# 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ inhibits the expression of microsomal prostaglandin E synthase type 2 in colon cancer cells

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**Abstract Prostaglandin (PG) $E_2$ ($PGE_2$) plays a predominant role in promoting colorectal carcinogenesis. The biosynthesis of $PGE_2$ is accomplished by conversion of the cyclooxygenase (COX) product $PGH_2$ by several terminal prostaglandin E synthases (PGES). Among the known PGES isoforms, microsomal PGES type 1 (mPGES-1) and type 2 (mPGES-2) were found to be overexpressed in colorectal cancer (CRC); however, the role and regulation of these enzymes in this malignancy are not yet fully understood. Here, we report that the cyclopentenone prostaglandins (CyPGs) 15-deoxy-$\Delta^{12,14}$-$PGJ_2$ and $PGA_2$ downregulate mPGES-2 expression in the colorectal carcinoma cell lines Caco-2 and HCT 116 without affecting the expression of any other PGES or COX. Inhibition of mPGES-2 was subsequently followed by decreased microsomal PGES activity. These effects were mediated via modulation of the cellular thiol-disulfide redox status but did not involve activation of the peroxisome proliferator-activated receptor γ or $PGD_2$ receptors. CyPGs had antiproliferative properties in vitro; however, this biological activity could not be directly attributed to decreased PGES activity because it could not be reversed by adding $PGE_2$. Our data suggest that there is a feedback mechanism between $PGE_2$ and CyPGs that implicates mPGES-2 as a new potential target for pharmacological intervention in CRC.**—Schröder, O., Y. Yudina, A. Sabirsh, N. Zahn, J. Z. Haeggström, and J. Stein. **15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ inhibits the expression of microsomal prostaglandin E synthase type 2 in colon cancer cells.** ***J. Lipid Res.*** **2006.** 47: **1071–1080.**

**Supplementary key words** colorectal cancer • cyclopentenone prostaglandins • feedback control • proliferation • prostaglandin $E_2$ • redox status

Population-based studies have demonstrated that long-term use of nonsteroidal antiinflammatory drugs (NSAIDs) reduces the relative risk of developing colorectal cancer (CRC) by 40–50% (1). In addition, increased levels of cyclooxygenase type 2 (COX-2) are found in colorectal adenomas and adenocarcinomas compared with normal mucosa (2, 3). NSAIDs inhibit the enzymatic activity of both isoforms of COX (COX-1 and COX-2). Because prolonged use of NSAIDs is associated with considerable side effects, which are believed to arise from the inhibition of the constitutively expressed COX-1 (4), selective COX-2 inhibitors were developed. Indeed, this new class of drugs retains the anti-inflammatory activity and antitumoral effects of the NSAIDs while reducing gastrointestinal toxicity by up to 50% (5). Unfortunately, prolonged use of higher doses of selective COX-2 inhibitors was recently shown to be associated with an increase in adverse cardiovascular events, resulting in the withdrawal of rofecoxib and valdecoxib.

Prostaglandins are formed from a common unstable endoperoxide intermediate, prostaglandin (PG) $H_2$ ($PGH_2$), which in turn is generated via enzymatic oxygenation of arachidonic acid (AA) catalyzed by COX-1 or COX-2. Among the various downstream metabolites of COX-2-derived $PGH_2$, $PGE_2$ and its receptors have been demonstrated to play a predominant role in the promotion of colorectal carcinogenesis. This prostaglandin, which is found at increased levels in human colorectal adenomas and cancer (2, 6), promotes a multitude of biologic actions related to colorectal carcinogenesis. $PGE_2$ facilitates tumor progression by stimulation of cellular proliferation and angiogenesis, inhibition of apoptosis, and modulation of immunosuppression (for

Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; cPGES, cytosolic prostaglandin E synthase; CRC, colorectal cancer; CyPG, cyclopentenone prostaglandin; DP, prostaglandin $D_2$ receptor; 15d-$PGJ_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$; EP, prostaglandin $E_2$ receptor; mPGES, microsomal prostaglandin E synthase; NSAID, nonsteroidal antiinflammatory drug; PGES, prostaglandin E synthase; PPARγ, peroxisome proliferator-activated receptor γ.

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*Manuscript received 9 January 2006 and in revised form 21 February 2006.*
*Published, JLR Papers in Press, March 2, 2006.*
*DOI 10.1194/jlr.M600008-JLR200*

review, see 7). Treatment of rodent models with $PGE_2$ has demonstrated increased cell proliferation and enhanced survival of epithelial cells in the gastrointestinal tract (8–10). Together, these findings suggest that the selective pharmacological inhibition of $PGE_2$ production downstream of COX-2 is the best treatment for inhibiting carcinogenesis and that this may result in fewer side effects. In addition to prostaglandin $E_2$ receptors (EPs), one such target for pharmacological intervention comprises the group of terminal prostaglandin E synthases (PGES). To date, three PGES isoforms have been identified, two perinuclear membrane-bound enzymes termed microsomal prostaglandin E synthase-1 (mPGES-1) and mPGES-2 and a cytosolic isoform [cytosolic prostaglandin E synthase (cPGES)]. Both microsomal proteins have been found to be overexpressed in human colorectal adenoma and cancer (11, 12), emphasizing their importance as drug targets. In contrast, there are no data suggesting that cPGES contributes to colorectal carcinogenesis.

Unlike $PGE_2$, which has been demonstrated to definitely contribute to the promotion and survival of CRC, cyclopentenone prostaglandins (CyPGs), especially 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$), are emerging as potent antitumor agents. In a variety of malignancies including CRC, 15d-$PGJ_2$ displays growth-inhibitory and proapoptotic actions (13, 14). 15d-$PGJ_2$ is a CyPG of the $J_2$ series derived from $PGD_2$. Interestingly, the dependence of $PGJ_2$ synthesis on $PGD_2$ production suggests that the formation of $PGJ_2$ adducts is delayed relative to the synthesis of other prostaglandins and that 15d-$PGJ_2$ may participate in the resolution of $PGE_2$-mediated inflammation (15). In addition, 15d-$PGJ_2$ itself has been shown to regulate COX-2 expression via both peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$)-dependent and -independent mechanisms (16, 17). Finally, in vitro studies have demonstrated that 15d-$PGJ_2$ can bind to mPGES-1, resulting in decreased PGES activity (18).

Both $PGE_2$ and 15d-$PGJ_2$ are derived from the same precursor by the action of COX, but they have opposing effects on inflammatory processes and tumorigenesis, so some additional means to regulate the production of these prostaglandins relative to each other must exist. Primary candidates are the PGES. It can be hypothesized that new insights into the regulation of these enzymes may result in novel approaches for the treatment and perhaps also the prevention of cancer. In this study, therefore, we aimed to elucidate the regulatory mechanisms of 15d-$PGJ_2$ on terminal PGES, in particular mPGES-1 and mPGES-2, in CRC.

## MATERIALS AND METHODS

### Materials

15d-$PGJ_2$ was purchased from Alexis Co. (Carlsbad, CA). All other prostaglandins and MCC555 were obtained from Cayman Chemical Co. (Ann Arbor, MI). All other reagents were from Sigma (Deisenhofen, Germany) and were of the highest analytical grade. Cell culture media and supplements were purchased from Gibco BRL (Lofer, Austria). Oligonucleotides were from Biospring (Frankfurt, Germany). RT-PCR reagents were obtained from Applied Biosystems (Branchburg, NJ). Secondary horseradish peroxidase-conjugated antibodies were from Vector Laboratories (Burlingame, CA), and the chemiluminescence reagent (ECL) and Hyperfilm-MP were from Amersham Pharmacia Biotech (Buckinghamshire, UK).

### Cell culture

Human colon cancer cell lines (Caco-2 and HCT 116) were obtained from the European Animal Cell Culture Collection. PPAR$\gamma$ dominant-negative mutant Caco-2 cells were kindly provided by V. K. Chatterjee (Department of Medicine, University of Cambridge, Cambridge, UK). Construction of plasmids as well as transfection of cells were performed as described previously (19). Before experiments, diminished transcriptional activity of the mutant was routinely checked by comparing the transactivation of wild-type and transfected cells to increasing doses of the PPAR$\gamma$ ligand BRL49653. All cell lines were maintained in DMEM containing 4.5 g/l glucose and 25 mM HEPES supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% nonessential amino acids, and 1% pyruvate. The medium was changed every second day. Cells were checked for *Mycoplasma* infection at monthly intervals.

To investigate the effect of prostaglandins and MCC555 on mPGES-2 expression in colon cancer cells, $2.5 \times 10^3$ cells/100 $\mu$l were seeded and incubated at 37°C under 6% $CO_2$ and 94% air until the cells were ~50% confluent. The new medium containing the respective ligand or solvent vehicle was then added, and cells were incubated for the indicated periods of time.

### Cellular proliferation

Cellular proliferation/survival was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) colorimetric method (Roche, Mannheim, Germany) according to the manufacturer's instructions. In brief, after defined periods of culture, cells grown in DMEM were harvested using a 10% trypsin-PBS solution and resuspended in DMEM. Cell suspensions ($2.5 \times 10^3$ cells/100 $\mu$l) were plated onto 96-well plates in DMEM with 15d-$PGJ_2$ (10 $\mu$M), $PGE_2$ (1 nM to 1 mM), or combinations of 15-$PGJ_2$ (10 $\mu$M) and various concentrations of $PGE_2$ as indicated. The plates were incubated for 12, 24, 48, or 72 h at 37°C before the addition of MTT solution, which was followed by spectrophotometric measurement of absorbance at 570 nm. Changes in cell number were deduced from the absorbance data using the linear part of standard absorbance curves produced with predetermined cell numbers.

### Cytotoxicity

Cytotoxicity was excluded using a lactate dehydrogenase release assay (LDH kit; Roche).

### Preparation of cellular fractions

Cultured cells were washed with PBS and trypsinated in 1× trypsin/EDTA for 10 min at 37°C. Thereafter, culture medium was added and cells were centrifuged at 500 *g* for 10 min, followed by two additional washing steps in PBS. The cell pellets were then resuspended in 1 ml of homogenization buffer consisting of potassium phosphate buffer (0.1 M, pH 7.4), 1× Complete™ protease inhibitor cocktail, and sucrose (0.25 M). The samples were then sonicated for 3 × 20 s at 100 W with a ultrasonic cell disruptor (Microson™; SPI Supplies, West Chester, PA) and subjected to differential centrifugation at 1,000 *g* for 10 min, 10,000 *g* for 15 min, and 100,000 *g* for 1.5 h at 4°C. After

the last centrifugation step, microsomal fractions were resuspended in 100 μl of homogenization buffer, and total protein concentration in cytosolic and microsomal fractions was determined by the Coomassie protein assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA).

### Analysis of mRNA by semiquantitative RT-PCR

RNA isolation was conducted with RNAzol (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's protocol. Briefly, total RNA (1 μg) in water was heated (65°C, 12 min), cooled, and reverse-transcribed (20 min, 42°C) in PCR buffer [5 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates, 1 U/μl Moloney murine leukemia virus (MuLV) reverse transcriptase, 5 μM oligo d(T)16, and 0.5 U/μl RNase inhibitor]. After denaturation (1.5 min, 95°C), samples were amplified in PCR buffer (1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.2 μM primer, and 0.05 U/μl Taq polymerase) with the primers and conditions described in **Table 1** using a Perkin-Elmer/GeneAmp PCR system 2400 (Applied Biosystems, Branchburg, NJ). Aliquots of the PCR mixtures (10 μl) were analyzed by electrophoresis using a 1% agarose gel containing 0.5 μg/ml ethidium bromide. For semiquantitative analysis of amplified PCR products, the fluorescent dye Pico Green® (dsDNA Quantitation Kit; Molecular Probes/Invitrogen, Karlsruhe, Germany) was used according to the manufacturer's instructions (20).

### Immunoblot analysis

Aliquots (15 μg of protein) of samples in loading buffer were separated by SDS-PAGE on a 12% Tris-glycine, precasted, linear-gradient polyacrylamide gel and electroblotted onto nitrocellulose membranes. Transfer efficiency was visualized using Ponceau S stain (Sigma-Aldrich, St. Louis, MO). Membranes were then blocked overnight using Tris-HCl, pH 7.5, containing 100 mM NaCl, 0.1% Tween-20 (TBS-T), and 3% nonfat dry milk. After washing the membranes with TBS-T, polyclonal antiserum against mPGES-2 was added at a 1:5,000 dilution in TBS-T and incubated for 2.5 h. After three washing steps, the membranes were incubated for 2 h at 25°C with a horseradish peroxidase-linked goat anti-rabbit IgG antibody (1:5,000 dilution) in TBS-T. The washing steps were repeated, and subsequently, enhanced chemiluminescence detection was performed. SDS-PAGE immunoblots were quantitated with scanning densitometry using a Desaga CabUVIS scanner and Desaga ProViDoc software (Wiesloch, Germany).

### PGES enzyme assay

PGES enzyme activity was determined according to Thorén and Jakobsson (21). Microsomal or cytosolic fraction samples were diluted in potassium phosphate buffer (0.1 M, pH 6.5) containing 0.5 mM DTT. $PGH_2$ (4 μl) dissolved in acetone (0.28 mM) was kept in separate vials at −80°C. Before incubation, both the substrate and samples were equilibrated at 4°C for 2 min. The reaction was started by the addition of the sample (100 μl) to the tubes containing $PGH_2$ (final concentration, 10 μM) and then terminated by the addition of 400 μl of stop solution (25 mM $FeCl_2$, 50 mM citric acid), decreasing the pH to 3, giving a total concentration of 20 mM $FeCl_2$ and 40 mM citric acid. The reaction mixture was then diluted and assayed for $PGE_2$ using an enzyme immunoassay kit (Cayman Chemical Co.).

### Statistical analysis

If not stated otherwise, data are expressed as means ± SD of three independent experiments performed in duplicate. Data were analyzed by one-way ANOVA and Student's *t*-test. $P < 0.05$ was considered statistically significant.

## RESULTS

### Effects of 15d-$PGJ_2$ on mPGES-2 mRNA and protein expression in Caco-2 and HCT 116 cells

To investigate the effect of 15d-$PGJ_2$ on mRNA and protein expression of cPGES, mPGES-1, mPGES-2, COX-1, and COX-2 in CRC cell lines HCT 116 and Caco-2 were treated with increasing concentrations of 15d-$PGJ_2$. Whereas mRNA expression of cPGES, mPGES-1, COX-1 (expressed in HCT 116), and COX-2 (expressed in Caco-2) remained unchanged after treatment with this CyPG (data not shown), 15d-$PGJ_2$ downregulated mPGES-2 mRNA in both cell lines compared with unstimulated or vehicle-treated cells. As can be seen in **Fig. 1A, B**, the suppressive effect of 15d-$PGJ_2$ was time- and dose-dependent. Maximal reduction in mPGES-2 mRNA expression was observed after 4 h of treatment, which gradually leveled off to baseline levels thereafter. At 4 h, a 50% suppression of mPGES-2 mRNA was observed at ~10 μM 15d-$PGJ_2$ in Caco-2 cells and at ~5 μM 15d-$PGJ_2$ in HCT 116 cells. The downregulation of mPGES-2 mRNA expression was followed by a subsequent transient reduction of this enzyme at the protein level. Thus, at a concentration of 1 μM 15d-$PGJ_2$, the suppressive effect on protein expression in HCT 116 cells was observed after an incubation period of 12 h, whereas in the Caco-2 cell line a similar response was found after 36 h of stimulation (**Fig. 2**). When challenged with 10 μM 15d-$PGJ_2$, inhibition of

TABLE 1. Sequences of oligonucleotides and PCR conditions

| Gene | Primer Sequence | Annealing Temperature | No. of Cycles |
|---|---|---|---|
| GAPDH | Forward: 5′-GCACCGTCAAGGCTGAGAAC-3′<br>Reverse: 5′-CCACCACCCTGTTGCTGTAG-3′ | 45°C | 24 |
| Microsomal prostaglandin E synthase-1 | Forward: 5′-GCACGCTGCTGGTCATCAAGATGTA-3′<br>Reverse: 5′-CCGCTTCCCAGAGGATCTGCAGA-3′ | 49.5°C | 38 |
| Microsomal prostaglandin E synthase-2 | Forward: 5′-CCTGCAGCTGACCCTGTACCAGTA-3′<br>Reverse: 5′-CCCACTTGTCAGCAGCCTCATAGA-3′ | 51°C | 31 |
| Cytosolic prostaglandin E synthase | Forward: 5′-GCAAAGTGGTACGATCGAAGGGACTAT-3′<br>Reverse: 5′-CCCAGTCTTTCCAATTATTGAAGTCGA-3′ | 48°C | 33 |
| Cyclooxygenase-1 | Forward: 5′-GTGGGCTCCCAGGAGTACAGCTAC-3′<br>Reverse: 5′-GCAATCTGGCGAGAGAAGGCATC-3′ | 48°C | 37 |
| Cyclooxygenase-2 | Forward: 5′-CCCTTCTGCCTGACACCTTTCAAATT-3′<br>Reverse: 5′-GCTCTGGATCTGGAACACTGAATGAAGT-3′ | 48°C | 35 |

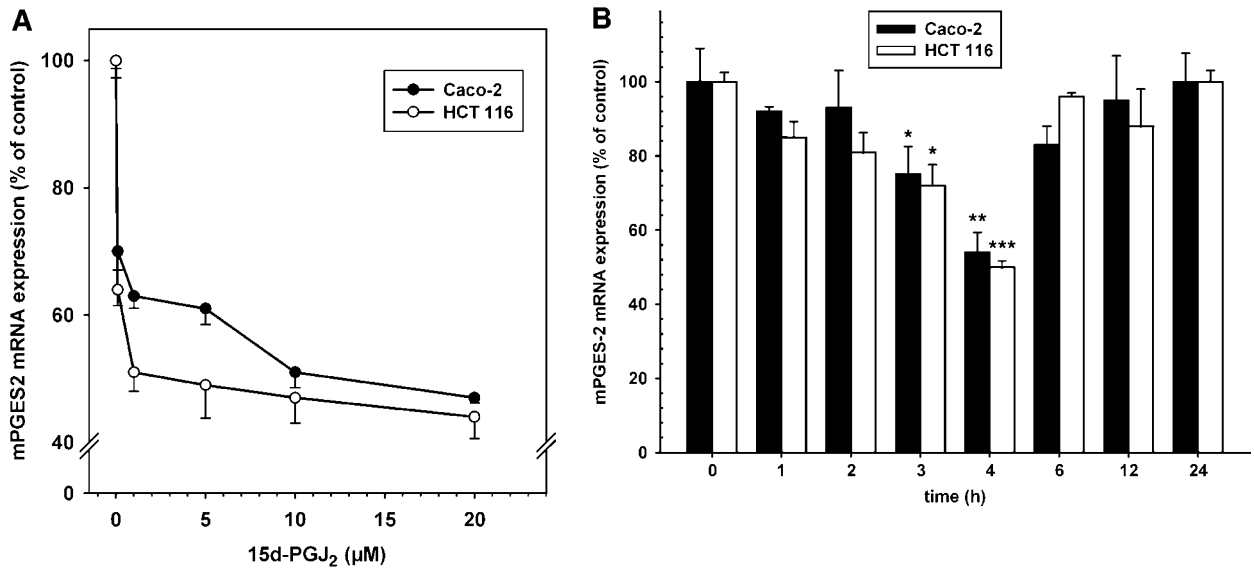


**Fig. 1.** Inhibition of microsomal prostaglandin E synthase-2 (mPGES-2) mRNA expression by 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$(15d-$PGJ_2$) in the colorectal cancer (CRC) cell lines Caco-2 and HCT 116. A: Caco-2 (closed circles) and HCT 116 (open circles) cells were incubated for 4 h in the absence or presence of 15d-$PGJ_2$ at concentrations of 0.1, 1, 5, 10, and 20 μM. B: Time course of mPGES-2 mRNA expression in Caco-2 cells (black bars) treated with 10 μM 15d-$PGJ_2$ and in HCT 116 cells (white bars) stimulated with 5 μM 15d-$PGJ_2$ for the indicated incubation periods. Total RNA was isolated as described in Materials and Methods and subjected to semiquantitative RT-PCR with the fluorescent dye PicoGreen®. All values for mRNA were normalized to the corresponding mRNA amount for the housekeeping gene GAPDH and represent means ± SD. The statistical significance of changes relative to vehicle-treated controls is expressed as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

mPGES-2 protein expression reached its maximum at 6 h (HCT 116) and 24 h (Caco-2), followed by a return to baseline levels thereafter (Fig. 2). HCT 116 and Caco-2 cells were also challenged with 10 μM 15d-$PGJ_2$ and $PGE_2$ as well as 16,16-dimethyl-$PGE_2$, a more stable analog of $PGE_2$, at concentrations ranging from $10^{-9}$ to $10^{-6}$ M for 4 h. However, the inhibitory effect of 15d-$PGJ_2$ on mPGES-2 mRNA and protein expression persisted in the presence of $PGE_2$ or 16,16-dimethyl-$PGE_2$ at any concentration used, thus excluding a potential counteractive effect of the product of PGES activity, $PGE_2$, on the downregulation of mPGES-2 mRNA and protein expression by 15d-$PGJ_2$ (data not shown).

### Alterations of spatial PGES activity after treatment with 15d-$PGJ_2$

In accordance with the downregulation of mPGES-2 at the mRNA and protein levels, a substantial decrease in enzyme activity was observed after treatment with 10 μM 15d-$PGJ_2$. As demonstrated in **Fig. 3**, enzymatic activity was found to be reduced only in the microsomal fraction, whereas the cytosolic PGES activity remained unchanged. The notable discrepancy between the two cell lines regarding the reduction in mPGES-2 activity can be explained, most likely, by a different sensitivity of HCT 116 and Caco-2 cells to 15d-$PGJ_2$, which is reflected in the mRNA and protein expression studies.

### Involvement of PPARγ activation in 15d-$PGJ_2$-mediated regulation of mPGES-2

Direct interaction of 15d-$PGJ_2$ with PPARγ to regulate gene transcription has been demonstrated to be one mode of action of this CyPG. To elucidate the signaling mechanism responsible for 15d-$PGJ_2$-mediated regulation of mPGES-2, Caco-2 cells, transfected with a mutant receptor to inhibit wild-type PPARγ action, were subjected to 15d-$PGJ_2$ treatment. As shown in **Fig. 4**, mPGES-2 mRNA

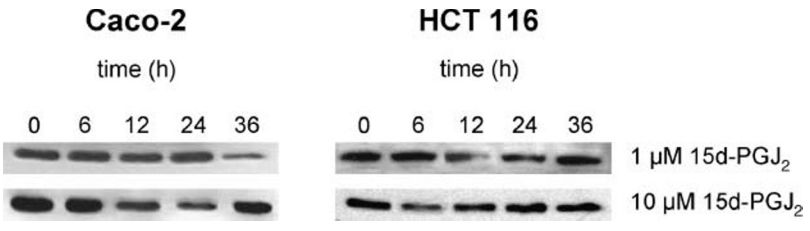


**Fig. 2.** Downregulation of mPGES-2 protein expression by 15d-$PGJ_2$ in Caco-2 and HCT 116 cells. Western blot analysis of mPGES-2 protein expression in Caco-2 and HCT 116 cells incubated in the absence and presence of 1 or 10 μM 15d-$PGJ_2$ for the times indicated. In all lanes, 15 μg of protein from the microsomal fraction of cells was analyzed. The results shown are representative of three separate experiments.

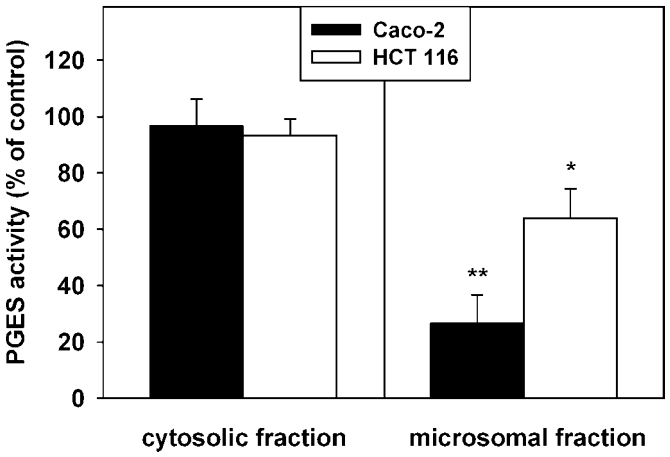


**Fig. 3.** PGES activity in the microsomal and cytosolic fraction of Caco-2 and HCT 116 cells treated with 15d-$PGJ_2$. Caco-2 and HCT 116 cells were treated with 10 μM 15d-$PGJ_2$ for 12 h. Cytosolic and microsomal fractions were separated, and enzymatic activity was determined as described in Materials and Methods. The relative amount of activity compared with vehicle-treated controls in Caco-2 (black bars) and HCT 116 (white bars) is depicted. Values shown represent means ± SD. The statistical significance of changes relative to unstimulated controls is expressed as follows: * $P < 0.05$, ** $P < 0.01$.

and protein expression were reduced to a similar extent compared with nontransfected Caco-2 cells or the HCT 116 cell line. In addition, PGES activity in PPARγ dominant-negative Caco-2 cells was found to be decreased in the same manner as in nontransfected Caco-2 or HCT 116 cells (data not shown). This finding suggested that the effect of 15d-$PGJ_2$ on mPGES-2 expression might be independent of PPARγ. To further corroborate this hypothesis, Caco-2 and HCT 116 cells were additionally stimulated with the thiazolidinedione homolog MCC555, a synthetic PPARγ agonist, at a concentration of 50 μM and cultured for various incubation periods (0–24 h). No changes in mRNA expression of mPGES-2 or any of the other enzymes examined (COX-1, COX-2, mPGES-1, and cPGES) were observed upon treatment with MCC555 (data not shown).

### Contribution of $PGD_2$ receptors in 15d-$PGJ_2$-mediated regulation of mPGES-2

One mechanism for 15d-$PGJ_2$ action may involve the activation of prostaglandin $D_2$ receptors (DPs). Previous studies indicate that 15d-$PGJ_2$ has a weak agonist activity on DP1 receptors in certain cell types of hematopoietic origin (22). Recently, a second high-affinity receptor (DP2), also designated CRTH2, was identified, thus far exclusively expressed in Th2 cells, T cytotoxic cells, eosinophiles, and basophiles (23). From a recent study it was known that HCT 116 does not express either DP receptor (24), and our own preliminary results indicated that Caco-2 is equipped with DP1 mRNA but not DP2 mRNA (data not shown).

Given that activation of DP1 leads to an increase in intracellular cAMP levels, we investigated the effect of increasing intracellular cAMP levels on mPGES-2 protein expression. Caco-2 cells were treated with forskolin, which directly stimulates adenylyl cyclase and thus increases in-

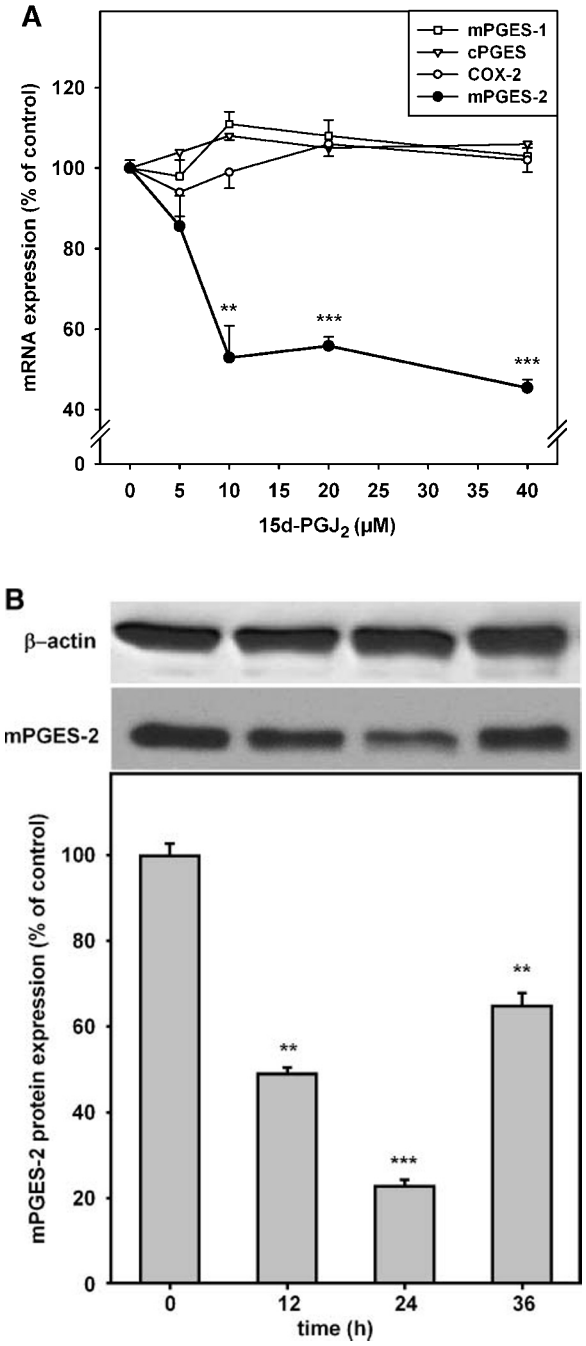


**Fig. 4.** Inhibition of mPGES-2 mRNA and protein expression by 15d-$PGJ_2$ in peroxisome proliferator-activated receptor γ (PPARγ) dominant-negative mutant Caco-2 cells. A: PPARγ dominant-negative mutant Caco-2 cells were treated for 4 h in the absence or presence of the indicated concentrations of 15d-$PGJ_2$. RT-PCR was performed on total RNA for cyclooxygenase-2 (COX-2), mPGES-1, mPGES-2, cytosolic prostaglandin E synthase (cPGES), and GAPDH during the linear phase of amplification. All values for mRNA are normalized to the corresponding mRNA amount for GAPDH. B: Immunoblot analysis of mPGES-2 protein expression in PPARγ dominant-negative mutant Caco-2 cells incubated in the absence or presence of 10 μM 15d-$PGJ_2$ for the times indicated. The top panel shows a series of immunoreactive bands corresponding to mPGES-2 and β-actin (serving as an internal control). The bottom panel depicts a histogram obtained by densitometric analysis of immunoblots from three independent experiments normalized to protein expression of β-actin. All values shown represent means ± SD. The statistical significance of changes relative to vehicle-treated controls is expressed as follows: ** $P < 0.01$, *** $P < 0.001$.

tracellular cAMP levels. However, mPGES-2 protein expression was not affected after treatment with various concentrations of forskolin (data not shown), thus excluding the participation of DP1 in the regulation of mPGES-2 in Caco-2 cells.

### Changes in the cellular redox status induced by 15d-$PGJ_2$

Biologic actions of 15d-$PGJ_2$ have also been linked to changes in the intracellular thiol-disulfide redox status. To investigate whether oxidative stress may be involved in 15d-$PGJ_2$-induced suppression of mPGES-2, Caco-2 and HCT 116 cells were preincubated with or without the antioxidant DTT for 2 h before stimulation with 15d-$PGJ_2$ for 12 h. As shown in **Fig. 5A**, the inhibitory effect of 15d-$PGJ_2$ on mPGES-2 protein expression was completely reversed by DTT at concentrations of 2 mM in both cell lines. The possible role of changes in the redox potential in mediating the downregulation of mPGES-2 by 15d-$PGJ_2$ was further investigated by treating HCT 116 cells with arsenite, which among other molecular events has been demonstrated to attack critical thiols (25). Arsenite dose-dependently reduced mPGES-2 protein expression in HCT 116 cells, which was statistically significant at concentrations of 50 μM, thereby mimicking the effect of 15d-$PGJ_2$. Again, attenuation of mPGES-2 protein expression could be abolished by preincubation of cells with 2 mM DTT for 2 h before stimulation of cells with 50 μM arsenite (Fig. 5B).

### Effects of $PGA_2$ on mPGES-2 expression

Finally, to determine whether or not 15d-$PGJ_2$ was selective for mPGES-2, HCT 116 and Caco-2 cells were treated with $PGA_2$, another representative CyPG. $PGA_2$ displayed a similar inhibitory effect on mPGES-2 protein expression. The minimal effective concentration was found to be 1 μM in both Caco-2 (**Fig. 6A**) and HCT 116 (Fig. 6B) cells, and maximum changes in protein level appeared at 12 h before gradually returning to baseline levels (data not shown).

To ascertain whether the effect of prostaglandins on mPGES-2 expression was specific for CyPG, CRC cells were also treated with AA and $PGD_2$. In contrast to 15d-$PGJ_2$ and $PGA_2$, no change in mPGES-2 protein expression was observed upon treatment with AA and $PGD_2$ (Fig. 6A, B).

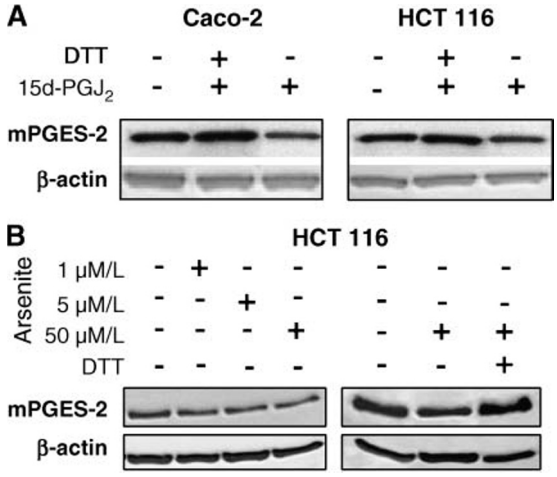


**Fig. 5.** Influence of the thiol-reducing agent DTT on the inhibitory effects of 15d-$PGJ_2$ and arsenite on mPGES-2 expression in CRC cells. A: HCT 116 and Caco-2 cells were pretreated for 2 h in the presence or absence of 2 mM DTT. After washing, cells were then stimulated with 10 μM 15d-$PGJ_2$ for the next 12 h. B: HCT 116 cells were stimulated with the indicated concentrations of arsenite for 12 h. Additionally, cells pretreated with 2 mM DTT for 2 h were also incubated with 50 μM arsenite for the subsequent 12 h. Protein amount was analyzed by immunoblotting of 15 μg of protein from the microsomal fraction of cells, with β-actin serving as an internal control. The results shown are representative of three separate experiments.

### Changes in cell proliferation induced by CyPGs and involvement of $PGE_2$

Both 15d-$PGJ_2$ and $PGA_2$ have been shown to decrease the growth of cancer cell cultures, but the molecular mechanisms are not completely understood. Although $PGE_2$-mediated processes have been found to play an essential role in tumor cell proliferation (26, 27), there are other lines of evidence indicating that $PGE_2$ itself does not directly stimulate cell growth in CRC. Among those are studies in several CRC cell lines, including Caco-2, demonstrating that proliferation is not directly sensitive to $PGE_2$ (28, 29). Based on these findings, we evaluated the influence of CyPGs on cell proliferation in both Caco-2 and HCT 116 cells as well as the potential contribution of the inhibition of mPGES-2 in the antiproliferative action of CyPGs in HCT 116 cells.

As shown in **Table 2**, exogenously added 15d-$PGJ_2$ exerted a growth-inhibitory effect on HCT 116 cells, which resulted in a time-dependent growth reduction of up to 62% at 72 h. A similar decrease in cell proliferation was also observed for Caco-2 cells (data not shown). $PGE_2$ as well as 16,16-dimethyl-$PGE_2$ failed to stimulate the proliferation of HCT 116 cells for up to 72 h of incubation. As expected, exogenous $PGE_2$ did not rescue 15d-$PGJ_2$-mediated growth inhibition in HCT 116 cells when both lipid mediators were added simultaneously to the medium (Table 2).

## DISCUSSION

In addition to the well-known role of COX-2 in CRC (30), recent findings also suggest an involvement of terminal PGES in this type of malignancy. mPGES-1 was found to be upregulated in colorectal adenomas and cancer (11). Kamei et al. (31) showed that overexpression of mPGES-1 accelerated not only $PGE_2$ production but also the proliferation rate of cultured cells, an effect that was attributed to changes in the expression of a variety of genes related to proliferation, morphology, adhesion, and the cell cycle. Moreover, cotransfection of COX-2 and mPGES-1 into HEK 293 cells resulted in cellular transformation manifested by colony formation in soft agar culture and tumor formation when implanted subcutaneously into nude mice (31). In contrast, little is known regarding the regulation and potential contribution of mPGES-2 in tumorigenesis, although

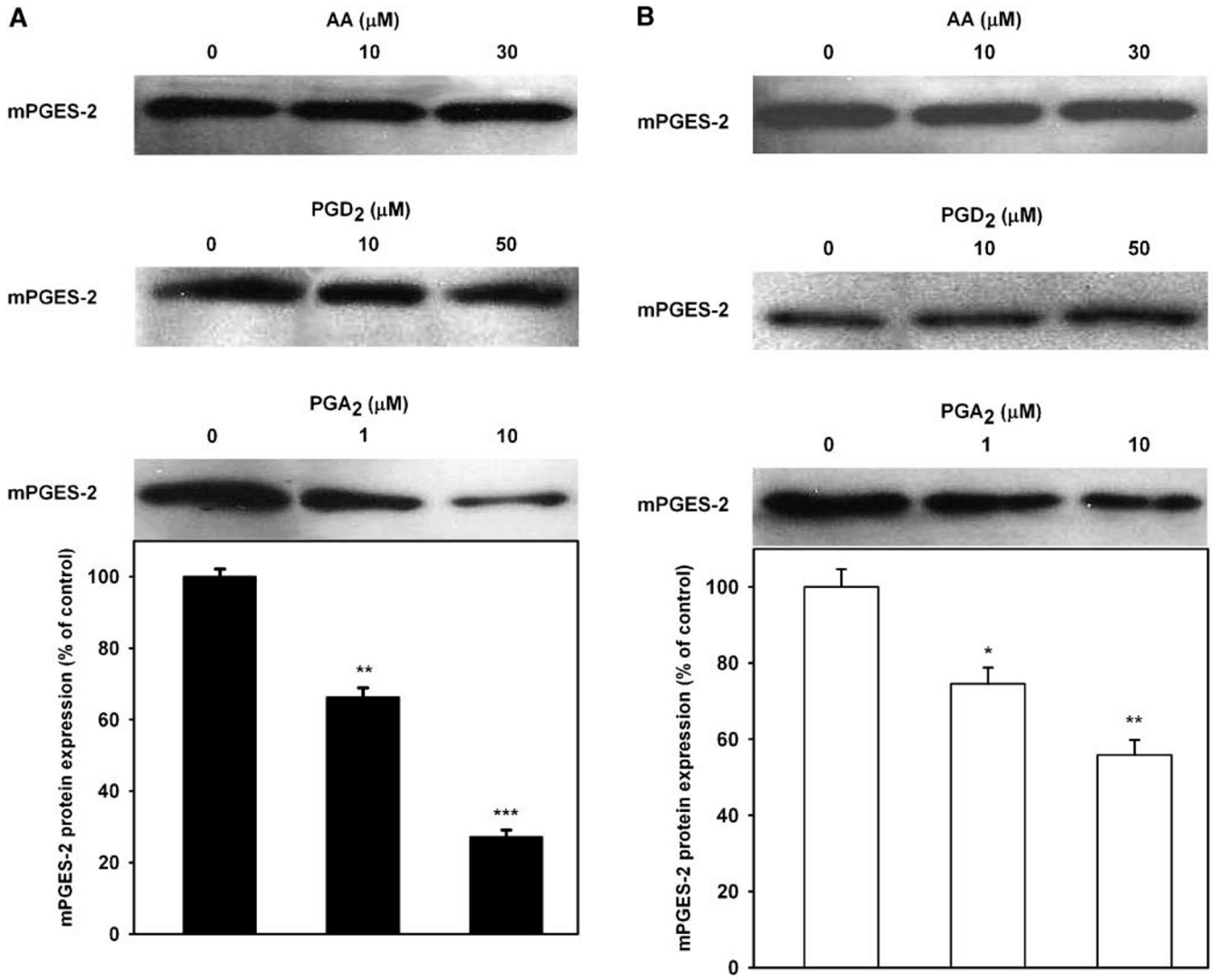


**Fig. 6.** Effects of arachidonic acid (AA), $PGA_2$, and $PGD_2$ on mPGES-2 protein expression. Caco-2 (A) and HCT 116 (B) cells were incubated for 12 h in the presence or absence of the indicated eicosanoids in various concentrations. Western blot analysis was performed as described in Materials and Methods. The results in the top panels show a series of immunoreactive bands corresponding to mPGES-2. The bottom panels depict histograms obtained using densitometric analysis of the immunoblots for each cell line from three independent experiments normalized to protein expression of β-actin. Values shown represent means ± SD. The statistical significance of changes relative to vehicle-treated controls is expressed as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

high expression levels of this enzyme have also been observed in colorectal carcinoma (12).

In light of recent findings regarding the role of the CyPG 15d-$PGJ_2$ both as a key regulator of negative feedback of the COX pathway during the resolution of inflammation and as a potent antineoplastic agent (13, 14, 16, 17), we sought to further determine its potential role in $PGE_2$-mediated CRC promotion. To our surprise, we found that

TABLE 2. Effects of 15d-$PGJ_2$, $PGE_2$, and combinations of both prostaglandins on the proliferation of HCT 116 cells

| | | | | | | 15d-$PGJ_2$ (10 μM) + $PGE_2$ | | | |
|---|---|---|---|---|---|---|---|---|---|
| Time | 15d-$PGJ_2$ (10 μM) | $PGE_2$ ($10^{-9}$ M) | $PGE_2$ ($10^{-8}$ M) | $PGE_2$ ($10^{-7}$ M) | $PGE_2$ ($10^{-6}$ M) | ($10^{-9}$ M) | ($10^{-8}$ M) | ($10^{-7}$ M) | ($10^{-6}$ M) |
| 12 h | 90 ± 6 | 93 ± 9 | 102 ± 4 | 96 ± 5 | 98 ± 7 | 92 ± 4 | 94 ± 3 | 95 ± 7 | 100 ± 8 |
| 24 h | 83 ± 5[a] | 95 ± 6 | 91 ± 9 | 100 ± 2 | 106 ± 9 | 85 ± 5[a] | 79 ± 7[a] | 80 ± 10[b] | 87 ± 9[b] |
| 48 h | 73 ± 4[c] | 103 ± 8 | 97 ± 6 | 101 ± 3 | 100 ± 4 | 72 ± 7[c] | 76 ± 9[c] | 70 ± 3[c] | 75 ± 8[c] |
| 72 h | 38 ± 3[c] | 101 ± 5 | 104 ± 3 | 99 ± 6 | 92 ± 10 | 34 ± 4[c] | 35 ± 8[c] | 38 ± 9[a] | 40 ± 2[c] |

15d-$PGJ_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$; $PGE_2$, prostaglandin $E_2$. Cell proliferation was performed as indicated in Materials and Methods and plotted after normalization to the proliferation of cells before stimulation.

[a] $P < 0.01$ relative to vehicle-treated controls.

[b] $P < 0.05$ relative to vehicle-treated controls.

[c] $P < 0.001$ relative to vehicle-treated controls.

15d-$PGJ_2$ selectively downregulated mPGES-2 in the CRC cell lines Caco-2 and HCT 116. In contrast, mPGES-1 and cPGES were not affected by this CyPG. Inhibition of mPGES-2 mRNA expression was time- and dose-dependent, and reduction in mRNA expression was followed by a delayed decrease in mPGES-2 protein levels. Several reports have demonstrated that gene expression of PGES and COX enzymes is coregulated. In general, these data indicate a preferred coupling of COX-2 and mPGES-1 expression (32), whereas cPGES has been postulated to be linked with COX-1 (33). To our knowledge, no such association between the COX isoforms and mPGES-2 has ever been described. To elucidate a possible coupling between mPGES-2 and COX-1 or COX-2, we performed studies in cell lines with different COX isoform phenotypes: Caco-2 cells, which do not express COX-1, and HCT 116 cells, which do not express COX-2. Both cell lines displayed similar downregulation of mPGES-2 mRNA and protein expression upon challenge with 15d-$PGJ_2$. COX-1 and COX-2 expression levels remained unaffected, thus excluding a coregulation of mPGES-2 and COX isoforms by 15d-$PGJ_2$. Furthermore, feedback control of COX-2 by this CyPG could be ruled out in these two CRC cell lines.

Downregulation of mPGES-2 by 15d-$PGJ_2$ resulted in a distinct decrease in PGES activity in the microsomal fraction of both Caco-2 and HCT 116 cells associated with a reduced net $PGE_2$ synthesis. Together with the unaltered expression of cPGES and mPGES-1 in response to 15d-$PGJ_2$, this finding provides good evidence that at least in this in vitro setting inhibition of mPGES-2 by this CyPG cannot be counteracted by compensatory mechanisms to increase PGES activity.

We were not able to demonstrate any effect of this enzymatic downregulation on cell proliferation, however, because stimulation of growth in Caco-2 and HCT 116 cells was found to be insensitive to $PGE_2$. Similar observations, under comparable experimental conditions, have been described for Caco-2 cells (28). Indeed, the direct causal relationship between $PGE_2$ and proliferation is a source of controversy in the literature. Contradictory results using various models have been obtained by many laboratories (25–28). Our data demonstrating that $PGE_2$ both failed to stimulate proliferation in Caco-2 and HCT 116 cells and was unable to rescue the growth-inhibitory effect of 15d-$PGJ_2$ support the idea that $PGE_2$ does not play a direct role in the proliferation of human colon adenocarcinoma cells.

Because $PGE_2$ exerts its biological activity through binding to its cognate receptors (EP1–EP4), a different expression repertoire might explain the conflicting results with respect to proliferation in CRC cell lines. Studies with transgenic mice lacking the genes encoding these receptors have revealed that $PGE_2$ promotes tumor growth using EP1, EP2, and EP4 (8, 9, 34, 35). In contrast, the EP3 subtype seems to play an important role in the suppression of cell growth (36). Indeed, expression of EP3 is downregulated in colon carcinogenesis at a later stage, whereas the EP1 and EP2 subtypes were found to be increased in colon cancer tissues (33, 36). Caco-2 cells express EP1 and EP2, and the EP receptor repertoire of the HCT 116 cell line includes EP1, EP2, and EP4 (36). These findings, together with the results presented here, suggest that in CRC cells, at least in the HCT 116 cell line, proliferation rates are not sensitive to $PGE_2$ unless some other as yet undefined EP can modulate cell growth. Thus, it has to be assumed that a complex network of intracellular and intercellular interactions involving various cell types is involved in $PGE_2$-induced growth stimulation during colorectal carcinogenesis. Indeed, in recent years, substantial amounts of data have been obtained that permit the delineation of signaling pathways and the identification of downstream targets involved in $PGE_2$-mediated carcinogenesis. These include the Raf/MEK/ERKs and PI3K/Akt pathways, interactions with the nuclear receptor PPARδ, angiogenic factors such as VEGF, the anti-apoptotic Bcl-2 protein, chemokines (e.g., RANTES, MIP-1α, and MIP-1β), and cytokines (37).

CyPGs exert their actions through complex mechanisms that are not completely understood. Best studied among the intracellular targets of 15d-$PGJ_2$ is the nuclear receptor PPARγ. This transcription factor has been demonstrated to regulate the gene expression of target proteins associated with lipid homeostasis and inflammation but also cell proliferation and malignancies (38–40). The downregulation of mPGES-2 by 15d-$PGJ_2$, however, was not affected in Caco-2 cells transfected with a PPARγ dominant-negative mutant receptor. Furthermore, the synthetic PPARγ agonist MCC555 had no effect on the expression of mPGES-2 in both Caco-2 and HCT 116 cells, clearly indicating that the regulation of mPGES-2 by 15d-$PGJ_2$ is not under the control of the PPARγ pathway.

Possible extracellular targets for 15d-$PGJ_2$ may be the DP receptors, at which 15d-$PGJ_2$ displays agonistic activity. The involvement of DP2 as one of the molecular mechanisms responsible for the regulation of mPGES-2 in Caco-2 and HCT 116 cells could be excluded because this receptor is not expressed in either of these CRC cell lines, as determined by RT-PCR. In addition, HCT 116 cells do not express the other known DP receptor, DP1 (24), whereas our data revealed the expression of DP1 mRNA in Caco-2 cells. If one assumes that the same biological effect in both cell lines does not occur via different mechanisms, then 15d-$PGJ_2$ binding to DP1 appeared to be an unlikely event in the downregulation of mPGES-2 in Caco-2 cells. Indeed, an increase in cAMP levels did not display any regulatory effect on mPGES-2 protein expression, suggesting that DP1 signaling is not involved in the 15d-$PGJ_2$-induced suppression of mPGES-2.

These findings clearly indicated that the control of mPGES-2 by 15d-$PGJ_2$ is not related to a mechanism specific for this CyPG but must be attributable to a mode of action inherent to CyPGs in general. The first indication of this was that another CyPG, $PGA_2$, could mimic the effects of 15d-$PGJ_2$ on mPGES-2, whereas eicosanoids not containing the cyclopentenone structure, such as AA and $PGD_2$, did not affect mPGES-2 protein expression. CyPGs are able to form adducts with cellular thiols, both in glutathione and proteins, as a result of the presence of an unsaturated carbonyl group in the cyclopentenone moiety

(41, 42). Modification of functionally important sulfhydryl groups in these proteins can be attributed to some of the biological effects of these prostanoids. Modulation of the cellular thiol-disulfide redox status may also influence the intensity and specificity of CyPG action (43). Indeed, downregulation of mPGES-2 by 15d-$PGJ_2$ could be reversed by the thiol-reducing agent DTT. Furthermore, arsenite, whose biochemical and molecular mechanisms of toxicity can be explained at least in part by the reaction of this compound with protein thiols, also downregulated mPGES-2 protein expression in a dose-dependent manner. From these results, it can be hypothesized that cyPGs exert a prooxidant effect, resulting in the conversion of a sulfhydryl group into an oxidized disulfide in cellular protein(s) (e.g., transcription factors), which in turn may lead to the downregulation of mPGES-2, as proposed previously by Liu et al. (44). However, identifying the proteins that participate in this signaling cascade will require further investigation.

In conclusion, the data presented here provide evidence for the control of mPGES-2 expression by CyPGs independently of upstream COX enzymes. The clinical relevance for CRC of the regulation of this terminal PGES could not be explored using our in vitro model. Nevertheless, in light of the need for new therapeutic strategies regarding the prevention and treatment of CRC, our findings warrant further assessment of the role of mPGES-2 and CyPGs as well as their interplay in this malignancy.

The authors acknowledge the advice received during discussions with Professor Mats Hamberg as well as the excellent technical assistance of S. Loitsch, R. Schmidt, and S. Ulrich. This work was financially supported by a graduate scholarship grant from the Deutsche Forschungsgemeinschaft to Y.Y., the Swedish Research Council and the 6th Framework programme of the European Community (LSHM-CT-2004-005033), the Else-Kröner Fresenius Foundation, and the AFA Health Foundation.

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