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# CREB-Dependent Cyclooxygenase-2 and Microsomal Prostaglandin E Synthase-1 Expression Is Mediated by Protein Kinase C and Calcium

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**Abstract** Cellular production of prostaglandins (PGs) is controlled by the concerted actions of cyclooxygenases (COX) and terminal PG synthases on arachidonic acid in response to agonist stimulation. Recently, we showed in an ileal epithelial cell line (IEC-18), angiotensin II-induced COX-2-dependent $PGI_2$ production through p38MAPK, and calcium mobilization (J. Biol. Chem. 280: 1582–1593, 2005). Agonist binding to the $AT_1$ receptor results in activation of PKC activity and $Ca^{2+}$ signaling but it is unclear how each pathway contributes to PG production. IEC-18 cells were stimulated with either phorbol-12,13-dibutyrate (PDB), thapsigargin (TG), or in combination. The PG production and COX-2 and PG synthase expression were measured. Surprisingly, PDB and TG produced $PGE_2$ but not $PGI_2$. This corresponded to induction of COX-2 and mPGES-1 mRNA and protein. PGIS mRNA and protein levels did not change. Activation of PKC by PDB resulted in the activation of ERK1/2, JNK, and CREB whereas activation of $Ca^{2+}$ signaling by TG resulted in the delayed activation of ERK1/2. The combined effect of PKC and $Ca^{2+}$ signaling were prolonged COX-2 and mPGES-1 mRNA and protein expression. Inhibition of PKC activity, MEK activity, or $Ca^{2+}$ signaling blocked agonist induction of COX-2 and mPGES-1. Expression of a dominant negative CREB (S133A) blocked PDB/TG-dependent induction of both COX-2 and mPGES-1 promoters. Decreased CREB expression by siRNA blocked PDB/TG-dependent expression of COX-2 and mPGES-1 mRNA. These findings demonstrate a coordinated induction of COX-2 and mPGES-1 by PDB/TG that proceeds through PKC/ERK and $Ca^{2+}$ signaling cascades, resulting in increased $PGE_2$ production. J. Cell. Biochem. 98: 1653–1666, 2006. 

**Key words:** cyclooxygenase-2; microsomal Prostaglandin E synthase-1; gene expression; protein kinase C; $Ca^{2+}$ signaling; IEC-18

Prostaglandins (PGs) play a fundamental role in a broad number of physiological processes in the gastrointestinal tract [Wang et al., 2005]. Alterations in PG production are implicated in pathological conditions including acute and chronic inflammation, angiogenesis, and cancer [DeWitt, 1991; Smith and Marnett, 1991]. The rate-limiting step of PG synthesis is the production of the unstable PG endoperoxide intermediate, $PGH_2$ from arachidonic acid by

Abbreviations used: PG, prostaglandin; AA, arachidonic acid; COX, cyclooxygenase; AngII, angiotensin II; mPGES-1, microsomal prostaglandin E synthase-1; PGIS, prostaglandin I synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IEC-18, intestinal epithelial cells; PKC, protein kinase C; ERK, extracellular regulated kinase; JNK, c-Jun N-terminal kinase; CREB, cAMP response element binding protein; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; PDB, phorbol 12,13-dibutyrate; TG, thapsigargin; RT-PCR, reverse transcriptase polymerase chain reaction; 2-APB, 2-aminoethoxydiphenylborate; GFX, bisindolylmaleimide; PD98059, 2′-amino-3′-methoxyflavone; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline; TBS, Tris buffered saline; BSA, bovine serum albumin

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cyclooxygenase (COX) [Chandrasekharan and Simmons, 2004]. $PGH_2$ is then converted to one of a several structurally related PGs, including $PGE_2$, $PGI_2$, $PGD_2$, $PGF_{2\alpha}$, and Thromboxane $A_2$, by specific PG synthases that are present in the cell. Acute changes in PG levels in cells are controlled by COX-2 (prostaglandin-endoperoxidase synthase, EC 1.14.99.1), which is rapidly induced as an immediate-early gene in response to pro-inflammatory cytokines, tumor promoters, and growth factors [Xie et al., 1994; Xie and Herschman, 1996; Morita, 2002]. COX-2 is overexpressed in cancers of the colon [Kawai et al., 2002], stomach [Ristimaki et al., 1997], lung [Brown and DuBois, 2004], and breast [Subbaramaiah et al., 1996]. Overexpression of human COX-2 in mammary glands of transgenic mice induces tissue-specific carcinogenesis with COX-2 derived $PGE_2$ mediating angiogenesis [Liu et al., 2001]. Chronic inhibition of COX activity by non-steroidal anti-inflammatory drugs has been associated with chemopreventative effects on colon cancer [Marnett and DuBois, 2002]. Consequently, the identification of the pathways and regulatory elements that control both COX-2 and PG synthase expression is the subject of major interest.

Recently, we demonstrated that angiotensin II (Ang II), a G-protein coupled receptor (GPCR) agonist, and epidermal growth factor (EGF), a receptor tyrosine kinase agonist, both induce COX-2-dependent $PGI_2$ production in IEC-18 cells [Slice et al., 2005], a non-tumorigenic epithelial cell line derived from rat ileal crypts [Quaroni et al., 1979]. Ang II binding to the $AT_1$ receptor, which is coupled to both the Gq and G12 families of heterotrimeric G-proteins [Chiu and Rozengurt, 2001] results in activation of protein kinase C (PKC) cascades and signaling by $Ca^{2+}$ [Chiu et al., 2003]. Ang II-dependent COX-2 expression in IEC-18 cells requires $Ca^{2+}$ signaling but the role of PKC is unclear [Slice et al., 2005]. Agonist binding to the EGFR results in activation of the Ras/Raf/MEK/ERK signaling cascade but it is unclear which components of this pathway leads to PG production. Additionally, Ang II/EGF induced, COX-2-dependent increases in PG suggests a functional link between COX-2 and PG synthases.

In this study, we use pharmacological agents to activate PKC and $Ca^{2+}$ signaling cascades in IEC-18 cells independent of agonist binding to cell surface receptors. We found that treatment of IEC-18 cells with phorbol-12,13-dibutyrate (PDB) or thapsigargin (TG) resulted in an increase in $PGE_2$ production with a corresponding increase in COX-2 and mPGES-1 (microsomal PGE synthase-1, EC: 5.3.99.3) mRNA expression. COX-2 and mPGES-1 protein expression was induced through PKC and $Ca^{2+}$ signaling. The expression of COX-2 and mPGES-1 proceeds through PKC signaling with the activation of extracellular regulated kinase-1 and -2 (ERK1/2), c-Jun N-terminal kinase (JNK), and cAMP response element binding protein (CREB) whereas $Ca^{2+}$ signaling results in the delayed activation of ERK1/2. The combined effect of PKC and $Ca^{2+}$ activation prolongs signaling resulting in sustained COX-2 and mPGES-1 mRNA and protein expression. Furthermore, expression of COX-2 and mPGES-1 via PKC and $Ca^{2+}$ is dependent on CREB. Stimulation of cells by either pharmacological agents or by specific receptor agonists can result in the production of different PGs.

## MATERIALS AND METHODS

### Materials

PDB, TG, cyclohexamide, 2-aminoethoxydiphenylborate (2-APB), bisindolylmaleimide (GFX, 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide), 2′-amino-3′-methoxyflavone (PD98059), calcium ionophore (A23187), dexamethasone (DEX), NS-398 (N-[2-(cyclohexyloxy)-4-nitro-phenyl]methanesulfonamide), and actinomycin D were acquired from Sigma (St. Louis, MO). JNK inhibitor II (SP600125) and 4-(4-flurophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole (SB 202190) were acquired from Calbiochem (San Diego, CA).

### Cell Culture

Rat intestinal epithelial cells IEC-18 (CRL-1589, American Type Culture Collection, Rockville, MD) were propagated with Dulbecco's modified Eagle's medium (DMEM) containing 4 mM L-glutamine, 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 10% fetal bovine serum (Sigma) and 1% antibiotic/antimycotic (Invitrogen, Carlsbad, CA) in 100-$mm^2$ tissue culture dishes (Nunc, Napierville, IL) at 37°C with 5% $CO_2$. The cells were allowed to grow to confluence (5–7 days) and serum starved for 24 h with 4 ml of Opti-MEM (Gibco, Grand Island, NY) prior to experiments.

### RNA Isolation and TaqMan Assay

Total RNA from the cells was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The isolated RNA was stored in 30 μl of RNA Storage Solution at −20°C for subsequent analysis. RNA concentration was determined from diluted purified RNA samples using a spectrophotometer (GeneQuant Pro, Amersham Biotechnology, Piscataway, NJ). Total RNA samples (25 ng) were reverse transcribed and cDNAs amplified using TaqMan Gold RT-PCR kit (Applied Biosystem, Foster City, CA) according to the manufacturer's protocol. Transcripts encoding COX-2, COX-1, mPGES-1, PGIS, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard were quantified by real-time PCR using an ABI Prism 7700 Sequence Detection System (PE Biosystems, Foster City, CA). The primers and probes used are as follows: rat COX-2 sense primer 5′-GGC ACA AAT ATG TTC GCA-3′, COX-2 anti-sense primer 5′-CCT CGC TTC TGA TCT GTC TTG A-3′, COX-2 probe 5′-$^{FAM}$TCT TTG CCC AGC ACT TCA CTC ATC AGT TT$^{TAMRA}$-3′, mPGES-1 sense primer 5′-GCG AAC TGG GCC AGA ACA-3′, mPGES-1 anti-sense primer 5′-GGC CTA CCT GGG CAA AAT G-3′, mPGES-1 probe 5′-$^{FAM}$CCC CGG AGC GAA TGC GTG G$^{TAMRA}$-3′, PGIS sense primer 5′-GAC GTT TTC CGC ACC TTC C-3′, PGIS anti-sense primer 5′-ACT GAC AAG GAG CCT CGA GC-3′, PGIS probe 5′-$^{FAM}$CCA GCT GGA TCT GAT GCT CCC CA$^{TAMRA}$-3′, COX-1 sense primer 5′-CAG TCC TTC AAT GAA TAC CGA AA-3′, COX-1 anti-sense primer 5′-GCG GCC ATC TCC TTC TCT-3′ and Universal ProbeLibrary probe #124 (Roche Applied Science, Indianapolis, IN). The thermal cycling conditions for reverse transcription and amplification activation were set at 48°C for 30 min and 95°C for 10 min, respectively. PCR denaturing was set at 95°C at 15 s and annealing/extending at 60°C at 60 s for 40 cycles.

### Protein Expression

IEC-18 cells were serum starved in Opti-MEM (Gibco) for 24 h prior to treatment with 100 nM of PDB, 100 nM of TG, or 100 nM of PDB/TG dissolved in DMSO, with the final DMSO concentration <0.1% (v/v). After incubation, the media were aspirated and the cells washed with ice-cold phosphate-buffered saline. The cells were solubilized in LDS loading buffer (Invitrogen) and protein scraped from the culture dish. The lysates were heated at 95°C for 5 min prior to loading. Samples were loaded with See Blue molecular weight standards (Invitrogen), subjected to a 4–12% SDS–polyacrylamide gel electrophoresis (NuPAGE Novex Bis-Tris Gels, Invitrogen) for 45 min at 200 V and transferred to a PVDF membrane (90 min at 30 V). After transfer, the membrane was washed with phosphate-buffered saline followed with Tris-buffered saline (TBS, Sigma) and incubated in blocking solution (5% dried non-fat milk and 5% BSA in 1% Tween-TBS). The membrane was probed with rabbit polyclonal antibody raised against either COX-2, mPGES-1, PGIS, phospho-CREB, phospho-JNK, phospho-p38MAPK, or phospho-ERK1/2 (1:1,000) followed by anti-rabbit IgG linked to alkaline phosphatase conjugate (1:2,500) and visualized by enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, England). For internal standards, the blot was stripped with Restore Western Blot stripping buffer (Pierce, Rockford, IL) for 10 min and probed for ERK2 (1:2,500) as the internal standard and visualized by enhanced chemiluminescence.

### Prostaglandin Levels

Cells were grown in 24-well plates to confluence and serum starved for 24 h with Opti-MEM prior to experiment. $PGE_2$ and $PGI_2$ were quantified according to EIA kit instructions (STAT-Prostaglandin $E_2$ EIA kit and STAT-Prostaglandin $F_{1\alpha}$ EIA kit, Cayman Chemical, Ann Arbor, MI). Duplicate sample culture medium of 50 μl each were assayed for either $PGE_2$ against appropriate standard curve. Absorbance readings were set between 405 and 420 nm on a microplate reader model 680 (BioRad, Hercules, CA).

### Transfection and Luciferase Assay

Cells were plated at $5 \times 10^4$ cells per 35-mm dish a day before transfection. The cells were depleted of media and incubated for 5 h with a transfection mixture containing 1 μg of the COX-2 reporter (pTIS-10s) or mPGES-1 reporter (sequenced −500 to +1) vector, 0.25 μg of phRG-TK (Promega, Madison, WI) and either 0.75 μg of pcDNA3 vector, CREB wild type vector, or S133A CREB vector (BD Bioscience, Mountain View, CA) combined with 5 μg of Lipofectin (Invitrogen) in 1 ml of Opti-MEM.

After removal of the transfection mixture, the cells were replenished with complete DMEM and allowed to grow to confluency (4 days). The transfected cells were depleted of complete media and replaced with opti-MEM 24 h prior to experiments. Following experiments, the cells were washed with ice-cold PBS, lysed with 150 μl of passive lysis buffer (Promega) and collected in eppendorf tubes as previously described [Slice et al., 2005]. The relative light units from firefly and *Renilla* luciferases (10 μl) were measured using the Dual-Luciferase activity assay kit (90 μl; Promega) on a Turner TD20/20 luminometer. The ratio of firefly to Renilla luciferase activity was measured and reported as relative changes in the ratio. Each transfection was done in triplicate and are reported as mean ± SEM or as percent ± SEM relative to the control. All experiments were independently performed at least twice.

### Electrophoretic Mobility Shift Assay

Proteins were extracted from the nuclei of IEC-18 cells according to manufacturer's protocol (Panomics, Redwood City, CA). Proximal, rodent COX-2 promoter (−200 to +1) was generated using PCR, gel purified, and then radiolabeled using T4 polynucleotide kinase and [$^{32}$P]-γ-ATP. Radiolabeled probe (10,000 cpm) was incubated with nuclear extract proteins (10 to 15 μg) in 15 μl containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 10 μg poly dI-dC). Bound probe was separated from unbound probe by a 4% polyacrylamide gel. Radioactivity was measured using a PhoshoImager. Competition experiments used unlabeled double stranded oligonucleotides that were purchased from Promega Corporation. Supershift experiments used antibodies from Cell Signaling (Beverly, MA).

### Electroporation

IEC-18 cells were harvested and suspended in hypoosmolar buffer (Eppendorf) at a density of $2 \times 10^6$ cells per 1 ml for 15 min at room temperature. The cells were electroporated (320 V, 100 μs, 2 pulses) at $8 \times 10^5$ cells in the presence of 100 nM of non-specific siRNA or 100 nM of CREB siRNA (Smartpool, Dharmacon, Lafayette, CO) and allowed to recover for 5 min at room temperature prior to plating in 35-mm dishes. After 72 h, total RNA was isolated as previously described and analyzed by real-time RT-PCR for COX-2 and mPGES-1.

### Statistics

Data are expressed as mean ± SEM. Two-way ANOVA was performed using Sigma Plot (SPSS, Chicago, IL) and $P < 0.05$ was considered statistically significant.

## RESULTS

### PDB and TG-Induced $PGE_2$ Secretion in IEC-18 Cells

Previously, we have determined that PGs are produced by IEC-18 cells in response to Ang II via the $AT_1$ receptor [Slice et al., 2005]. Ligand binding to the $AT_1$ receptor activates the Gq family of heterotrimeric G-proteins, resulting in PKC and calcium signaling. In order to determine whether activation of PKC and calcium signaling is sufficient for PG production, IEC-18 cells were treated with the PDB and TG. PDB, which is a potent activator of PKC, induced a rapid increase in the level of $PGE_2$ in a time-dependent manner (Fig. 1), reaching a maximum (1,020 ± 50 pg/ml) at 4 h and then decreasing to basal levels by 16 h. TG, which causes the release of intracellular calcium by inhibition of endoplasmic reticulum $Ca^{2+}$-ATPases [Thastrup et al., 1990], showed a gradual increase in $PGE_2$ levels that were maximal (705 ± 55 pg/ml) at 6 h and then decreased to basal levels by 24 h. When cells were treated with the combination of PDB and TG, $PGE_2$ levels reached a maximum (1,530 ± 45 pg/ml) by 4 h and then decreased at 20 h. To confirm $PGE_2$ generation in IEC-18 cells is COX-2-dependent, the cells were pre-treated with 5 μM of NS-398 (COX-2-specific inhibitor) for 1 h prior to stimulation with PDB, TG, and PDB/TG. Pre-treatment with NS-398 completely suppressed $PGE_2$ production by PDB, TG, and PDB/TG (Fig. 1 inset). The level of prostacyclin, $PGI_2$, was not increased in IEC-18 cells in response to PDB, TG, or PDB/TG (data not shown).

### PDB and TG Induce COX-2 and mPGES-1 Expression

Since PGs are products of the actions of COX and PG synthases on arachidonic acid, we wanted to determine whether PDB and TG increase COX-2 and PG synthase expression. PDB rapidly induced COX-2 mRNA levels in

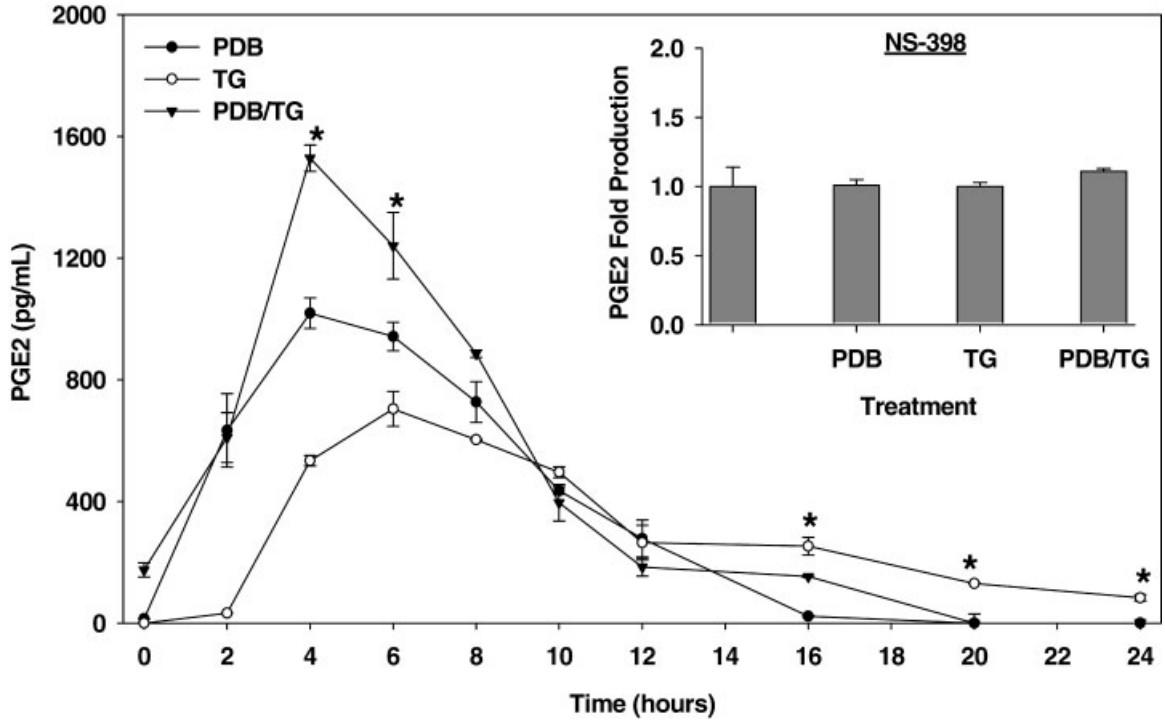


**Fig. 1.** Time-dependent induction of $PGE_2$ by PDB and/or TG. Confluent IEC-18 cells were serum starved for 24 h prior to stimulation with 100 nM of PDB (●), 100 nM of TG (○), or 100 nM of both PDB/TG (▼). Prostaglandin $E_2$ ($PGE_2$) levels were quantified by EIA from the medium samples and the results are expressed as pg/ml of $PGE_2 \pm$ SEM. The asterisk (*) indicates a statistical significance of $P < 0.05$ between the combined PDB/TG and PDB, or TG. Inset: IEC-18 cells were pre-treated with 5 μM of NS-398 (COX-2-specific inhibitor) for 1 h prior to stimulation with 100 nM of PDB, TG, or PD/TG and $PGE_2$ levels were quantified by EIA 4 h later.

IEC-18 cells (Fig. 2A). COX-2 mRNA levels reached a maximum of $43 \pm 7$-fold at 1 h and then decreased to 22-fold above basal levels for up to 8 h. PDB also increased mPGES-1 mRNA levels that peaked after 2 h ($6.3 \pm 0.5$-fold) and then decreased back to basal levels by 8 h. Pre-treatment with cyclohexamide (inhibitor of protein synthesis) followed by the addition of PDB, TG, or PDB/TG resulted in elevated COX-2 and mPGES-1 mRNA expression (data not shown), indicating that COX-2 and mPGES-1 are immediate early genes that do not require de novo protein synthesis.

PDB induced both COX-2 and mPGES-1 protein levels. Increases in COX-2 protein were apparent after 2 h of treatment with PDB. The levels peaked at 8 h and remained elevated up to 24 h. PDB induction of mPGES-1 protein revealed a biphasic response. The initial increase was seen after 1 h, reached a maximum at 2 h, and then decreased by 8 h. A second increase in mPGES-1 protein levels was observed after 8 h that remained elevated up to 24 h.

TG rapidly induced COX-2 mRNA levels that were maximal ($39 \pm 2$-fold) at 4 h and then decreased to $21.6 \pm 1.3$-fold by 8 h (Fig. 2B). Compared to the induction profile by PDB, the maximal level of COX-2 mRNA was delayed but remained higher at the later time points. TG induced a sustained increase in COX-2 protein beginning at 4 h and reaching maximal levels after 12 h.

TG induced mPGES-1 mRNA and protein levels in IEC-18 cells. The mPGES-1 mRNA increased to $4.4 \pm 0.7$-fold after 1 h followed by a gradual decrease to basal levels after 8 h. The mPGES-1 protein showed a biphasic induction pattern that was similar to that observed with cells treated with PDB. There was an initial increase in mPGES-1 protein at 1 h followed by a decrease in protein levels by 6 h. There was a subsequent increase in mPGES-1 protein levels by TG that reached a maximum after 20 h.

Combined treatment with PDB/TG resulted in a synergistic induction of COX-2 mRNA (compared to PDB or TG) with enhanced COX-2 protein expression (Fig. 2C). PDB/TG

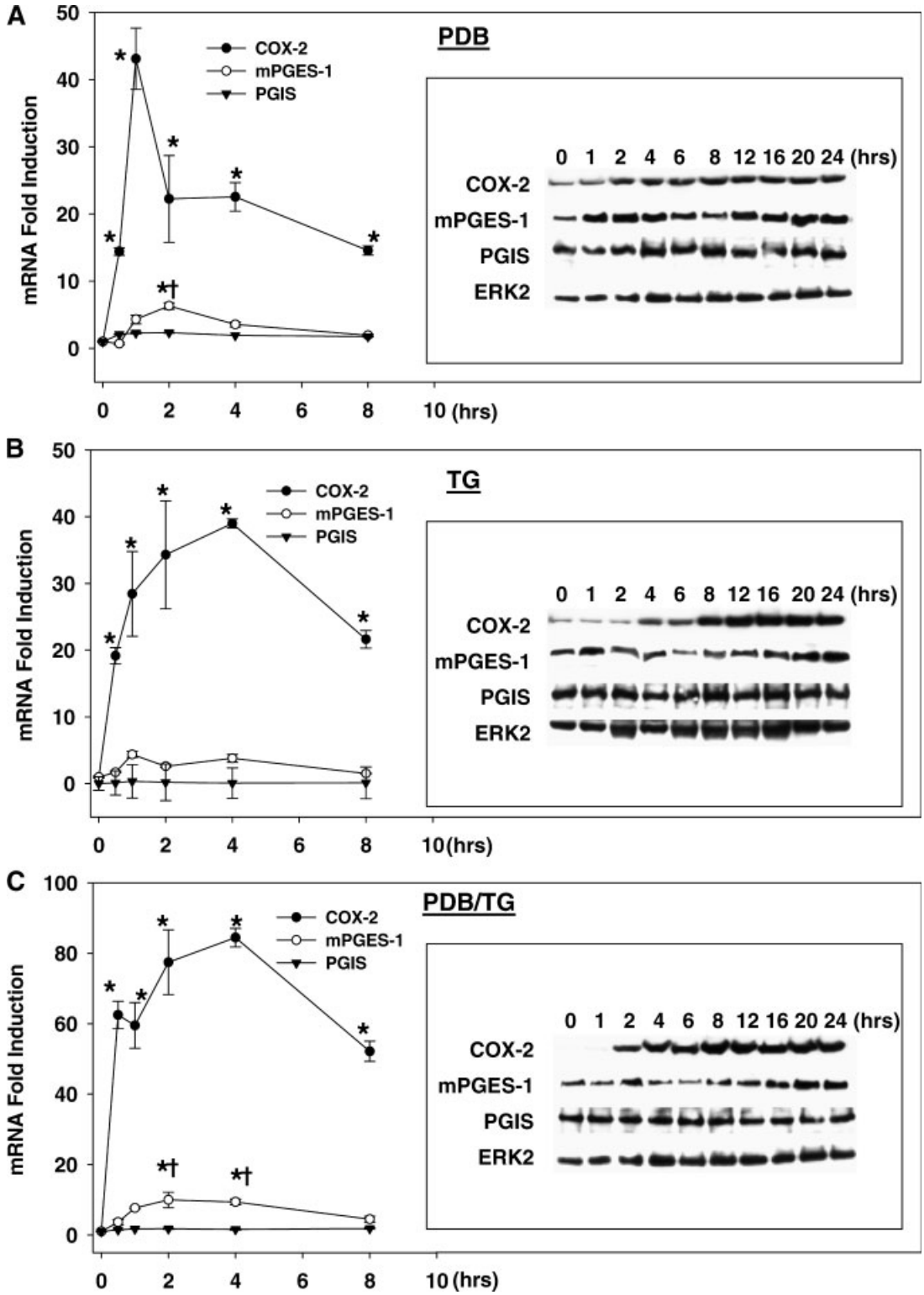


**Fig. 2.** Time-dependent induction of COX-2 and mPGES-1 mRNA and protein expression by PDB, TG, and PDB/TG. Confluent IEC-18 cells serum starved for 24 h were incubated at the indicated times with (**A**) 100 nM of PDB, (**B**) 100 nM of TG, or (**C**) 100 nM of both PDB/TG. Total RNA was purified and the relative expression levels of mRNAs for COX-2 (●), mPGES-1 (○), and PGIS (▼) were quantified by real-time RT-PCR. The data are expressed as fold induction above time 0 and the asterisk (*) indicates a statistical significance of $P < 0.05$ for COX-2. The asterisk and dagger (*†) indicates a statistical significance of $P < 0.05$ for mPGES-1. Inset: Total cellular protein extract from stimulated cells was examined by Western blot. Antibodies specific for COX-2 (72 kDa), mPGES-1 (16 kDa), and PGIS (56 kDa) were used. Protein loading was standardized using an anti-ERK 2 antibody (42 kDa).

produced a biphasic increase in COX-2 mRNA ($62.5 \pm 3.9$-fold, 30 min) followed by a slight decline with a second maximum of $84.5 \pm 2.6$-fold by 4 h. By 8 h, COX-2 mRNA levels were $52.2 \pm 2.9$-fold above basal levels. COX-2 protein levels were significantly induced 2 h after treatment with PDB/TG with maximal induction occurring after 8 h.

Co-treatment with PDB/TG resulted in an additive increase in the level of induced mPGES-1 mRNA (Fig. 2C). The maximal level was $10.0 \pm 1.2$-fold at 2 h followed by a gradual decrease by 8 h. The level of mPGES-1 protein showed a biphasic response to PDB/TG with the first maximum after 2 h, a decrease by 6 h followed by a second maximal increase in protein levels after 20 h.

Stimulation of cells with PDB, TG, or PDB and TG did not induce PGIS mRNA or protein in IEC-18 cells. Additionally, COX-1 mRNA expression levels did not significantly change in cells treated with PDB/TG (data not shown).

To determine that the concentrations of PDB and TG (100 nM) were sufficient to elicit maximal expression of COX-2 and mPGES-1, serum-starved IEC-18 cells were treated with increasing doses (Fig. 3). Induction of COX-2 mRNA was minimal at low concentrations and reached a maximum between 50 and 100 nM of PDB, TG, and PDB/TG. Induction levels of mPGES-1 mRNA were minimal at low concentrations of agonists and reached a maximum at 100 nM of PDB, TG, and PDB/TG. The combined effect of PDB/TG did not result in an additive induction of mPGES-1 mRNA.

In order to confirm that TG-dependent $Ca^{2+}$ signaling is mediating the induction of COX-2 and mPGES-1 mRNA expression, IEC-18 cells were treated with the calcium ionophore, A23187. Cells treated with A23187 with and without PDB showed a similar pattern of both time- and dose-dependent expression of COX-2 and mPGES-1 mRNA compared to cells that were treated with TG/PDB (data not shown).

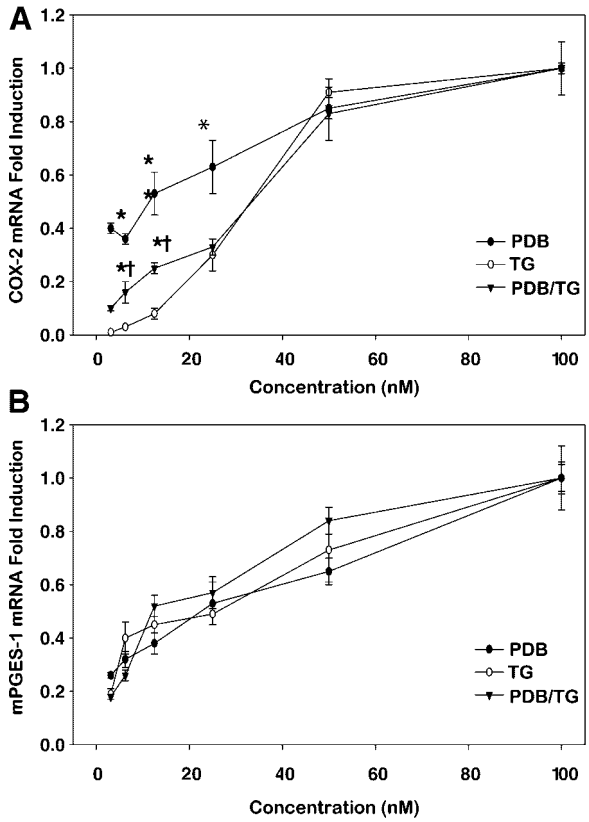


**Fig. 3.** Concentration-dependent induction of COX-2 and mPGES-1 mRNA by PDB, TG, and PDB/TG. Confluent IEC-18 cells serum starved for 24 h were incubated with 3.125, 6.25, 12.5, 25, 50, or 100 nM of PDB (●), TG (○), or PDB/TG (▼) for 1 h. Total RNA were isolated with TRIzol and quantified by real-time RT-PCR for (**A**) COX-2 and (**B**) mPGES-1 with GAPDH mRNA as the internal standard. The data are expressed as fold induction above time 0 and the asterisk (*) indicates a statistical significance of $P < 0.05$. The asterisk and dagger (*†) indicate a statistical significance of $P < 0.05$ between PDB/TG and PDB or TG.

### Phosphorylation of ERK and CREB in Cells Treated With PDB and TG

Prior studies have shown that PDB stimulation of IEC-18 cells results in PKC-dependent activation of ERK [Chiu and Rozengurt, 2001]. Our studies have shown COX-2 induction by MAP kinases and CREB in IEC-18 cells [Shafer and Slice, 2005] but the effects of PDB and/or TG on CREB activation is not known. Therefore, we investigated specific intracellular mediators that are activated in IEC-18 cells by PDB and/or TG. Addition of PDB to serum-starved cells resulted in maximal presence of activated JNK and CREB at 5 min followed by reduction to basal level at 60 min (Fig. 4A). Activation of JNK and CREB was not responsive to the addition of TG (Fig. 4B) whereas PDB/TG combination activated JNK and CREB at 5 min followed by a decrease to basal level at 60 min (Fig. 4C), indicating that PDB was the stimuli that activated JNK and CREB. Maximal activation of ERK1/2 occurred at 5 min and decreased to basal level by 10 to 60 min with PDB. TG activation of ERK1/2 occurred at 10 and 20 min without subsequent activation at 30 or 60 min.

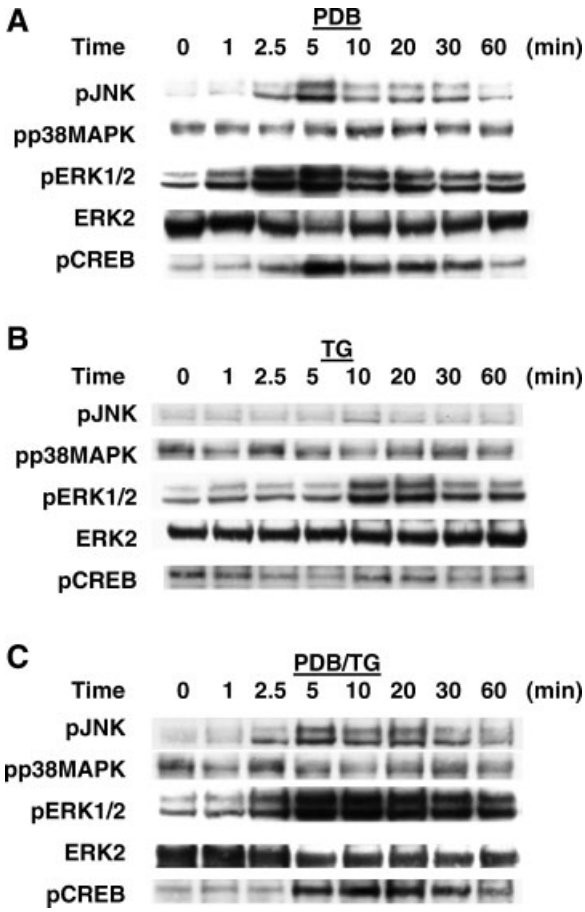


**Fig. 4.** Effects of PDB and/or TG on JNK, ERK1/2, CREB, and p38MAPK. Serum-starved confluent IEC-18 cells were stimulated with either (**A**) 100 nM of PDB, (**B**) 100 nM of TG, or (**C**) 100 nM of PDB/TG for 0, 1, 2.5, 5, 10, 20, 30, and 60 min. Total cellular protein extracted in loading buffer were subjected to SDS–PAGE, Western blotting, and probed for phosphorylated JNK, p38MAPK, ERK1/2, and CREB. Protein loading was standardized using an anti-ERK 2 antibody. This is representative of three independent experiments.

The combination of PDB/TG activated ERK1/2 at 5 min and remained activated at 60 min. The level of phosphorylation of p38MAPK did not significantly change after addition of PDB, TG, or PDB/TG.

Next, we used selective inhibitors to PKC and $Ca^{2+}$ signaling pathways to identify the intracellular mediators involved in COX-2 and mPGES-1 mRNA expression. IEC-18 cells treated with cells with the selective PKC inhibitor, bisindolylmaleimide (GFX), or the selective MEK inhibitor, PD98059, showed a significant decrease in PDB/TG induced COX-2 and mPGES-1 mRNA (Fig. 5A,B). Activation of PKC by PDB in IEC-18 cells leads to MEK-dependent ERK phosphorylation [Chiu and Rozengurt, 2001], suggesting that inhibition of COX-2 and mPGES-1 expression by GFX and PD98059 could be through PKC/MEK/ERK signaling.

TG-induced $Ca^{2+}$ signaling has been shown to modulate PKC activity in intestinal epithelia [Thastrup et al., 1990]. We treated IEC-18 cells with 2-APB, which antagonizes $IP_3$ receptors and blocks the influx of $Ca^{2+}$ from channels. This resulted in a significant decrease in PDB/TG-dependent COX-2 and mPGES-1 mRNA expression (Fig. 5A,B).

To test the specificity of these inhibitors to block targeted signaling events, IEC-18 cells were pre-treated with GFX, PD98059, or 2-APB, prior to stimulation with PDB and/or TG for 5 min. GFX, PD98059, and 2-APB had no effect on PDB and/or TG activation of JNK (Fig. 5C); however, GFX, PD98059, and 2-APB, significantly suppressed PDB and/or TG activation of CREB (Fig. 5D). Pre-treatment with 2-APB had no effect on PDB and/or TG activation of ERK1/2 (Fig. 5E) whereas GFX suppressed ERK1/2 activation. The presence of PD98059 suppressed ERK1/2 activation by PDB but not by TG or the combination of PDB/TG. COX-2 and mPGES-1 expression by PDB and/or TG does not appear to be mediated by JNK because treatment with the selective JNK inhibitior (SP600125, 2–20 μM) did not inhibit induction of COX-2 or mPGES-1 (data not shown). These findings suggest that signaling by PKC, ERK1/2 and CREB, but not JNK, play a major role in COX-2 and mPGES-1 expression by PDB and TG.

### COX-2 and mPGES-1 mRNA Stability and Promoter Activity

The mRNA expression levels of COX-2 and mPGES-1 are affected by gene transcription rates and mRNA stability. We measured the half-life of COX-2 and mPGES-1 mRNAs in IEC-18 cells using actinomycin D. The COX-2 mRNA decay rate for TG was $\tau_{1/2} = 29.3$ min whereas decay rates for PDB and PDB/TG were $\tau_{1/2} = 36.4$ min and $\tau_{1/2} = 42.9$ min, respectively. The decay rate for mPGES-1 mRNA with TG was $\tau_{1/2} = 7.6$ min compared to $\tau_{1/2} = 23.2$ min and $\tau_{1/2} = 13.8$ min for cells that were stimulated with PDB and PDB/TG, respectively. Post-transcriptional stability of mPGES-1 mRNA was significantly reduced under all conditions compared to COX-2 mRNA.

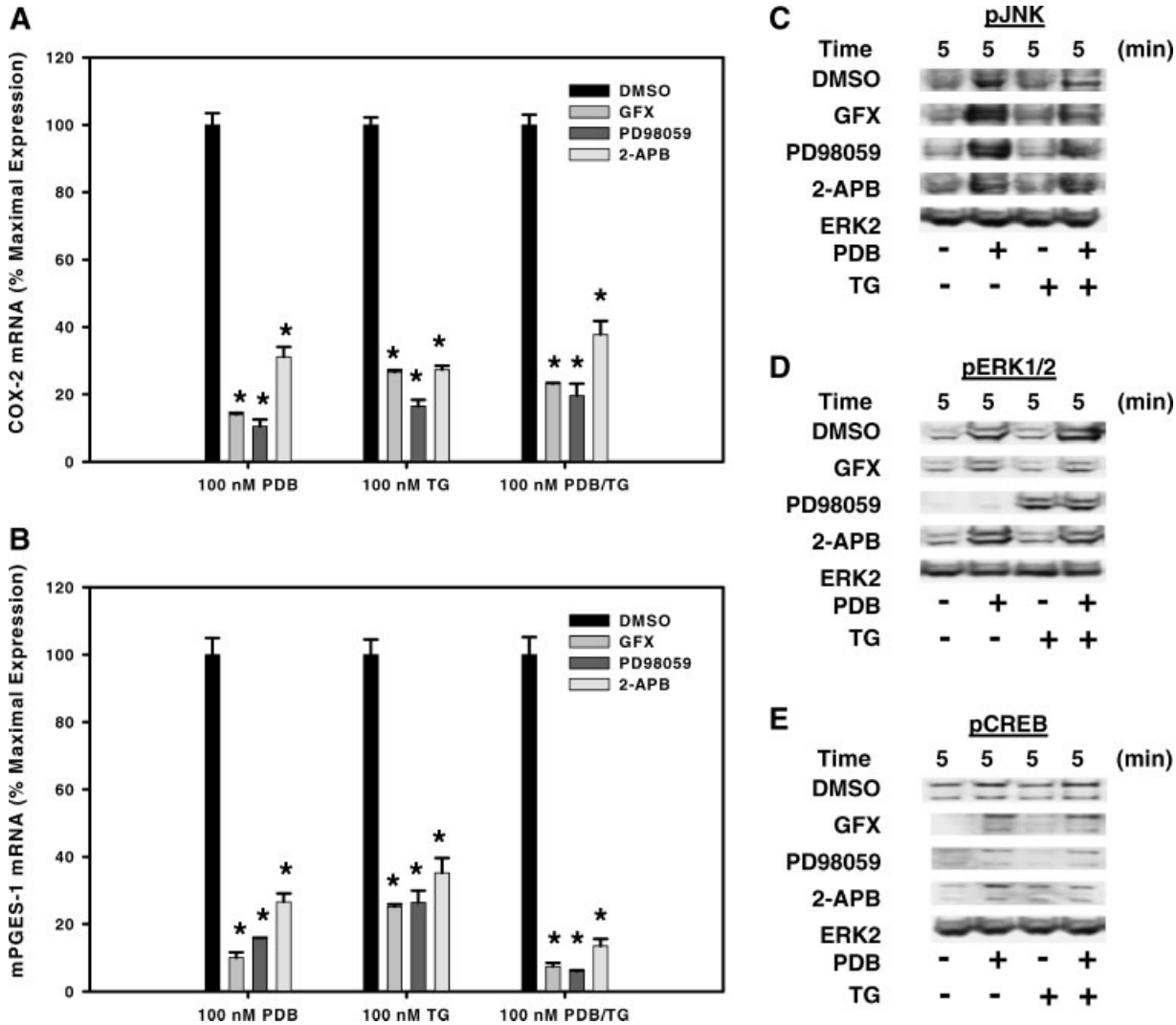


**Fig. 5.** Attenuation of COX-2 and mPGES-1 mRNA induction by inhibitors to PKC, MEK/ERK, and $Ca^{2+}$ signaling. Serum-starved confluent IEC-18 cells were pre-incubated with 1 μl of DMSO (vehicle), 3.5 μM of GFX, 10 μM of PD98059, and 75 μM of 2-APB for 1 h followed with the addition of 100 nM of PDB, 100 nM of TG, or 100 nM of PDB/TG for 1 h. Total RNA were isolated using TRIzol and (**A**) COX-2 and (**B**) mPGES-1 mRNA were quantified by real-time RT-PCR. The data are expressed as fold induction above control ± SEM with the asterisk (*) indicating a statistical significance of $P < 0.05$. Serum-starved confluent IEC-18 cells were pre-treated with 1 μl of DMSO, 3.5 μM of GFX, 10 μM of PD98059, or 75 μM of 2-APB followed by stimulation with either 100 nM of PDB, 100 nM of TG, or 100 nM of PDB/TG for 5 min. Total protein extracted in loading buffer were subjected to SDS–PAGE, Western blotting and probed for phosphorylated (**C**) JNK, (**D**) ERK1/2, and (**E**) CREB. Protein loading was standardized using antibody to ERK2.

The potential for activation of COX-2 and mPGES-1 promoters by PDB and/or TG was measured by transient transfection of IEC-18 cells using luciferase reporter plasmids containing either the COX-2 (−371 to +70) or mPGES-1 (−500 to +1) promoters. Treatment with PDB and PDB/TG resulted in a three- and a fourfold increase in both COX-2 and mPGES-1 promoter activities, respectively (Fig. 6A). TG did not significantly induce either the COX-2 or the mPGES-1 promoters (data not shown). Inhibition of PKC activity by GFX, MEK/ERK signaling by PD98095 and calcium signaling by 2-APB attenuated both COX-2 and mPGES-1 promoter activation by PDB and PDB/TG.

Early studies have shown Ras- and ERK-dependent transcriptional activation of the COX-2 promoter through CREB binding to the CRE/ATF *cis*-acting element [Xie et al., 1994; Xie and Herschman, 1995]. Since we have demonstrated CREB phosphorylation in response to PDB and the combination of PDB/TG in

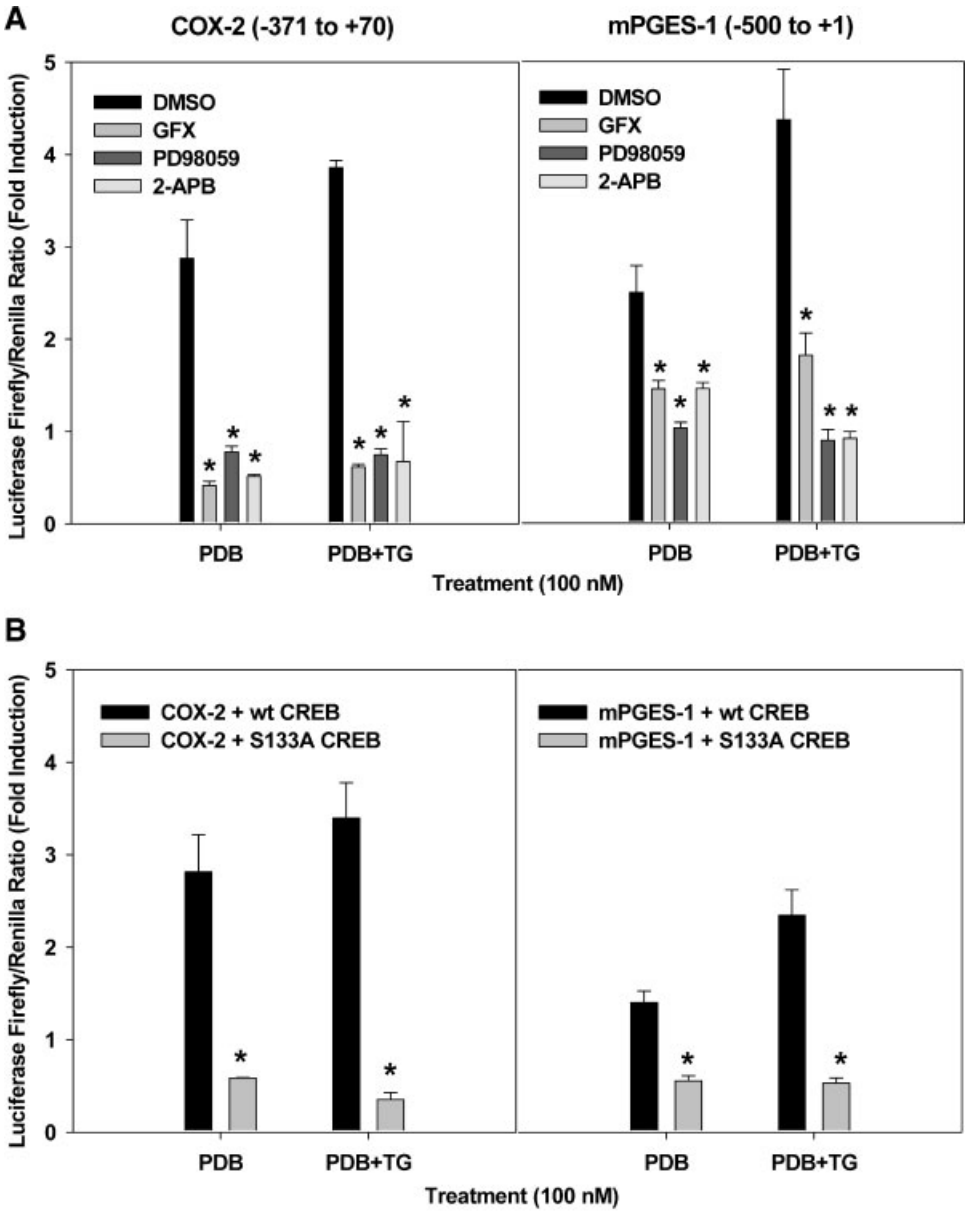


**Fig. 6.** Transcriptional activation of COX-2 and mPGES-1 promoter by PDB and PDB/TG. IEC-18 cells were transiently transfected with (**A**) COX-2 (−371 to +70) promoter- or mPGES-1 (−500 to +1) promoter-luciferase plasmids and grown to confluency. The confluent cells were serum starved overnight and pre-incubated with 1 μl of DMSO, 3.5 μM of GFX, 10 μM of PD98059, or 75 μM of 2-APB for 1 h. Following pre-treatment, the transfected cells were stimulated with 100 nM of PDB or 100 nM of PDB/TG for 5 h and luciferase expression relative to Renilla luciferase expression (marker of transfection efficiency) were measured. **B**: The COX-2 promoter and mPGES-1 promoter were coupled with either wild type CREB or S133A CREB and transiently transfected into IEC-18 cells and grown to confluency prior to stimulation with either 100 nM of PDB or 100 nM of PDB/TG. Data are expressed as fold induction ± SEM from triplicate experiments. The asterisk (*) indicates a statistical significance of $P < 0.05$.

IEC-18 cells, we wanted to determine the role of CREB activation in COX-2 and mPGES-1 expression. IEC-18 cells were transiently transfected with COX-2 or mPGES-1 promoter vectors and either expression vectors for wild type CREB or a dominant negative mutant S133A CREB [Mayr and Montminy, 2001]. Addition of PDB or PDB/TG increased COX-2 and mPGES-1 promoter activity in cells that were expressing wild type CREB whereas COX-2 and mPGES-1 promoter activity were significantly inhibited in cells that were co-transfected

with the S133A CREB expression vector (Fig. 6B). This result demonstrates the involvement of CREB in the activation of COX-2 and mPGES-1 promoters by PDB and TG.

### CREB siRNA Suppression of COX-2 and mPGES-1 mRNA Expression

To confirm that CREB has a role in PDB, TG, and PDB/TG-dependent expression of COX-2 and mPGES-1 mRNA, the cells were transfected with either CREB-specific or random sequence siRNA. After 72 h, confluent cells were serum starved overnight and treated with 100 nM of PDB, TG, or PDB/TG. Inhibition of CREB expression blocked COX-2 and mPGES-1 expression by PDB and/or TG (Fig. 7A). Cells pre-treated with non-specific siRNA did not suppress the induction of COX-2 or mPGES-1 mRNA expression by PDB, TG, and PDB/TG. Cells treated with specific siRNA targeting CREB did show reduced levels of CREB protein expression (Fig. 7B).

Electrophoresis mobility shift assays confirmed that CREB interacts with the COX-2 promoter. Multiple shifted bands are apparent with nuclear extracts irrespective of treatment with PDB/TG (Fig. 7C). Competition binding with unlabeled CRE dsDNA eliminated a specific band (indicated by an arrow). Incubation of the nuclear extract with an antibody to CREB showed a shift of the same band that was not present in the lane corresponding to nuclear extract incubated with control antibody. This demonstrates that CREB directly interacts with a specific site on the COX-2 promoter.

## DISCUSSION

Previously, we have shown Ang II-dependent induction of COX-2 expression in non-transformed intestinal epithelial cells (IEC-18) that resulted in the production of $PGI_2$ [Slice et al., 2005]. Agonist binding to the $AT_1R$ activated PKC, and $Ca^{2+}$ signaling via Phospholipase Cβ in addition to p38MAPK through a small GTPase-dependent mechanism. Ang II-induced $PGI_2$ production and COX-2 expression were dependent on $Ca^{2+}$ signaling and p38MAPK but were not dependent on either PKC or ERK. In order to further elucidate the role of post $AT_1R$ signaling events in COX-2 expression and PG production, we used PDB and TG as activators of PKC and $Ca^{2+}$ signaling, respectively. Treatment of IEC-18 cells with PDB and/or TG resulted in a rapid induction of COX-2 mRNA without de novo protein synthesis. Surprisingly, the expected production of $PGI_2$ did not occur, but rather, $PGE_2$ was produced. The increase in $PGE_2$ production was COX-2-dependent and correlated with induction of mPGES-1 mRNA and protein. We were not able to detect any differences in the expression levels of PGIS mRNA or protein or COX-1 mRNA in cells treated with PDB and/or TG. Eventhough PKC activation and $Ca^{2+}$ signaling result from agonist binding to the $AT_1R$, our demonstration that different PGs are produced by Ang II in IEC-18 cells compared to stimulation with PDB and/or TG indicates that other signaling pathways play a significant role in PG production. One possibility could include activation of signaling pathways that are unique to the prolonged cellular effects of treating cells with PDB and/or TG. Alternatively, the $AT_1R$ activates parallel signaling pathways (i.e., p38MAPK) through other G-proteins that modulate PG synthase expression and thus change the type of induced PG. $PGI_2$ induction by Ang II correlates with activation of p38MAPK activation, whereas PDB and/or TG did not activate p38MAPK.

Studies have reported the coordinated expression of COX-2 and mPGES-1 in macrophages and osteoblasts induced with lipopolysaccharide [Murakami et al., 2000]. Induction of COX-2 and mPGES-1 by PDB and/or TG could also represent coordinated expression in IEC-18 cells resulting in $PGE_2$ production. Both COX-2 and mPGES-1 mRNAs are induced by PDB or TG with maximal expression in cells treated with both PDB and TG. This correlated with $PGE_2$ production. PDB and PDB/TG induction of COX-2 and mPGES-1 was dependent on PKC activity, MEK-ERK signaling and $Ca^{2+}$ signaling. PDB stimulated phosphorylation of ERK1/2, JNK, and CREB with TG co-stimulation significantly enhancing ERK1/2 phosphorylation. Selective inhibitors to PKC (GFX), MEK (PD98059), and $Ca^{2+}$ (2-APB) signaling blocked both expression of agonist-induced COX-2 and mPGES-1 mRNAs and agonist-induced transcription from COX-2 and mPGES-1 promoters as demonstrated by the luciferase reporter assays. COX-2 and mPGES-1 induction by PDB and/or TG was dependent on CREB. Overexpression of a dominant negative CREB mutant (S133A) blocked PDB and PDB/TG activation of COX-2 and mPGES-1 promoters

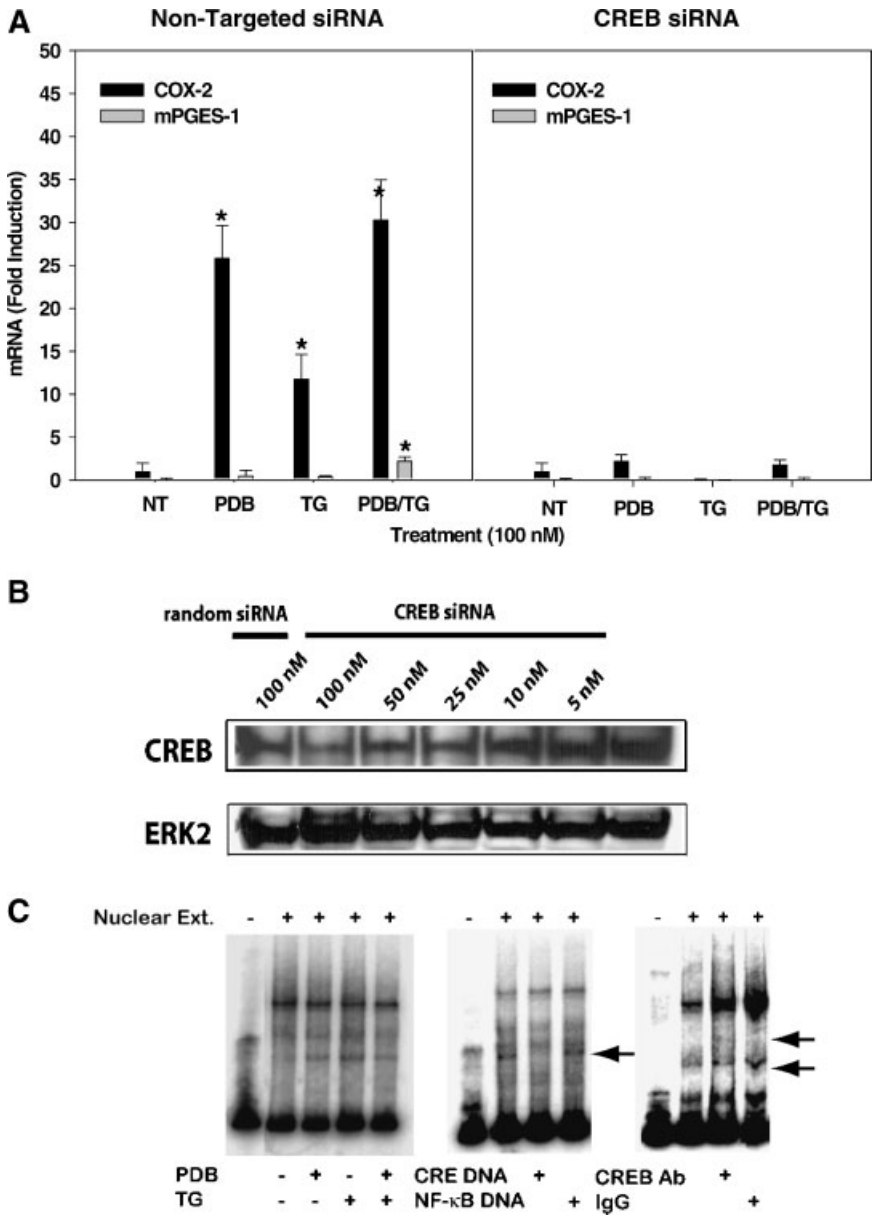


**Fig. 7.** CREB-dependent expression of COX-2 and mPGES-1 mRNA. IEC-18 cells were transfected with (**A**) 100 nM of non-targeting siRNA, or 100 nM of CREB siRNA by electroporation. The transfected cells were grown to confluency and serum starved prior to stimulation with 100 nM of PDB, TG, or PDB/TG for 1 h. Total RNA was purified and COX-2 and mPGES-1 mRNA and quantified by real-time RT-PCR. The data are expressed as fold induction above control ± SEM with the asterisk (*) indicating a statistical significance of $P < 0.05$. **B**: IEC-18 cells were transfected with CREB siRNA or non-targeted siRNA. Western blot analysis was used to measure the level of CREB in the total cellular proteins. **C**: EMSA using radiolabeled COX-2 promoter (−200 to +1) and nuclear extract proteins from IEC-18 cells that were either serum starved or treated with PDB, TG, or both (**left panel**). Unlabeled double stranded oligonucleotides containing consensus sequences to CRE or NF-κB was used for competition binding with nuclear extract proteins from PDB/TG-treated IEC-18 cells (**middle panel**). CREB-specific antibody was used with nuclear extract from PDB/TG-treated IEC-18 cells to detect a shift of the CRE-specific band (**right panel**).

in IEC-18 cells. Inhibition of CREB expression using CREB-specific siRNA blocked PDB, TG, and PDB/TG-induced COX-2 and mPGES-1 expression in IEC-18 cells. Lastly, the CREB transcription factor from IEC-18 cells binds to a specific site on the COX-2 promoter. These results indicate that in IEC-18 cells, PDB activates PKC, which leads to MEK-dependent ERK phosphorylation. CREB is phosphorylated which mediates transcriptional activation of

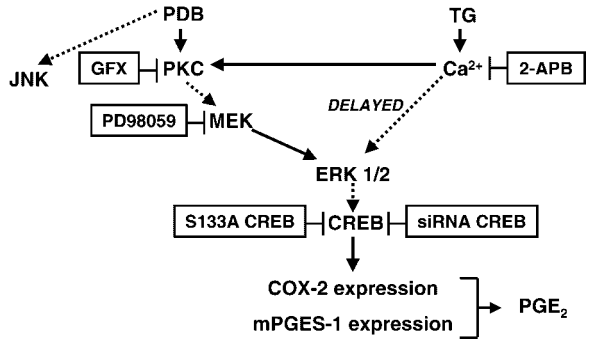


**Fig. 8.** PDB/TG induction of COX-2 and mPGES-1 expression. This scheme summarizes the generation of $PGE_2$ via the induction of COX-2 and mPGES-1 expression by PDB and TG through CREB. PDB initiates the activation of PKC leading to the activation of MEK and ERK1/2 with the subsequent activation of CREB. Blocking PKC activation with (⊥) GFX or MEK activation with PD98059 abolishes ERK1/2 and CREB activation resulting in the suppression of COX-2 and mPGES-1 expression. PDB also activates JNK but it is unclear how JNK activates CREB leading to the transcriptional activation of COX-2 and mPGES-1 mRNA and protein expression. TG activates the $Ca^{2+}$ signaling cascade that exhibits a delayed activation of ERK1/2 with the subsequent activation of CREB leading to COX-2 and mPGES-1 mRNA and protein expression. Inhibiting $Ca^{2+}$ influx with 2-APB suppresses COX-2 and mPGES-1 expression. The dominant negative mutant S133A CREB abolishes COX-2 and mPGES-1 transcriptional activation by PKC and $Ca^{2+}$ signaling. The *dashed arrows* imply the existence of intermediary steps leading to the subsequent event whereas the *solid arrows* represent direct consequences of the previous effector.

COX-2 and mPGES-1 promoters. $PGE_2$ is produced by the concerted actions of COX-2 and mPGES-1 on Arachidonic acid (Fig. 8). TG stimulates $Ca^{2+}$ signaling and delayed ERK phosphorylation. The combination of PDB and TG act synergistically to prolong ERK and CREB phosphorylation resulting in enhanced COX-2 and mPGES-1 expression. It appears that JNK signaling does not play a significant role in PDB and/or TG induction of COX-2 and mPGES-1 because the JNK selective inhibitor (SP600125) did not block induction of COX-2 or mPGES-1. These results and our previous studies demonstrating that Ang II-dependent COX-2 expression in IEC-18 cells is mediated by CREB via p38MAPK [Shafer and Slice, 2005], indicate that multiple kinase cascades converge onto CREB. This suggests that CREB could be a linchpin in COX-2-dependent PG production in these cells. Of interest, is the recent focus on the association between CREB expression and cancer [Conkright and Montminy, 2005; Shankar et al., 2005].

COX-2-derived prostanoids are key mediators of acute inflammatory responses [Portanova et al., 1996], arthritis [Anderson et al., 1996], and IBD [Gould et al., 1981]. The excessive generation of $PGE_2$ through the COX pathway contributes to the development and progression of colorectal carcinogenesis [Williams et al., 1999]. The overexpression of mPGES-1 has been reported in colorectal carcinogenesis and is functionally linked to COX-2 [Yoshimatsu et al., 2001]. Conversely, $PGI_2$ and overexpression of PGIS has been associated with antiproliferative properties [Hara et al., 1995]. This suggests that a change in the type of PG produced by epithelial cells from $PGI_2$ to $PGE_2$ could strongly promote carcinogenesis. Our results demonstrating a change from $PGI_2$ to $PGE_2$ from IEC-18 cells through MEK/ERK-dependent COX-2 and mPGES-1 expression could represent one mechanism used by Ras mutations in promoting colorectal carcinogenesis. Supporting this hypothesis is a recent report showing the oncogenic potential of MEK1 in rat intestinal epithelial cells that was mediated by induction of COX-2 [Komatsu et al., 2005]. This study showed activation of ERK signaling by expression of active MEK induced the production of $PGE_2$ compared to $PGI_2$. We are currently studying the role of k-Ras expression in epithelial cells in the context of CREB-dependent COX-2 and PG synthase expression and its effects on PG production and carcinogenesis.

## ACKNOWLEDGMENTS

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