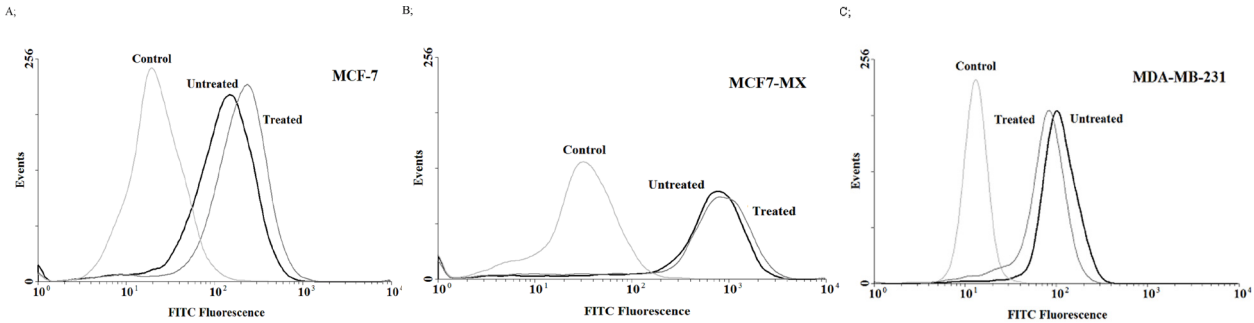

Figure 5. Effect of TPA on ABCG2 Protein Levels in A, MCF-7, B, MCF-7-MX and C, MDA-MB-231 Cells. After 48-h incubation with TPA (10 nM), expression of ABCG2 protein was measured by flow cytometry. Each histogram shows the overlay of the treated sample (dark gray), untreated sample (black) and secondary antibody as negative control (light gray).

expression of ABCG2 mRNA in MCF-7 and MCF-7/MX breast cancer cell lines. In addition, the level of ABCG2 protein expression and function was decreased in dexamethasone-treated MCF-7/MX cells (Elahian et al., 2009: 2010). During the last decade, numerous studies have reported that enforced expression of COX-2 causes enhancement of multidrug resistance proteins expression in different cancer cell lines (Patel et al., 2002; Sorokin, 2004; Surowiak et al., 2005; 2006; 2008; Lee et al., 2007;). In the present study, we aimed to evaluate the existence of a positive correlation between COX-2 and ABCG2 gene expression using COX-2 inducer TPA in three breast cancer cell lines.

The results indicated that TPA could induce COX-2 expression in all tested cell lines. Our report is consistent with of Liu et al. who showed that in contrast to MCF-7 cells, the biologically aggressive, invasive MDA-MB-23l cell line possesses a high constitutive level of COX-2 mRNA and also displays a further and prolonged increase in the expression of the COX-2 protein in response to TPA (Liu and Rose, 1996). Furthermore, the amount of COX-2 mRNA induction and production of COX-2 protein were closely related in MCF-7 and MCF7/MX cells.

In the MDA-MB-231 cell line, real-time RT-PCR revealed a 6-fold increase in COX-2 mRNA in response to TPA 10 nM but only a 60% increase in the protein level was shown by flow cytometry. These results are in agreement with the reports by liu et al. (Liu & Rose, 1996), Lee et al. (Lee et al., 1992) and Kargman et al. (Kargman et al., 1995) that high COX-2 mRNA expression may occur in human breast cancer cells and colorectal adenomas without a associated increase in COX-2 protein. It seemed that COX-2 may go through complex posttranscriptional and posttranslational modification to yield the active enzyme.

In MCF-7 cells, TPA treatment increased ABCG2 expression up to 9-fold. While, TPA treatment could not significantly stimulate ABCG2 expression in MCF7-MX. In the drug resistant MCF7/MX cells, ABCG2 is already overexpressed, and its expression may be at a threshold maximum level. Perhaps an induction with TPA treatment may not be causing any detectable increase in mRNA level (Elahian et al., 2009; Mosaffa et al., 2009).

We previously reported that TPA enhanced ABCG2 activity in breast cancer cell lines (Kalalinia et al., 2010). In the present study, we showed that TPA increases the ABCG2 expression in MCF-7 cell line while it significantly decreased ABCG2 expression in MDA-MB-231 cells. Considering what was mentioned earlier, TPA increased COX-2 expression several fold to control level in MDA-MB-231 cell which was significantly greater than the effect observed in other tested cell lines. So overall it seems that the observed effect of TPA on ABCG2 expression may be COX-2 independent. Different studies showed that incubation of MDR cells with protein kinase C (PKC) activator TPA stimulate P-glycoprotein (P-gp) phosphorylation, reduce drug accumulation, and enhance drug resistance (Ramachandran et al., 1998). Fine et al. demonstrated that 12,13-dibutyrate [P(BtO)2] led to an increase in protein kinase C activity and induced a drug-resistance phenotype as a result of increased phosphorylation of an unknown 20-kDa particulate protein (Fine et al., 1988). Similar to TPA treatment, Diacylglycerol (DAG), a physiological stimulant of PKC, also increased the expression of MDR1 mRNA and protein. Whereas, protein kinase inhibitor staurosporine suppressed the induction of MDR1 expression by TPA and DAG (Chaudhary and Roninson, 1992). These reports suggest that MDR1 gene expression in different cell types is regulated by a PKC-mediated pathway. In addition, TPA induces several biological cellular procedures such as differentiation and causes reduced drug influx (Drew et al., 1996). On the other hand, Drew et al. proposed a P-gp independent drug resistance due to a PKC-mediated process, which is maintained after depletion of certain PKC subspecies or is due to activation of down regulation insensitive PKC subspecies (Drew et al., 1996). To our knowledge, there are no similar reports about ABCG2; further studies are needed to determine whether TPA induces PKC, COX-2 or any of the above-mentioned cellular events resulting in increased ABCG2 expression and function.

In conclusion, we found that TPA stimulate COX-2 mRNA expression up to 11-fold in MDA-MB-231 while it caused a slight induction of COX-2 expression in MCF-7 and MCF7/MX cell lines. TPA caused ABCG2 overexpression in drug sensitive and drug resistant MCF-7 cell lines, while it reduced the ABCG2 expression in MDA-MB-231 cells. Therefore, the existence of a positive correlation between COX-2 and ABCG2 gene expression remains to be questioned according to observed effects of the COX-2 inducer TPA in MDA-MB-231 cells. Further studies should be performed on COX-2 pathway to more clarify the observed effects. The discovery of this relationship will facilitate the development of strategies to fight multidrug resistance in tumors which overexpress the COX-2 protein.

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