# Regulation of Microsomal Prostaglandin E2 Synthase by Cyclopentenone Prostaglandins in Colon Cancer Cells

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***To my parents,***

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## Abbrevations

| | |
|---|---|
| 15d-$PGJ_2$ | 15-deoxy- $\Delta^{12,14}$-prostaglandin $J_2$ |
| | |
| AA | Arachidonic acid |
| ACF | Aberrant crypt foci |
| AOM | Azoxymethane |
| AP-2 | Activator protein -2 |
| APC | Adenomatous polyposis coli |
| ATM | Ataxia-telangectasia mutated kinase |
| ATP | Adenosine triphosphate |
| | |
| cDNA | Complementary deoxyribonucleic acid |
| COX | Cyclooxygenase |
| cPGES | Cytosolic prostaglandin E synthase |
| cPLA | Cytosolic phospholipase A |
| cAMP | Cyclic adenosine monophosphate |
| CRE | cAMP response element |
| CRTH2 | Chemoattractant receptor–homologous molecule expressed on TH2 cells |
| | |
| DEPC | Diethyl pyrocarbonate |
| DMEM | Dulbecco's modified Eagle medium |
| dNTPs | Deoxyribonucleotide triphosphates |
| DTT | Dithiothreitol |
| | |
| ECACC | European Animal Cell Culture Collection |
| EGFR | Epidermal growth factor receptor |
| Egr-1 | Early growth response |
| EIA | Enzyme immunoassay |
| ER | Endoplasmic reticulum |
| ERK1/2 | Extracellular signal-regulated kinase1/2 |

| | |
|---|---|
| FBS | Foetal Bovine serum |
| FLAP | 5-lipoxygenase-activating protein |
| Fura-2/AM | Fura-2 acetoxymethyl ester |
| | |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GSH | Glutathione |
| | |
| H-Ras | Homologous to the oncogene of the Harvey rat sarcoma virus |
| HSA | Human serum albumin |
| HSF-1 | Heat shock transcription factor 1 |
| HSP | Heat shock protein |
| | |
| IMN | Indomethacin |
| IP3 | Inositol (1,4,5)-trisphosphate |
| iPLA | Intracellular phospholipase A |
| | |
| JAK | Janus protein kinase |
| JNK | c-jun N-terminal kinase |
| | |
| LDH | Lactate dehydrogenase |
| LT | Leukotriene |
| | |
| MAPEG | Membrane Associated Proteins in Eicosanoid and Glutathione metabolism |
| MAPK | Mitogen-activated protein kinases |
| MGST1-L1 | Membrane-bound glutathione S-transferase 1-like-1 |
| *Min* | Multiple intestinal neoplasms |
| mPGES | Microsomal prostaglandin E synthase |
| mRNA | Messenger ribonucleic acid |
| | |
| NAC | *N*-acetylcysteine |
| NF-IL-6 | Nuclear factor for interleukin-6 |
| NF-κB | Nuclear factor-kappa B |
| NSAID | Non-steroidal anti-inflammatory drugs |

| | |
|---|---|
| PBS | Posphate buffered saline |
| PG | Prostaglandin |
| PGT | Prostaglandin transporter |
| PK | Protein kinase |
| PPAR | Peroxisome proliferator-activated receptor |
| PPRE | PPAR-response element |
| | |
| ROS | Reactive oxygen species |
| RT-PCR | Reverse transcription-polymerase chain reaction |
| RXR | Retinoid X receptor |
| | |
| SAPK | Stress-activated protein kinases |
| SDS-PAGE | Sodium dodecylsulfate polyacrylamide gel electrophoresis |
| Sp1 | Specificity protein 1 |
| sPLA | Secretory phospholipase A |
| | |
| TBS-T | Tween Tris Base Saline |
| Tx | Thromboxane |
| | |
| VEGF | Vascular endothelial growth factor |

# 1 Introduction

## 1.1 Prostaglandins

Prostaglandins, thromboxanes and leukotrienes, collectively referred to as 'eicosanoids', are the cyclooxygenase (COX) and lipooxygenase metabolites of arachidonic acid (AA) (Fig. 1.). Discovery of eicosanoids (from Greek *eicosa*=twenty; for twenty carbon fatty acid derivatives), was initiated in 1930. (Burr G. et al., 1930; Kurzrok R. et al., 1930; Euler U. 1934). First, it was found that exclusion of fat from the diet of rats led to growth retardation, reproductive disturbances, scaly skin, kidney lesions and excessive water consumption, which led to the discovery of essential fatty acids. Second, a factor with fatty acid properties and vasodepressor and smooth muscle-stimulating activity was identified that was termed "prostaglandin." Bergström and Samuelsson linked these observations when they elucidated the structures of the "classical" prostaglandins and demonstrated that they were produced from an essential fatty acid, AA (Bergström S. et al., 1964).

All prostaglandins exhibit roughly the same structure as all are oxygenated fatty acids composed of 20 carbon atoms and contain a cyclopentane ring, a C-13 > C-14 *trans*-double bond, and a hydroxyl group at C-15 (Fig. 5, chapter 1.3). They were classified into types A to I, dependent on the modifications of this cyclopentane ring. The abbreviations are commonly followed by an index (for instance $PGE_2$), which indicates the number of double bonds present in the various side chains attached to the cyclopentane ring. Based on the number of these double bonds, prostaglandins were classified into three series 1, 2, and 3.

Prostaglandins are formed by most cells of the body and act as autocrine and paracrine lipid mediators, signalling at or immediately adjacent to their site of synthesis. They are not only key mediators of inflammation and involved in apoptosis, cell differentiation and oncogenesis, but also play critical physiologic roles in tissue homeostasis and function. For example, gastric mucosal protective function, sleep induction and vascular smooth muscles contraction and relaxation are all dependent upon these compounds. (Wallance J. 1992; Funk C. 2001; Muller R. 2004). The actions of prostaglandins are summarized in Fig. 1.

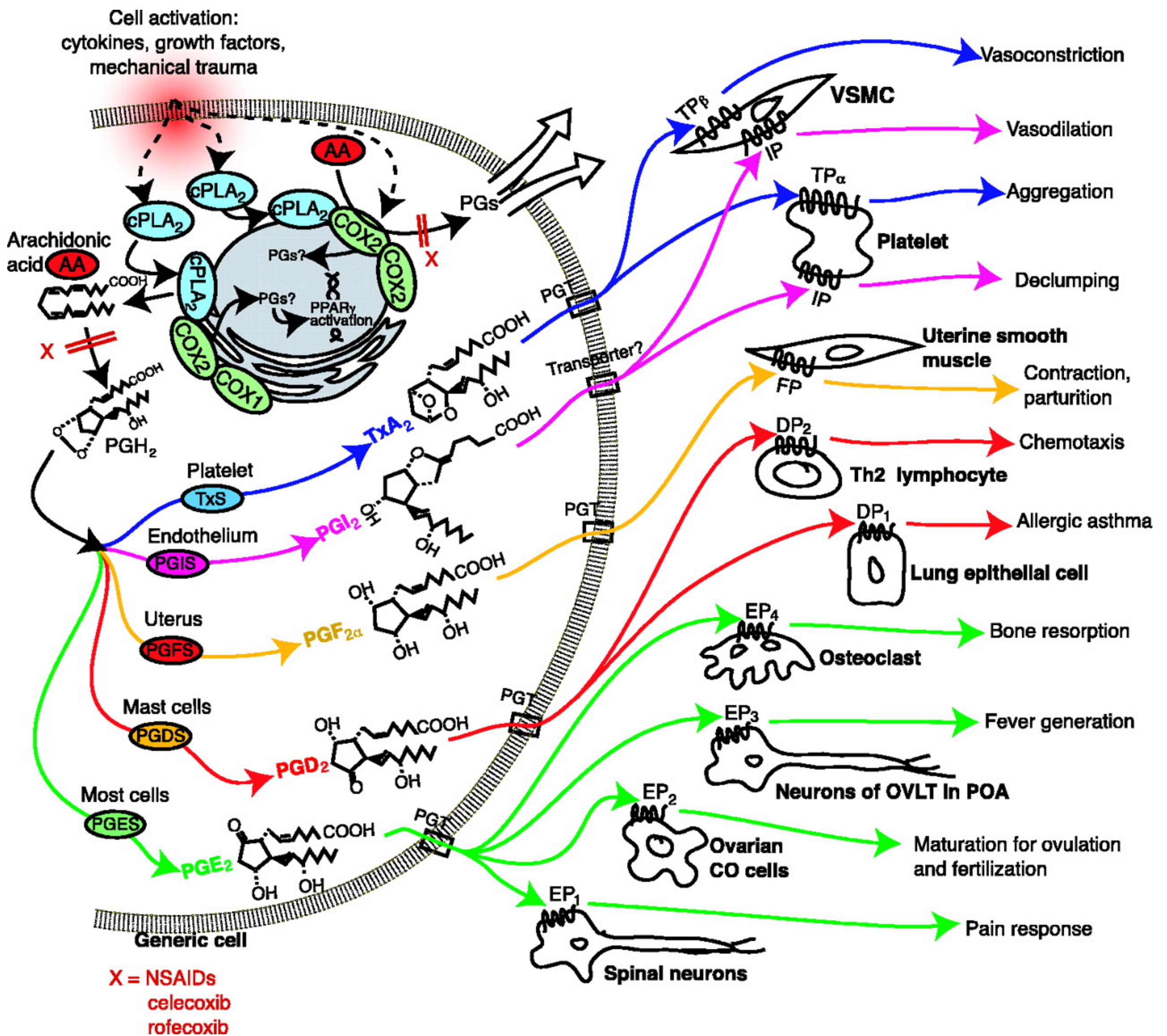


***Fig. 1. Prostaglandin actions and synthesis***. *Mechanical trauma, cytokines, growth factors, or various inflammatory stimuli activate cells, triggering signalling, including cytosolic phospholipase (cPLA$_2$) translocation to endoplasmatic reticulum and nuclear membranes, release of arachidonic acid from membrane lipids and production of PGH$_2$ intermediate by COX-2 or COX-1. Heterogeneous family of PGH2 metabolizing enzymes can form PGE$_2$, PGD$_2$, PGF$_{2\alpha}$, PGI$_2$ (prostacyclin) and TxA$_2$ (thromboxane). These prostaglandins may pass cell through a known prostaglandin transporter (PGT) to exert their actions on a family of prostaglandin receptors named EP1, EP2, EP3, EP4, DP1, DP2, FP, IP, TPα and TPβ. (Funk C., 2001)*

### 1.1.1 Biological functions of prostaglandin $E_2$

Prostaglandin $E_2$ ($PGE_2$) is a major cyclooxygenase product in a number of physiological settings. It was first isolated and its structure determined in the 1960-s. (Samuelsson B. 1963). Thereafter, this prostaglandin was found in many different tissues. $PGE_2$ plays a protective role in maintaining the integrity of the gastric mucosa (Woo S. et al. 1986). The $PGE_2$ production in the kidney is critical for normal renal function by preserving renal blood flow and glomerular filtration rate in settings of physiological stress, modulating salt and water transport in the distal tubule, and stimulating renin release from the juxtaglomerular apparatus (Breyer M. et al. 2001). $PGE_2$ was also shown to play a role in the maintenance of blood pressure, particularly in the setting of salt overload (Kennedy C. et al. 1999). In certain instances, $PGE_2$ was observed to have multiple and apparently opposing functional effects. For example, $PGE_2$ elicits both smooth muscle relaxation and constriction (Walch L. et al. 2001; Davis R. et al. 2004). Complexity was also observed in modulation of the immune response by $PGE_2$; it was shown that $PGE_2$ regulates the function of many cell types including macrophages, dendritic cells, T and B lymphocytes leading to both pro- and anti-inflammatory effects. (Hata A. et al. 2004). $PGE_2$ is also associated with oncogenesis. $PGE_2$ signalling promotes tumour angiogenesis (Kurie J. et al. 2001), increases cell proliferation and stimulates oncogenesis (Pai R. et al. 2002).

The diverse effects of $PGE_2$ may be accounted for in part by the existence of four G-protein-coupled receptors, EP1, EP2, EP3, and EP4, and heterogeneity in the coupling of these receptors to intracellular signal transduction pathways (Fig. 2). Synthesized $PGE_2$ is released mainly through a prostaglandin transporter (PGT) (Schuster V. 1998) out of the cells and then binds to receptors in the vicinity of the site of $PGE_2$ production. Of the four known EP receptors, EP3 and EP4 receptors bind $PGE_2$ with the highest affinity ($K_d \sim 1$ nM), whereas EP1 and EP2 receptors bind with lower affinity ($K_d > 10$ nM) (Abramovitz 2000). The EP1 receptor mediates $PGE_2$-induced elevation of free $Ca^{2+}$ concentration, whereas the EP2 and EP4 receptors are coupled to $G_s$-type G protein and their activation leads to an increase in cyclic AMP (cAMP) levels. The signalling pathway of the EP3 receptor mediates inhibition of adenylate cyclase via $G_i$ (Narumiya S. et al. 1999).

Among the prostanoid receptors EP3 and EP4 receptors are widely distributed throughout the body. In contrast, the distribution of EP1 is restricted to several organs, such as the kidney, lung, and stomach, and EP2 is the least abundant among the EP receptors.

However, EP2 is effectively induced in the response to inflammatory stimuli (Narumiya S. et al. 1999).

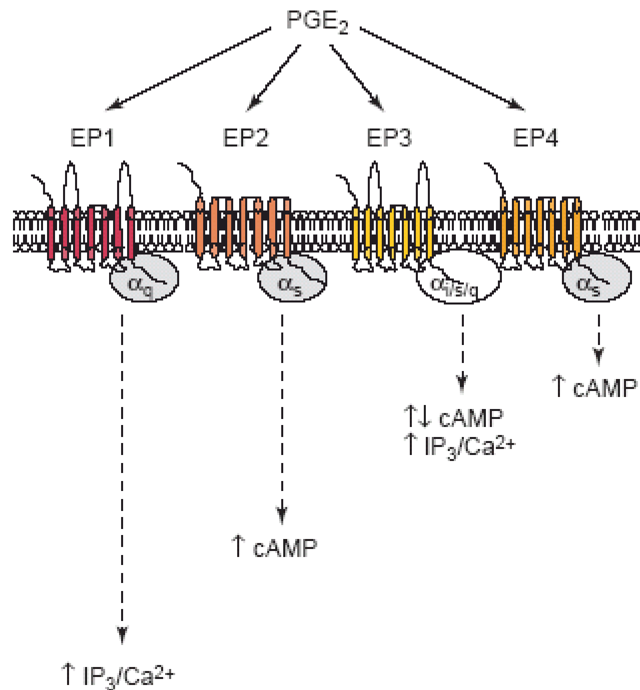


***Fig. 2. Signal transduction of prostaglandin $E_2$ receptors****. $PGE_2$ is actively transported out of the cell, where it exerts the effect by coupling to its heptahelical transmembrane receptors, EP1, EP2, EP3 and EP4 to activate second messengers, such as cAMP and inositol (1,4,5)-trisphosphate (IP3), and intracellular signalling cascades (Jabbour H. et al. 2001).*

### 1.1.2 Prostaglandin $E_2$ and cancer

During the last years COX-2, a subset of prostanoids and prostanoids receptors was clearly established as crucial players in oncogenesis that regulate, and are regulated by, pathways with essential functions in oncogenesis (Muller R. 2004). The use of non-steroidal anti-inflammatory drugs (NSAID), which inhibit COX enzymes, was linked to reduced cancer risk in multiple tissues including those of the breast, colon, prostate, and lung (Zha S. et al. 2004). Among the various downstream prostaglandins, $PGE_2$ has long been suggested as the key player. Stimulatory effect of prostaglandin E was first observed in dog kidney cells. These cells required $PGE_2$ or $PGE_1$ for growth in serum-free media (Taub M. et al. 1979, Taub M. et al. 1983). Furthermore, increased amount of $PGE_2$ was found in breast and colon

cancer (Karmali R. et al. 1983, Yoshimatsu K. et al. 2001) and inducible microsomal prostaglandin E synthase-1 was described to be constitutively expressed in several cancers, most of which also express COX-2 constitutively. (Yoshimatsu K. et al. 2001, Jabbour H. et al. 2001, Yoshimatsu K., Golijanin D. et al. 2001).

Several reports have demonstrated increased $PGE_2$ levels in neoplastic colorectal lesions compared with normal mucosa (Rigas B. et al. 1993, Pugh S. et al. 1994;Yoshimatsu K. et al. 2001). There is a body of evidence, beyond the association between a cancer phenotype and increased levels of $PGE_2$, suggested that $PGE_2$ also contributes to the development and progression of colorectal cancer. The $PGE_2$ level increases in a size-dependent manner in colorectal adenomas in familial adenomatous polyposis patients (Hull M. et al. 2004). $PGE_2$ can induce angiogenesis *in vitro* and increases cellular resistance to apoptosis (Ben-Av P. et al. 1995), enhancing the survival and motility (Sheng H. et al. 1998, Sheng H. et al. 2001) in colon cancer. Immune surveillance is also inhibited by $PGE_2$ (Balch C. et al. 1984). Cumulative evidence indicated that COX-2-derived $PGE_2$ provides growth advantage to colorectal carcinomas through transactivation of the epidermal growth factor receptor (EGFR) signalling system (Sheng H. et al. 2001; Pai R. et al. 2002). On the other hand, $PGE_2$ exposure induces the expression of VEGF (vascular endothelial growth factor) in colon cancer cells (Fukuda R. et al. 2003).

In addition to these findings, studies with experimental animals suggested that $PGE_2$ promotes tumorigenesis. Treatment with anti-$PGE_2$ monoclonal antibody retards the growth of a transplantable lung tumour (Stolina M. et al. 2000). Knockout mouse studies shown that only the prostaglandin E receptors, but not any other single prostaglandin receptor knockout mice, show a significant decrease in the number of aberrant crypt foci when compared with the wild type controls (Zha S. et al. 2004). Three of four identified G-protein coupled $PGE_2$ receptors are involved in enhancement of colon carcinogenesis in different mouse models of oncogenesis. Disruption of both EP2 alleles was reported to inhibit tumour cell proliferation, tumour growth, and led to reduced polyp incidence and size in $Apc^{\Delta 716}$ mice, employed as a model of familial adenomatous polyposis (Seno et al. 2002; Sonoshita et al. 2001). These tumours show decreased microvessel density indicating a critical role for EP2 in tumour angiogenesis (Seno et al. 2002). The role of EP4 was studied in another mouse model of intestinal oncogenesis, i.e., the formation of polyps in mice treated with the chemical carcinogen, azoxymethane (AOM) (Mutoh et al. 2002). Genetic inactivation of EP4 led to a 44% reduction in the numbers of polyps. Antagonists of EP1 and EP4, similarly suppressed

aberrant crypt foci (ACF) formation in this mouse model. An EP1-selective antagonist reduced the colon cancer incidence, multiplicity and volume, along with cell proliferation in colon tumour cells (Niho N. et al. 2005). In contrast, expression of EP3 mRNA is markedly decreased in colon cancer tissues, compared with normal colon mucosa. Deficiency of EP3 receptor had no effect on the early stages of cancer in AOM treated mice; however, long term *in vivo* examination of AOM induced colon tumour development demonstrated enhancement of tumour incidence and multiplicity. Moreover, the size of the tumours was significantly increased. Thus, suggesting that the EP3 receptor does not influence the early stage of colon carcinogenesis, including ACF formation, but could play an important role in cancer development at a later stage (Shoji Y. et al. 2004). Some studies have also shown that EP3 and EP2 receptors have been implicated in tumor-associated angiogenesis via VEGF production (Hull M. et al. 2004).

## 1.2 Cyclopentenone prostaglandins

The cyclopentenone prostaglandins $PGA_2$, $PGA_1$, and $PGJ_2$ are formed by dehydration within the cyclopentane ring of $PGE_2$, $PGE_1$, and $PGD_2$, respectively. $PGJ_2$ is metabolized further to yield $\Delta^{12}$-$PGJ_2$ and 15-deoxy- $\Delta^{12,14}$-$PGJ_2$ (15d-$PGJ_2$). Cyclopentenone prostaglandins contain a cyclopentenone ring structure, which is characterized by the presence of a chemically reactive α,β-unsaturated carbonyl. The α,β-unsaturated carbonyl group contains an electrophilic centre which makes these prostaglandins susceptible to undergo addition reactions with nucleophiles such as the free sulfhydryl group of cysteine residues located in reduced glutathione or cellular proteins (Fig. 3) (Honn K. et al. 1985, Atsmon J. et al. 1990, Parker J. et al. 1992). Alkylation of the exposed cysteine residues on key cellular target proteins results in changes of function of the targets, thus explaining the bioactivity of cyclopentenone prostaglandins (Straus D. et al. 2001).

Although prostaglandins in general was shown to stimulate cell proliferation, the cyclopentenone prostaglandins were reported to induce apoptosis in various malignant and transformed cells (Kim I. et al. 1993; Joubert A. et al. 2003; Chen Y. et al. 2003; Straus D. et al. 2001). In addition to their proapoptotic properties, various compounds within the

cyclopentenone prostaglandin family possess potent anti-inflammatory and anti-viral activity. Most actions of the cyclopentenone prostaglandins do not appear to be mediated by binding to G-protein coupled prostanoid receptors (Straus D. et al. 2001).

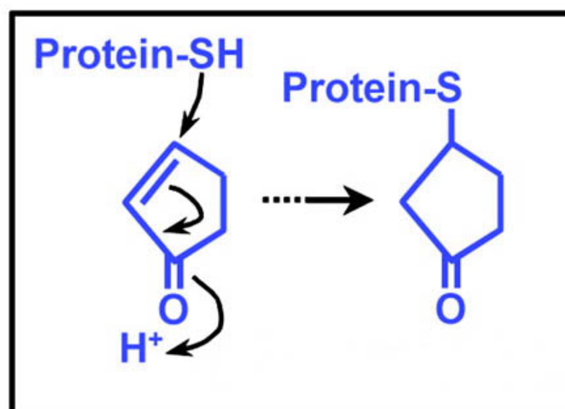


***Fig. 3. Covalent binding of cyclopentenone ring to signalling molecules**. The specific effect of cyclopentenone prostaglandins are mediated through their covalent binding to intracellular proteins. This is due to their reactive cyclopentenone ring (Powell W. 2003).*

### 1.2.1 15-deoxy- $^{\Delta 12,14}$-$PGJ_2$

15d-$PGJ_2$ was initially identified as a product of albumin-catalyzed transformation of $PGD_2$ *in vitro* (Fitzpatrick F. et al. 1983). Later on, it was discovered that $PGD_2$ is converted to $PGJ_2$, a direct precursor of $\Delta^{12}$-$PGJ_2$ and 15d-$PGJ_2$. Serum albumin is involved only in the process leading from $PGJ_2$ to $\Delta^{12}$-$PGJ_2$ (Fig. 4) (Shibata T. et al. 2002). Considerable interest was generated in 1995 with the discovery that 15d-$PGJ_2$ is an activating ligand for peroxisome proliferator-activated receptor-γ (PPARγ) (Forman B. et al. 1995, Kliewer S. et al. 1995).

15d-$PGJ_2$ may alter cellular functions by multiple mechanisms. This cyclopentenone was shown to inhibit the expression of a variety of proinflammatory genes, including COX-2 (Straus D. et al. 2000; Subbaramaiah K. et al. 2001; Sanchez-Gomez F. et al. 2004; Straus D. et al. 2001). 15d-$PGJ_2$ was identified in inflammatory fluids and it was demonstrated that

levels of this compound increase during the resolution phase of inflammation (Gilroy D. et al. 1999). Together with the known potent anti-inflammatory activity of 15d-$PGJ_2$, these results suggest that 15d-$PGJ_2$ may play a role as a naturally occurring feedback inhibitor of inflammation *in vivo*.

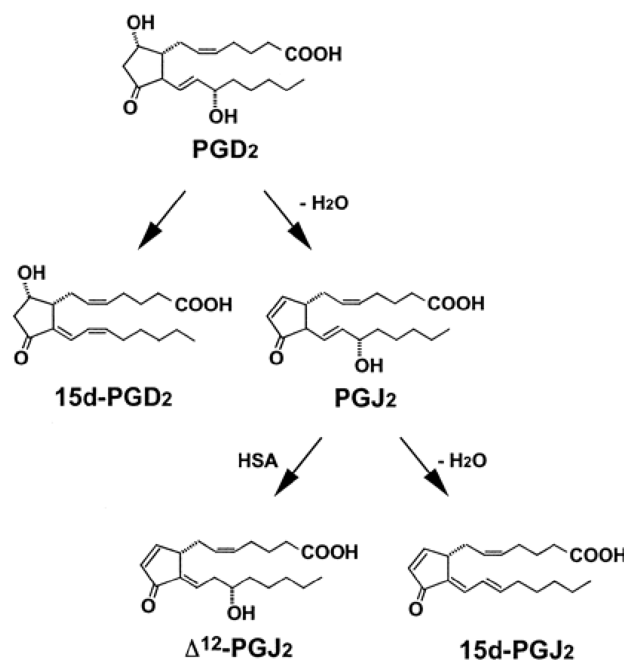


***Fig. 4. Generation of 15d-$PGJ_2$. $PGD_2$ metabolism.*** *$PGD_2$ is sequentially converted to $PGJ_2$ and 15d-$PGJ_2$ in an albumin-independent manner and human serum albumin (HSA) is involved only in the process leading from $PGJ_2$ to $\Delta^{12}$-$PGJ_2$.*

Some of the effects of 15d-$PGJ_2$ are mediated by its ability to bind and activate PPARγ (Straus D. et al. 2001; Inoue H. et al. 2000; Shimada T. et al. 2002) However, 15d-$PGJ_2$ can induce a variety of responses independently of PPARγ because of the reactive cyclopentenone ring of this prostaglandin (Nosjean O. et al. 2002). 15d-$PGJ_2$ regulates the activity of several signalling molecules and transcription factors including nuclear factor-kappa B (NF-κB), c-Jun, H-Ras (homologous to the oncogene of the Harvey rat sarcoma virus) and heat shock protein (HSP)70 (Straus D. et al. 2000, Rossi A. et al. 2000; Perez-Sala D. et al. 2003; Powell W. 2003; Cippitelli M. et al. 2003). In addition to the intracellular effects of 15d-$PGJ_2$ on transcription factors and signalling molecules, 15d-$PGJ_2$ can also act

on cell membrane receptors, DP1 and chemoattractant receptor–homologous molecule expressed on TH2 cells (CRTH2) (Monneret G. et al. 2002; Wright D. et al. 1998).

There is a body of evidence indicating that 15d-$PGJ_2$ possesses anti-neoplastic properties because of its inhibitory effects on tumour cell proliferation and angiogenesis. (Nencioni A. et al. 2003; Michalik L. et al. 2004; Straus D. et al. 2001). This cyclopentenone was shown to be the most active anti-tumour compound in the $PGJ_2$ series (Kato T. et al. 1986). Low levels of 15d-$PGJ_2$ were observed in metastatic tissues compared to non-metastatic tissues, suggesting that 15d-$PGJ_2$ may influence the development of cancer and its progression to metastasis *in vivo* (Badawi A. et al. 2002). However, the precise mechanisms underlying the ability of 15d-$PGJ_2$ to act as an anti-tumoral agent are yet poorly understood.

## 1.3 Enzymes involved in prostaglandin biosynthesis

### 1.3.1 Phospholipase $A_2$ enzymes

Arachidonic acid, the common precursor of eicosanoids, is stored at the sn-2 position of membrane glycerophospholipids, and released by the hydrolytic action of phospholipase A2 ($PLA_2$) enzymes (Fig. 1). Several $PLA_2$s were identified based on their nucleotide gene sequences. They were classified mainly into three groups: (i) cytosolic $PLA_2$ ($cPLA_2$), (ii) $Ca^{2+}$-dependent secretory $PLA_2$ ($sPLA_2$), and (iii) $Ca^{2+}$-independent intracellular $PLA_2$ ($iPLA_2$). Under stimulated conditions, $Ca^{2+}$-dependent cytosolic $PLA_2$s ($cPLA_2$s) and several secretory $PLA_2$s ($sPLA_2$s) are responsible for releasing AA from glycerol-based phospholipids to form PGs and related molecules (Jones R. et al. 2003). Mitogen-activated protein kinases (MAPK), protein kinase A (PKA) and C (PKC) were shown to regulate $PLA_2$ activity (Chakraborti S. 2003; Pinelli E. et al. 1999; Antonio V. et al. 2002; Thomas G. et al. 2000). The upregulation of $PLA_2$ enzymes results in the production of a free pool of AA that is required for the synthesis of various eicosanoids.

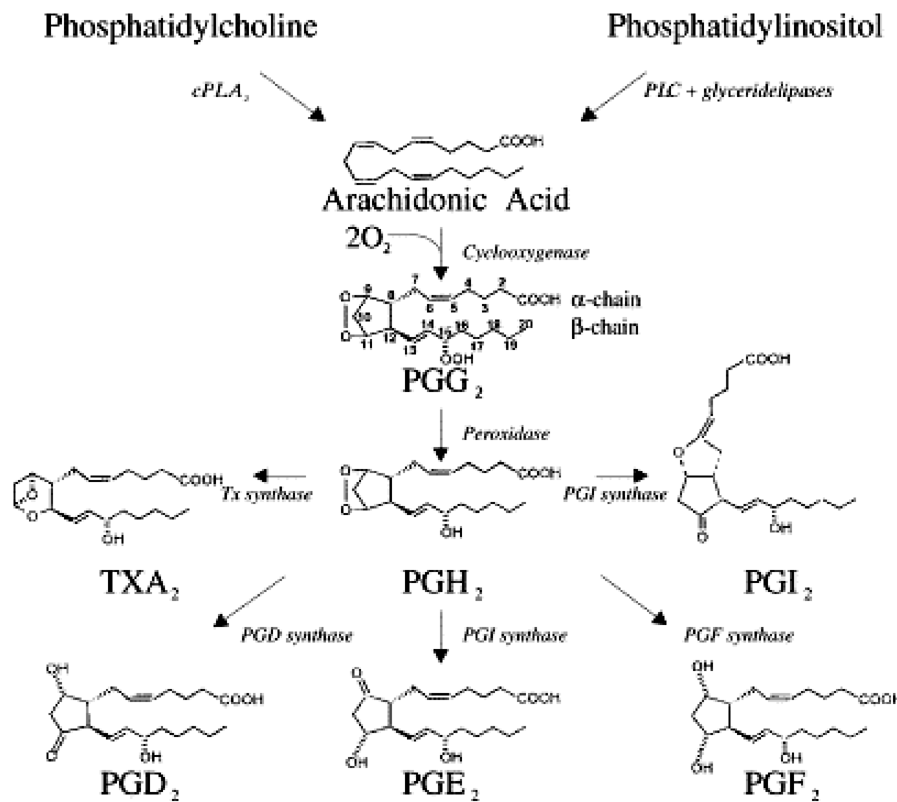


***Fig. 5. Biosynthetic pathways of prostanoids****. Arachidonic acid is converted to the prostanoid precursor PGG*$_2$*, which is subsequently peroxidized to PGH*$_2$*. Both enzymatic reactions are catalyzed by the COX protein, which consists of two forms: the endoplasmic reticulum-localized COX-1, and COX-2, that acts predominantly at the nuclear envelope. Prostaglandin H*$_2$ *spontaneously rearranges or is enzymatically isomerised, oxidized, or reduced to yield bioactive prostaglandin isomers, some of which are shown.*

### 1.3.2 Cyclooxygenase enzymes

AA is metabolized to the unstable intermediate prostanoid, $PGH_2$, by the action of COX enzymes. COX, also known as prostaglandin H synthase, is a heme-containing enzyme that catalyzes two sequential enzymatic reactions; first, the bis-oxygenation of AA leading to the production of $PGG_2$ (COX reaction) and second, reduction of 15-hydroperoxide of $PGG_2$ leading to the formation of $PGH_2$ (hydroperoxidase reaction) via separate active sites of the enzyme (Smith W. et al. 2000). Two COX isoforms, COX-1 and COX-2, were found in mammals. Both enzymes are encoded by separate genes on different chromosomes: COX-1 is located on chromosome 9, COX-2 on chromosome 1. COX-1 and COX-2 display ~ 60% sequence identity and both possess homodimeric structure. Both COX have a molecular

weight of 72 kDa made up of a dimeric complex of two polypeptides, each of which requires one molecule of heme for maximal catalytic activity (Roth G. et al. 1981; Brown J. et al. 2005).

It is commonly considered that COX-1 is constitutively expressed in a wide variety of cells and plays a housekeeping role, whereas COX-2 is a stimulus-inducible enzyme that is implicated in pathological conditions, such as inflammation, pain, fever and cancer (Morita I. 2002). In general, COX-1 is more enriched in the Golgi, endoplasmic reticulum (ER) than in the perinuclear envelope and COX-2 is located predominantly in the perinuclear envelope. The promoter structure of the COX-1 gene suggests that specificity protein 1 (Sp1), activator protein -2 (AP-2), and nuclear factor for interleukin-6 (NF-IL-6) are involved in the regulation of COX-1 gene expression. The two Sp1 sites contribute to constitutive expression of COX-1 (Xu X. et al. 1997). The promoter region of the COX-2 gene reveals a typical feature of the immediate early genes, which contains various transcription elements, such as NF-IL-6, AP-2, Sp1, NF-kB and cAMP response element (CRE). Three mitogen-activated protein kinase (MAPK) cascades: (i) extracellular signal-regulated kinase1/2 (ERK1/2), (ii) c-jun N-terminal kinase/stress-activated protein kinases (JNK/SAPK), (iii) p38 (Su B. et al. 1996; Simmons D. et al. 2004; Murakami M. et al. 2004) contribute to the induction of COX-2 gene concertedly or independently. The nuclear receptor, peroxisome proliferator-activated receptor (PPAR) $\gamma$ was also shown to regulate the expression of COX-2 (Inoue H. et al. 2000; Straus D. et al. 2000; Meade E. et al. 1999).

COX-2 induction is associated with various premalignant and malignant lesions of epithelial and nonepithelial origin. Gastric, hepatic, esophageal, pancreatic, head and neck, lung, breast, bladder, cervical, endometrial, skin, and colorectal cancers all shown to display elevated COX-2 expression when compared with nonmalignant tissue (Koki A. et al. 2002). In regard to experimental evidence, nonselective inhibitor of COX-2, NSAIDs, have been known to inhibit cancer formation in rodent models of colorectal cancer since the 1980s, and the development of selective COX-2 inhibitors has shown equal promise in rodent models and in several models of colorectal cancer (Brown J. et al. 2005). More compelling evidence for the role of COX-2 in the cancer formation was provided by genetic studies in mice. Genetic inhibition of COX-2 reduced the development of colonic polyps in the adenomatous polyposis coli$^{\Delta 716}$ (APC$^{\Delta 716}$) murine model (Oshima M. et al. 1996). In a model of mammary tumorigenesis, overexpression of COX-2 alone in mammary glands was sufficient to induce cellular transformation and resulted in the formation of breast carcinomas (Liu C. et al. 2001). Similarly, COX-2 overexpression in basal keratinocytes induced hyperplasia, skin

premalignancy and skin malignancy (Neufang G. et al. 2001; Muller-Decker K. et al. 2002). Together these studies underline the important role of COX-2 in the initiation and promotion of carcinogenesis.

In 2002, Chandrasekharan and colleagues reported the isolation of a splice-variant of COX-1 mRNA found in highest concentrations in the cerebral cortex and heart of the dog, which they termed ''COX-3''. COX-3 is made from the COX-1 gene but retains intron 1. This intron contains an open reading frame that introduces an insertion of 30–34 amino acids, depending on mammalian species. COX-3 was postulated to be a 65-kDa protein. COX-3 mRNA is mainly expressed in cerebral cortex and heart and exhibits glycosylation-dependent COX activity. Comparison of COX-3 with COX-1 and COX-2 demonstrates that this enzyme is selectively inhibited by analgesis/antipyretic drugs, such as acetaminophen and phenacetin (Murakami M. et al. 2004).

## 1.4 Prostaglandin E synthases

The final step of the $PGE_2$-synthesizing cascade is a nonoxidative rearrangement of the COX product, $PGH_2$, into $PGE_2$ (Fig. 5). Although this isomerization may also occur nonenzymatically, living cells produce $PGE_2$ via a catalytic reaction, which is attributed to several discovered proteins: cytosolic prostaglandin E synthase, microsomal prostaglandin E synthase-1 and microsomal prostaglandin E synthase -2 (Tanioka et al. 2000; Jakobsson PJ. et al. 2000; Watanabe et al. 1999).

### 1.4.1 Cytosolic prostaglandin E synthase

Cytosolic prostaglandin E synthase (cPGES) is a 23 kDa cytosolic protein that is identical to the Hsp90-associated protein p23 (Tanioka T. et al. 2000). The gene for cPGES is localized to human chromosome 12q13.13. cPGES is expressed ubiquitously and constitutively in the cytosol of a variety of tissues and cells, with an exception in rat brain in which LPS treatment increases its amount several-fold (Tanioka T. et al. 2000). cPGES resides predominantly in the cytosol and moves to the endoplasmic reticulum after $Ca^{2+}$ ionophore challenge. Cotransfection and antisense experiments indicated that cPGES is capable of converting COX-1-, but not COX-2- derived $PGH_2$ to $PGE_2$ in cells. Thus, in line

with the roles of COX-1 *in vivo*, cPGES may contribute physiologically to the production of $PGE_2$ required for the maintenance of tissue homeostasis. cPGES activation in cells requires its binding to Hsp90 (Tanioka T. et al. 2003) and phosphorylation by casein kinase 2 (Kobayashi T. et al. 2004).

cPGES requires glutathione (GSH) as an essential cofactor for its activity. The $K_m$ and $V_{max}$ values of recombinant cPGES for $PGH_2$ are estimated to be 14 μM and 0.19 μM/min/mg protein, respectively (Tanioka T. et al. 2000).

### 1.4.2 Microsomal prostaglandin E synthase-1

Membrane associated prostaglandin E synthase-1 (mPGES-1), originally designated MGST1-L1 (membrane-bound glutathione S-transferase 1-like-1) belongs to the Membrane Associated Proteins in Eicosanoid and Glutathione metabolism (MAPEG) family (Jakobsson PJ. et al. 1999). This protein family consists of six human proteins including 5-lipoxygenase-activating protein (FLAP), leukotriene $C_4$ ($LTC_4$) synthase, microsomal glutathione S-transferase 1 (MGST1), MGST2, MGST3, and MGST1-L1 (Jakobsson PJ. et al. 2000). All MAPEG proteins show significant homology and have similar molecular masses of 14–18 kDa.

mPGES-1 requires GSH as an essential cofactor for its activity. The $V_{max}$ value of purified $His_6$-mPGES-1 was 170 μM/min/mg for the conversion of $PGH_2$ to $PGE_2$ at 37°C and $K_m$ for $PGH_2$ was 160 μM. Two-dimensional crystal analysis revealed that mPGES-1 protein forms a trimer (Thoren S. et al. 2003).

The gene for human mPGES-1 maps to chromosome 9q34.3 (Forsberg L. et al., 2000). The promoter of the human mPGES-1 gene contains binding sites for AP-1 and early growth response (Egr-1) transcriptional factor. p38 and p42/44 MAPK, ERK as well as JNK are all involved in the regulation of mPGES-1 in human (Han R. et al. 2002; Giannico G. et al. 2005).

mPGES-1 is involved in various physiological and pathological events, such as inflammation, reproduction, bone metabolism, Alzheimer's disease and tumorigenesis (Murakami M. et al. 2004). Stimulation of different cultured cells with proinflammatory stimuli leads to a marked elevation of mPGES-1 expression with a concomitant elevation of COX-2 expression and $PGE_2$ production. There are some data indicating an important role for the transcription factors NF-κB and PPARγ in the co-ordinate regulation of the COX-

2/mPGES pathway (Catley M. et al. 2003; Mendez M. et al. 2003). However, the kinetic induction of COX-2 and mPGES-1 often differs significantly (Stichtenoth D. et al. 2001), suggesting distinct regulatory mechanisms for their expression. COX-2 and mPGES-1 are colocalized in the perinuclear membrane. Colocalization of the two sequential biosynthetic enzymes in the same subcellular compartment may allow efficient transfer of the unstable substrate $PGH_2$ from COX-2 to mPGES-1 (Murakami M. et al. 2002; Murakami M. et al. 2000; Mancini J. et al. 2001).

Involvement of mPGES-1 downstream of COX-2 in tumorigenesis has been suggested by the observation that transfection of mPGES-1 in combination with COX-2, but not with COX-1, into HEK293 cells leads to cellular transformation, which was manifested by aggressive growth, piling up and aberrant morphology (Murakami M. et al. 2000). The COX-2/mPGES-1-cotransfected cells formed a number of large colonies in soft agar culture and were tumorigenic when implanted into nude mice (Kamei D. et al. 2003). Furthermore, the expression of mPGES-1 as well as COX-2 is markedly elevated in several types of cancer, including human colon adenomas and cancers and overexpressing Ras, a well established signalling molecule in cancer, caused a several-fold increase in mPGES promoter activity (Yoshimatsu K. et al. 2001, Yoshimatsu K., Golijanin D. et al. 2001).

### 1.4.3 Microsomal prostaglandin E synthase -2

Membrane-associated, GSH-non specific, PGES-2 was originally purified from microsomal fraction of bovine heart (Watanabe K. et al. 1999), was cloned and termed mPGES-2. The gene for mPGES-2 is localized to human chromosome 9q33-34 in proximity to the genes for mPGES-1 and COX-1 (Tanikawa N. et al. 2002).

The cDNA encodes a 41 kDa protein that contains an N-terminal hydrophobic region, which is removed by proteolytic processing in the Golgi membrane. Proteolytic cleavage occurs presumably between amino acid residues 87-88 and then truncated protein is distributed in the cytosol with a trend to be enriched in the perinuclear region. Both, intact and the N-terminal truncated, mPGES-2 show similar catalytic activities (Tanikawa N. et al. 2002).

Most recently, N-terminal truncated mPGES-2 complexed with the non-steroidal anti-inflammatory drug indomethacin (IMN) has been crystallized and the complex structure has been determined (Fig. 6).

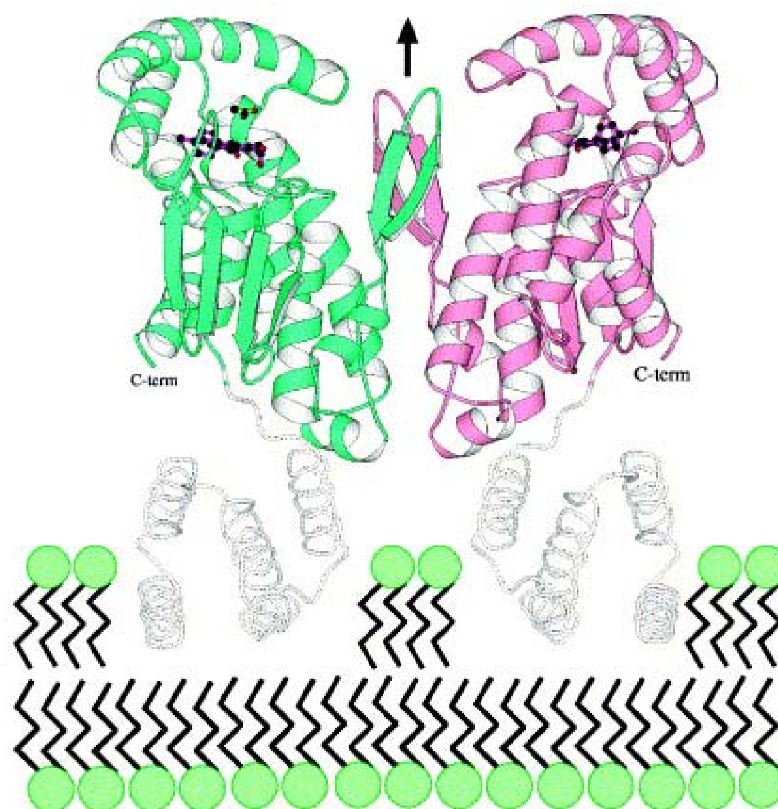


***Fig. 6. Overall structure of mPGES-2. A dimeric mPGES-2 sits on the lipid bilayer.*** *Two subunits are shown by aquamarine and light-pink, respectively. Parts of the truncated N-terminal section (residues 1–87) and disorder section (residues 88-99) shown by white are built based on a secondary structure prediction and a hydropathic index analysis, and are apparently associated with the lipid bilayer (Yamada T. et al. 2005).*

mPGES-2 forms a dimer being attached to the lipid membranes by anchoring the N-terminal section. Two hydrophobic pockets connected to form a V shape are located in the bottom of a large cavity. It was supposed, that when $PGH_2$ binds to the cavity in mPGES-2 and the endoperoxide moiety of $PGH_2$ is located in the catalytic site, the isomerization

reaction is initiated by a proton transfer from cystein110 to O11 of $PGH_2$. mPGES-2 catalyzes the isomerization of $PGH_2$ to $PGE_2$ without the presence of an R–SH compound (Yamada T. et al. 2005). The $V_{max}$ and $K_m$ values for $PGH_2$ of the purified recombinant mPGES-2 were about 3.3 μmol/min/mg of protein and 28 μM, respectively (Tanikawa N. et al. 2002).

mPGES-2 can be coupled with both COX-1 and COX-2 enzymes. mPGES-2 is expressed constitutively in several tissues in which mPGES-1 expression is relatively low and is not increased appreciably during tissue inflammation (Murakami M. et al. 2003, Guay J. et al. 2004). However, considerable elevation of mPGES-2 expression was observed in human colorectal cancer (Murakami M. et al. 2003). The regulation of mPGES-2 remains unclear. Nevertheless, there is evidence that neither MAPK kinases nor hormones are capable to regulate the expression of mPGES-2 (Giannico G. et al. 2005, Duffy D. et al. 2005).

## 2. Aim of the present investigation

$PGE_2$ is the major prostaglandin involved in colorectal carcinogenesis. $PGE_2$ induces cell proliferation, inhibits apoptosis as well as promotes motility and angiogenesis, moreover elevated levels of $PGE_2$ have been shown in colorectal cancer. The biosynthesis of $PGE_2$ is accomplished by several terminal prostaglandin E synthases through catalytical conversion of the COX product $PGH_2$. Among the known terminal prostaglandin E synthases, mPGES-1 and mPGES-2 were found to be overexpressed in colorectal cancer, however the role and regulation of these enzymes in this tumour entity are yet not fully understood. 15d-$PGJ_2$ is a cyclopentenone prostaglandin, which possesses anti-neoplastic, anti-inflammatory and anti-viral activities. The inhibition of cell growth and induction of apoptosis by this compound was previously described. Moreover, 15d-$PGJ_2$ was shown as a key regulator of negative feedback of the COX pathway in an inflammatory setting. Based on these data, it was planned to determine the potential implication of 15d-$PGJ_2$ in $PGE_2$ mediated colorectal cancer promotion.

It was demonstrated that mPGES-1 and COX-2 enzymes are co-regulated, however, in several cases, mPGES-1 was not found to be linked to COX-2, and $PGE_2$ production by mPGES-2 using $PGH_2$ supplied by both COX-1 and COX-2, has also been demonstrated. In contrast, expression of cPGES has been postulated to be linked with COX-1. There is also a body of evidence, indicating a link between PG biosynthesis and 15d-$PGJ_2$ by means of a feedback control of COX-2 by this PG metabolite. Moreover, 15d-$PGJ_2$ has been shown to inhibit mPGES-1 *in vitro*.

In the present study it was planned to investigate the effect of 15d-$PGJ_2$ on PGES expression in colorectal cancer cell lines and to find out which COX isoforme is linked to this regulation.

# 3. Materials and Methods

## 3.1 Materials

### 3.1.1 Chemicals

| | |
|---|---|
| 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ | Alexis, Carlsbad, CA, USA |
| 2-propanol<br>Acetic acid | Merck, Darmstadt, Germany |
| Acrylamid solution | AppliChem, Darmstadt, Germany |
| Agarose NEEO | Roth, Karlsruhe, Germany |
| Ammonium peroxodisulfate | Merck, Darmstadt, Germany |
| Arachidonic acid | Cayman Chemical, Ann Arbor, MI, USA |
| ATP | Sigma-Aldrich, St. Louis, MO, USA |
| BW245C | Cayman Chemical, Ann Arbor, MI, USA |
| $CaCl_2$<br>Chloroform<br>Citric acid | Merck, Darmstadt, Germany |
| Complete™ | Roche Diagnostics GmBH, Manheim, Germany |
| Coomassie protein assay, precision plus protein™ standards | Bio-Rad, Hercules, Ca, USA |
| Crystal violet | AppliChem, Darmstadt, Germany |
| Developer solution | Fujifilm, Tokyo, Japan |
| Diethyl pyrocarbonate (DEPC)<br>Dithiothreitol | Sigma-Aldrich, St. Louis, MO, USA |
| DNA gel loading buffer ( 6× ) | Novagen, Madison, WI, USA |
| dNTPs | Bioline, London, U.K. |
| Dulbecco's modified Eagle medium (DMEM)<br>Dulbecco's PBS (1×) | Gibco/ Invitrogen, Karlsruhe, Germany |
| Ethanol | Merck, Darmstadt, Germany |

| | |
|---|---|
| Ethidium bromide<br>$FeCl_2 \times 4H_2O$ | AppliChem, Darmstadt, Germany |
| Fixer solution | Fujifilm, Tokyo, Japan |
| Foetal Bovine serum (FBS) | Gibco/ Invitrogen, Karlsruhe, Germany |
| Formaldehyde | Otto Fishar GmBH, Saarbrueken, Germany |
| Forskolin<br>Fura-2 acetoxymethyl ester | Sigma-Aldrich, St. Louis, MO, USA |
| Geneticin | Gibco/ Invitrogen, Karlsruhe, Germany |
| Glucose | Sigma-Aldrich, St. Louis, MO, USA |
| Glycin | Roth, Karlsruhe, Germany |
| HCl | Merck, Darmstadt, Germany |
| HEPES | Sigma-Aldrich, St. Louis, MO, USA |
| Hyper film | Amersham Biosciences, Buckinghamshire, U.K. |
| KCl<br>$K_2HPO_4$<br>$KH_2PO_4$<br>KOH | Merck, Darmstadt, Germany |
| Lactate dehydrogenase kit | Roche, Mannheim, Germany |
| MCC-555 | Cayman Chemical, Ann Arbor, MI, USA |
| Methanol | Mallinckrodt Baker B. V., Deventer, Holland |
| $MgCl_2$ | Merck, Darmstadt, Germany |
| MuLV reverse transcriptase | Applied Biosystems, Branchburg, NJ, USA |
| *N*-acetylcysteine | Sigma-Aldrich, St. Louis, MO, USA |
| NaCl | Merck, Darmstadt, Germany |
| Nitrocellulose transfer membrane | BioSciense, San Jose, Ca, USA |
| Non-essential Amino Acid (100×) | Gibco/ Invitrogen, Karlsruhe, Germany |
| Oligo $d(T)_{16}$ | Applied Biosystems, Branchburg, NJ, USA |
| Oligonucleotides | BioSpring, Frankfurt, Germany |

| | |
|---|---|
| Penicillin/Streptomycin 10000U | Gibco/ Invitrogen, Karlsruhe, Germany |
| Pico Green (dsDNA Quantitation Kit) | MolecularProbes/Invitrogen, Karlsruhe, Germany |
| Ponceau S stain | Sigma-Aldrich, St. Louis, MO, USA |
| Probenecid | |
| Prostaglandin $A_2$ | |
| Prostaglandin $E_2$ enzyme immunoassay kit | Cayman Chemical, Ann Arbor, MI, USA |
| Prostaglandin $D_2$ | |
| Ready-Load™ 100 bp DNA Ladder | Invitrogen, Carlsbad, Ca, USA |
| RNase inhibitor | Applied Biosystems, Branchburg, NJ, USA |
| RNAzol | ISO-TEX Diagnostics, Friendswood, TX, USA |
| Roti Load 1 | Roth, Karlsruhe, Germany |
| SDS | AppliChem, Darmstadt, Germany |
| Sodium Pyruvate 100 mM | Gibco/ Invitrogen, Karlsruhe, Germany |
| Sucrose | AppliChem, Darmstadt, Germany |
| SuperSignal® West Pico Luminol/Enhancer Solution | Pierce, Rockford, IL, USA |
| SuperSignal® West Pico Stable Peroxide Solution | |
| Taq polymerase | Applied Biosystems, Branchburg, NJ, USA |
| TBE buffer (10 ×) | Invitrogen, Carlsbad, Ca, USA |
| TEMED | AppliChem, Darmstadt, Germany |
| TRIS | Sigma-Aldrich, St. Louis, MO, USA |
| Trypan blue stain, 0,4% | Gibco/ Invitrogen, Karlsruhe, Germany |
| Trypsin-EDTA (10×) | |
| Tween-20 | Sigma-Aldrich, St. Louis, MO, USA |

Prostaglandin $H_2$ was a generous gift from Prof. M. Hamberg, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden.

### 3.1.2 Antibodies

First antibodies

| | |
|---|---|
| Anti-β-actin monoclonal antibody produced in mouse | Sigma-Aldrich, St. Louis, MO, USA |
| Prostaglandin E synthase (cytosolic) polyclonal antibody, produced in rabbit<br>Prostaglandin E synthase-2 (microsomal) polyclonal antibody produced in rabbit | Cayman Chemical, Ann Arbor, MI, USA |

Prostaglandin E synthase-1 (microsomal) polyclonal antibody, produced in rabbit, was a kind gift from Prof. P-J Jakobsson, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden.

Secondary antibodies:

| | |
|---|---|
| Anti-mouse IgG, horse radish peroxidase, made in goat. | Santa-Cruz Biotechnology, Santa-Cruz, Ca, USA |
| Anti-rabbit IgG, horse radish peroxidase, made in goat | Vector Laboratories, Inc, Burlingame, Ca, USA |

### 3.1.3 Mediums and Solutions

Medium for Caco-2 and HCT 116 cells:

DMEM containing 4.5 g/L glucose and 25 mM Hepes, supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin, 1% non-essential amino acids and 1% pyruvate.

Culture medium for PPARγ dominant-negative mutant Caco-2 cells: DMEM + 400 µg/ml geneticin.

Buffer and solutions

*DEPC-water:* 0.1% DEPC solution was incubated at 37°C for 1 hour and then autoclaved.

*Tris-glycine buffer*: 25 mM Tris, 192 mM glycine, 0.1% SDS; pH 8.3

*Blot-buffer*: 25 mM Tris, 192 mM glycine, 16.5% methanol; pH 8.3

*Tween Tris Base Saline (TBS-T) buffer*: 10 mM Tris/HCl, 100 mM NaCl, 0.05% Tween-20; pH 7.5.

*Potassium phosphate buffer (0.1 M)*: 39 mM $KH_2PO_4$, 61 mM $K_2HPO_4$; pH 7.4
84 mM $KH_2PO_4$, 16 mM $K_2HPO_4$; pH 6.5

*Stop solution*: 25 mM $FeCl_2$, 50mM citric acid.

*FURA-buffer*: 135 mM NaCl, 4.6 mM KCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 11 mM glucose, 11 mM Hepes, 2.5 mM Probenecid; pH 7.4.

### 3.1.4 Cell lines

Human colon cancer cell lines (Caco-2 and HCT 116) were obtained from the European Animal Cell Culture Collection (ECACC).

**Table I**

| Cell line name | Morphology | Description | ECACC Nº |
|---|---|---|---|
| Caco-2 | Epithelial | Human Caucasian, well differentiated, colon adenocarcinoma. COX-1 non-expressing cell line. | 86010202 |
| HCT 116 | Epithelial-like | Human colon carcinoma. COX-2 non-expressing cell line. | 91091005 |

PPARγ dominant-negative mutant Caco-2 cells were kindly provided by VK Chatterjee (Department of Medicine, University of Cambridge, United Kingdom) (Gurnell M. et al. 2000). In PPARγ dominant-negative cells the highly conserved hydrophobic and charged residues ($Leu^{468}$ and $Glu^{471}$) in the ligand-binding domain of PPARγ were mutated to alanine. This PPARγ mutant retains ligand and DNA binding, but exhibits markedly reduced transactivation due to impaired coactivator recruitment (Gurnell M. et al. 2000)

### 3.1.5 Instruments

| Instrument | Supplier |
|---|---|
| Autoclave Steam-Sterilizer CV-EL18L | CertoClav Sterilizer GmbH, Traun, Austria |
| Bad-thermostat UC/5 | Julabo Labortechnik GmbH, Seelbach, Germany |
| Cell culture incubator BB6220 | Heraeus, Hanau, Germany |
| GelCamera DS-34 | Polaroid, Bedfordshire, U.K. |
| Gibco BRL horizontal gel electrophoresis apparatus Horizon 11-14 | Life technology, Carlsbad, Ca, USA |
| Laminar air flow HB2448 | Heraeus, Hanau, Germany |
| Megafuge 2.0, biofuge fresco | Heraeus, Hanau, Germany |
| Microcomputer electrophoresis power supply E443 | Consort, Turnhout, Belgium |
| Microscope, Axiovert 135 | Zeiss, Goettingen, Germany |
| Mini-centrifuge “Dual-rotor” | Roth, Karlsruhe, Germany) |
| Minigel electrophoresis apparatus | Biometra, Goettingen, Germany |
| Multiphor II IEF electrophoresis system | Amersham Biosciences, Buckinghamshire, U.K. |
| PerkinElmer/GeneAmp PCR system 2400 | Applied Biosystems Branchburg, NJ, USA |
| POLARstarOPTIMA plate reader | BMG LabTechnologies, Offenburg, Germany |
| Power supply EPS301 | Amersham Biosciences, Buckinghamshire, U.K. |
| Rocking platform WT16 | Biometra, Goettingen, Germany |
| SLT RainBow plate reader | Tecan, Maennedorf, Switzerland |
| SpectroFluor Plus plate reader | Tecan, Maennedorf, Switzerland |
| Spectrophotometer U-2000 | Hitachi, Tokyo, Japan |
| Thermomixer compact | Eppendorf, Hamburg, Germany |
| TL 100 ultracentrifuge | Beckman Instruments GmbH, Muenchen, Germany |
| Transilluminator Ti 1 | Biometra, Goettingen, Germany |
| Ultrasonic cell disruptor Microson™ | SPI Supplies, West Chester, USA |
| X-ray film processor Fuji FPM 800A | Fujifilm, Tokyo, Japan |

### 3.1.6 Software

GeneRunner 3.05 (Hasting Software Inc., Hasting, NY), Magellan™ comprehensive reader control and data reduction software (Tecan, Maennedorf, Switzerland), POLARstarOPTIMA software (BMG LabTechnologies, Offenburg, Germany), Desaga CabUVIS scanner and Desaga ProViDoc software (Desaga, Wiesloch, Germany), SigmaPlot 2001, Microsoft Office package.

## 3.2 Methods

### 3.2.1 Cell culture

Caco-2 and HCT 116 cells were maintained in DMEM medium containing 4.5 g/L glucose and 25 mM Hepes, supplemented with 10% FBS, 1% penicillin/streptomycin, 1% non-essential amino acids and 1% pyruvate. PPARγ dominant-negative mutant Caco-2 cells were cultured in the same medium, additionally containing 400 µg/ml geneticin. The medium was changed every second day. The cells were checked for Mycoplasma at monthly intervals.

For experiments, $13 \times 10^3$ cells/cm$^2$ cells were seeded and incubated at 37°C, 6% $CO_2$, until the cells were 40–50% confluent. The new medium with or without effectors was then added and the cells were incubated for the periods of time indicated.

### 3.2.2 Cell proliferation

Cell counting was used to evaluate proliferation by using a colorimetric assay following crystal violet staining (*Westergren-Thorsson, G et al. 1991*). The cells were seeded onto 96-well plates in a volume of 100 µL medium, incubated until 40–50% confluent and exposed to increasing concentrations of effectors. After incubation for 12-48 h, cells were fixed with 5% formaldehyde and the number of viable cells was evaluated by staining with 0.05% crystal violet in 2% ethanol. After washing with phosphate buffered saline (PBS), the dye was extracted using citrate buffer, and the absorbance at 620 nm was measured. The number of cells per well was determined in comparison to a standard growth curve determined separately for each cell line and each experiment.

In a limited number of experiments, the cells were harvested by trypsinisation, stained with trypan blue and counted in a haemocytometer. The correlation coefficient of the results from both methods was 95%.

### 3.2.3 Cytotoxicity

Cytotoxicity was excluded by the quantification of cell death and cell lysis. The test was performed using a commercial lactate dehydrogenase (LDH) kit, based on the measurement of LDH activity released from the cytosol of damaged cells into the supernatant. The cell culture supernatant was collected cell-free and incubated with the reaction mixture from the kit. LDH enzyme activity was measured using microplate reader SLT RainBow at 492 nm.

### 3.2.4 Preparation of cellular fractions

The cells were washed with cold PBS and trypsinated in 1 × trypsin/EDTA for 10 min at 37ºC. Thereafter, culture medium was added and cells were centrifuged at 500 × g for 10 min followed by two washes in PBS. The cell pellets were resuspended in 1.0 ml homogenization buffer consisting of potassium phosphate buffer (0.1 M, pH 7.4), 1 x Complete$^{TM}$ protease inhibitor cocktail and sucrose (0.25 M). The samples were sonicated for 3 × 20 s at 100 W with an ultrasonic cell disruptor ( Microson™ ) 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 homogenization buffer and total protein concentration in cytosolic and microsomal fractions was determined by the Coomassie protein assay according to the manufacturer’s instructions.

### 3.2.5 Analysis of mRNA levels using RT-PCR

Cells were homogenized with RNAzol and total RNA was isolated according to the manufacturer's protocol. Concentration of total RNA was measured spectrophotometrically at 260 nm (Spectrophotometer U-2000) and reverse transcription reaction was performed. Briefly, total RNA (1 µg) in water was heated (65°C, 12 min), slowly cooled down to 20ºC,

and reverse transcribed (20 min, 42°C) in PCR buffer (10 mM Tris/HCl, 50 mM KCl; pH 8.3), containing 5 mM $MgCl_2$, 1 mM dNTPs, 1 U × $\mu L^{-1}$ MuLV reverse transcriptase, 5 µM of oligo $d(T)_{16}$, 0.5 U × $\mu L^{-1}$ RNase inhibitor.

After reverse transcription, reaction samples were amplified using following polymerase chain reaction (PCR) mixture: cDNA in PCR buffer (10 mM Tris/HCl, 50 mM KCl, 1.5 mM $MgCl_2$; pH 8.3), containing 0.2 mM dNTPs, 0.2 µM primer and 0.05 U × $\mu L^{-1}$ Taq polymerase and then was denaturated 2 min at 94°C and amplified, using depicted primers and conditions as described in Table II (Appendix), using a PerkinElmer/GeneAmp PCR system 2400.

Aliqouts of the PCR mixtures (10 µl) were analysed by electrophoresis using a 1% agarose gel containing 0.5 µg/ml ethidium bromide. For semi-quantitative analysis of amplified PCR products the fluorescent dye Pico Green® was used according to the manufacturer's instructions (Singer V. et al. 1997).

### 3.2.6 Immunoblot analysis

Samples were diluted and boiled for 5 min in Roti Load 1 sample buffer. Proteins were separated on Tris-glycine polyacrylamide gels (15% for cPGES and mPGES-1, and 12% for mPGES-2). After electrophoresis gels were washed in blot-buffer for 15 min to remove SDS and electroblotted onto nitrocellulose membranes. Transfer efficiency was visualized using Ponceau S stain, then membranes were washed in TBS-T buffer. Membranes were blocked overnight using TBS-T, and 3% non-fat dry milk. After washing the membranes with TBS-T, antibodies against mPGES-1, mPGES-2 and cPGES were added at a 1:1,000, 1:5,000 and 1:500 dilution, respectively, in TBS-T and incubated for 2.5h. Membranes were incubated with antibodies against mPGES-1 and cPGES diluted in TBS-T buffer with 2% non-fat dry milk. After washing the membranes three times for 10 minutes with TBS-T, a horseradish peroxidase-conjugated goat antirabbit IgG was added at a 1:10,000 (mPGES-1) or 1:5,000 (mPGES-2 and cPGES) dilution in the same buffer and incubated for 1 h and 2 h, respectively. The washing steps were repeated before the protein bands were visualised using chemiluminescence. The washed membranes were incubated for 5 min in SuperSignal® West Pico Luminol/Enhancer Solution and SuperSignal® West Pico Stable Peroxide Solution. Then, blots were exposed to X-ray film and developed, using a X-ray film processor. SDS-

PAGE immunoblots were quantitated by scanning densitometry, using a Desaga CabUVIS scanner and Desaga ProViDoc software.

### 3.2.7 PGES enzyme assay

PGES enzyme activity was determined according to Thoren S. et al. 2000. Microsomal or cytosolic fraction samples were diluted in potassium phosphate buffer (0.1 M, pH 6.5) containing 0.5 mM dithiothreitol (DTT). $PGH_2$ (4 µl) dissolved in acetone (0.28 mM) was kept in separate vials at -80 °C. Prior to incubation, both the substrate and samples were equilibrated at 4 °C for 2 min. The reaction was started by the addition of the sample to the tubes containing $PGH_2$ (final concentration 10 µM) and then terminated by adding stop solution lowering the pH to 3, giving a total concentration of 20 mM $FeCl_2$ and 40 mM citric acid. The reaction mixture was then diluted in EIA buffer and assayed for $PGE_2$ using an enzyme immunoassay kit. The assay is based on the competition between $PGE_2$ and $PGE_2$-acetylcholinesterase conjugate for a limited amount of $PGE_2$ monoclonal antibody. The antibody-$PGE_2$ complex bound to goat polyclonal anti-mouse IgG and substrate for acetylcholinesterase was added. The absorbance was determined using 5,5'-dithio-*bis*-(2-nitrobenzoic acid) at 412nm.

### 3.2.8 Measurement of intracellular calcium level

Measurement of intracellular calcium was performed according to (Sabirsh A. et al. 2003). Caco-2, HCT 116 and Caco-2 PPARγ dominant-negative mutant cells were seeded onto black, clear bottomed 96-well plates (Greiner bio-one, Kremsmuenster, Austria). The plates were incubated for 4-5 days at 37°C, 6% $CO_2$, until the cells were 80–90% confluent. The growth medium was then exchanged for growth medium without FBS containing 4 µM Fura-2 acetoxymethyl ester (Fura-2-AM), 2.5 mM Probenecid; the cells were incubated for 1 h at 37°C and 6% $CO_2$. After that loading time, the cells were washed four times with 50 µl of FURA-buffer to remove free FURA-2AM. A final 50 µl volume of FURA-buffer was added to each well. Fluorescence was monitored following 120 s using a fluorometer POLARstarOPTIMA, after injection of 50 µl of buffer or solution containing test substances. The ratio of emitted fluorescence, following stimulation by 340- and 380-nm light, was

calculated following background subtraction at each wavelength. Cells not exposed to FURA-2 were used to subtract background auto-fluorescence.

### 3.2.9 Statistics

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

# 4. Results

## 4.1. 15d-$PGJ_2$ down-regulates mPGES-2 gene and protein expression

### 4.1.1 Effect of 15d-$PGJ_2$ on gene expression of the enzymes of prostaglandin E2 synthesis

Since cyclooxygenases and prostaglandin E synthases are known to be involved in colon cancer development and progression, the possible effect of 15d-$PGJ_2$, on the mRNA expression of these enzymes was first assayed. HCT 116 and differentiated Caco-2 cells were treated with increasing concentrations of 15d-$PGJ_2$ from 0.1 to 40 μM and cells were incubated during 4 hours.

Total RNA was isolated from samples and RT-PCR was performed. The amount of PCR products was examined using the Pico Green dsDNA quantitation kit. As a control of mRNA expression of target genes house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was analysed. Fig. 7A and B depict an expression of COX and PGES mRNA in Caco-2 and HCT 116 colon cancer cell lines. The levels of cPGES, mPGES-1, COX-1 (expressed in HCT 116) and COX-2 (expressed in Caco-2) remained unchanged following treatment with this cyclopentenone, while the expression of mPGES-2 mRNA was down-regulated by 15d-$PGJ_2$ in both cell lines tested. The effect of 15d-$PGJ_2$ on mPGES-2 mRNA levels was dose dependent. HCT 116 cells were more sensitive to 15d-$PGJ_2$ than Caco-2 cells; the 50% inhibition of mRNA expression was at 5 μM concentration of 15d-$PGJ_2$ for HCT 116 and at 10 μM for Caco-2 cells. Concentrations above 40 μM led to the death of HCT 116 cells within 4 hours of incubation.

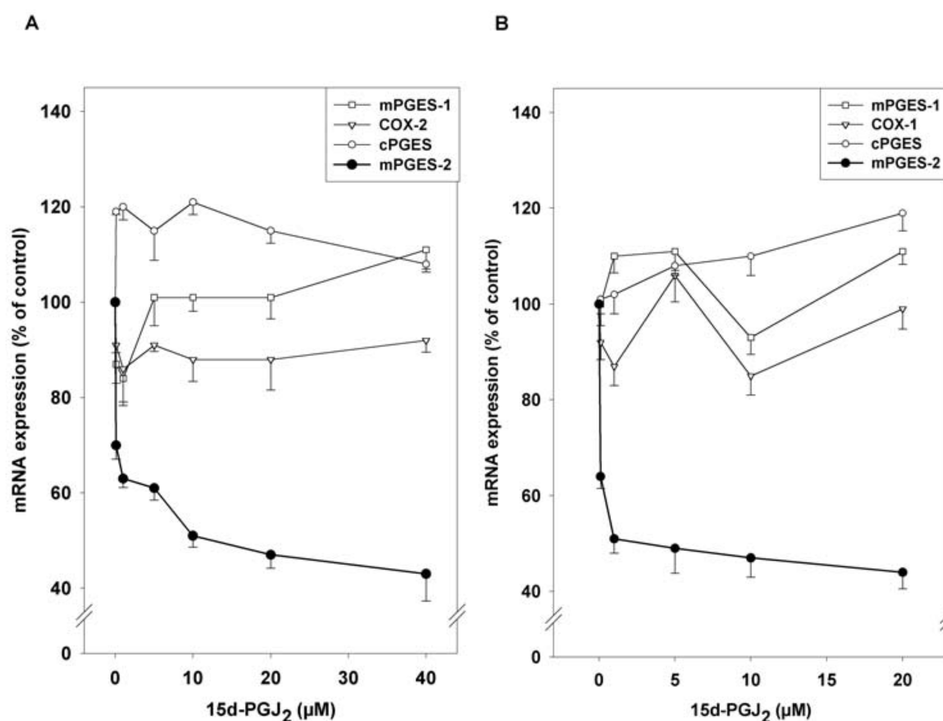


***Fig. 7. Inhibition of mPGES-2 mRNA expression by 15d-PGJ$_2$ in the colorectal cancer cell lines Caco-2 and HCT 116.*** *Caco-2 (A) and HCT 116 (B) cells were incubated for 4 h in the absence or presence of indicated concentrations of 15d-PGJ$_2$. RT-PCR was performed on total RNA for COX-2, COX-1, mPGES-1, mPGES-2, cPGES, and GAPDH during the linear phase of amplification. All values for mRNA are normalized to the corresponding mRNA amount for GAPDH.*

The concentrations of 15d-PGJ$_2$ induced 50% reduction of mPGES-2 mRNA expression were then used for a time course analysis. Colon cancer cells were treated with 15d-PGJ$_2$ for different time intervals, from 1 to 24 hours. (HCT 116 cells were stimulated with 5 µM and Caco-2 with 10 µM 15d-PGJ$_2$). mPGES-2 mRNA expression was analysed as described above. As shown in Fig. 8, 15d-PGJ$_2$ transiently inhibited mPGES-2 mRNA expression with a maximal suppression at 4 hours for both cell lines.

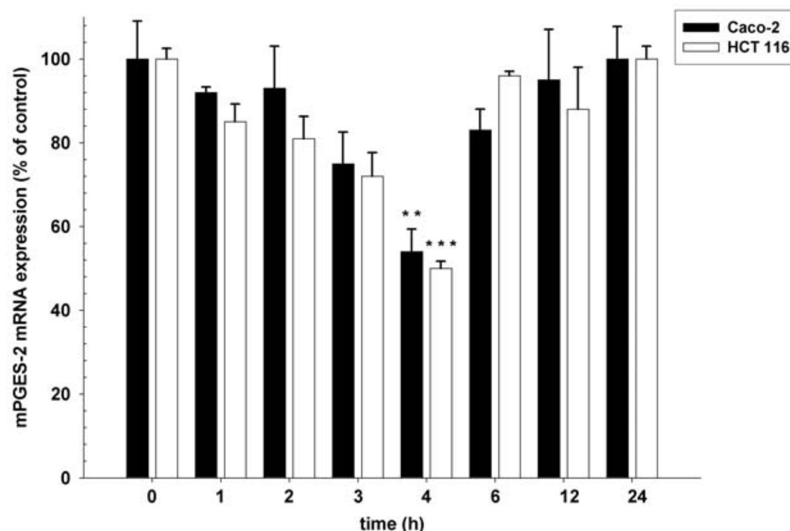


***Fig. 8. Transient effect of 15d-PGJ$_2$ on mPGES-2 mRNA expression in the colorectal cancer cell lines Caco-2 and HCT 116.*** *Time course of mPGES-2 mRNA expression in Caco-2 (black bars) cells treated with 10 µM of 15d-PGJ$_2$ and HCT 116 (white bars) cells stimulated with 5 µM of 15d-PGJ$_2$ for indicated incubation periods. Total RNA was isolated as described in the method section and subjected to semi-quantitative RT-PCR by the fluorescent dye PicoGreen®. All values for mRNA are normalized to the corresponding mRNA amount for the housekeeping gene GAPDH. The statistical significance of changes relative to untreated controls is expressed: **P < 0.01, ***P < 0.001.*

#### 4.1.2 15d-PGJ$_2$ decreases mPGES-2 protein expression

To determine if the reduction of mPGES-2 mRNA level correlated with decreases in protein expression cell lines were treated with 1 and 10 µM 15d-PGJ$_2$ for various time intervals. Since recent work by Murakami M. et al. could demonstrate that mPGES-2 is synthesized as a Golgi membrane-associated protein, and then additionally distributed in the cytosol with local enrichment in the perinuclear region, the subcellular distribution of this enzyme in response to 15d-PGJ$_2$ was also determined. Cell lysate was divided in microsomal and cytosolic fractions and the amount of mPGES-2 protein was analysed by immunoblotting as described in the method section. The intensity of protein spots was quantitated by scanning densitometry, using a Desaga CabUVIS. As a control of protein expression, the expression of the house-keeping gene β-actin was analysed.

As shown in Fig. 9, at a concentration of 1 µM 15d-$PGJ_2$, the suppressive effect on protein expression in HCT 116 was observed as early as 12 h after treatment, whereas in the Caco-2 cell line reduction of mPGES-2 protein expression only occurred after an incubation period of 36 h. At later time points, protein level gradually returned to baseline levels. When incubated with 10 µM 15d-$PGJ_2$, transient suppression of protein reached its maximum already at 6 h for HCT 116 and 12 h for Caco-2.

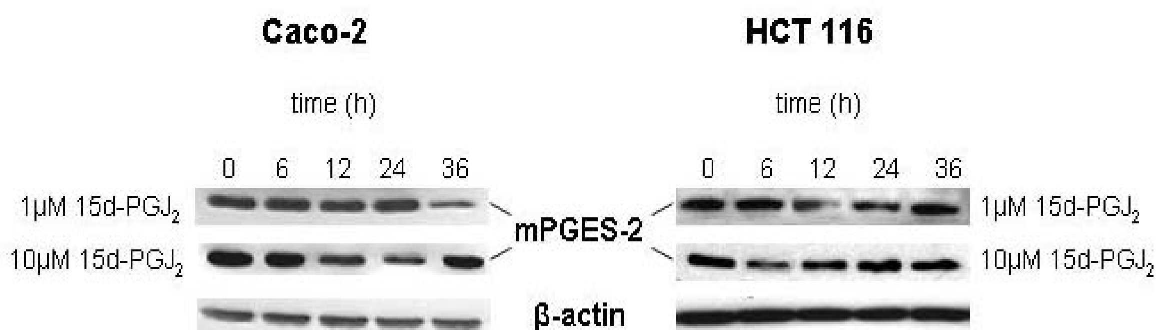


***Fig. 9. Down-regulation of mPGES-2 protein expression by 15d-$PGJ_2$ in Caco-2 and HCT 116 cells.*** *Western blot analysis of mPGES-2 protein expression in Caco-2 and HCT 116 cells incubated in the presence of 1 and 10 µM 15d-$PGJ_2$ for the times indicated. In all lanes, 15 µg of protein from the microsomal fraction of cells was analyzed. The results shown are representative of three separate experiments performed in duplicate.*

mPGES-2 could be detected in both, the cytosolic and membrane fraction. As demonstrated in Fig. 10, the concentration of mPGES-2 protein remained unchanged in cytosolic fraction.

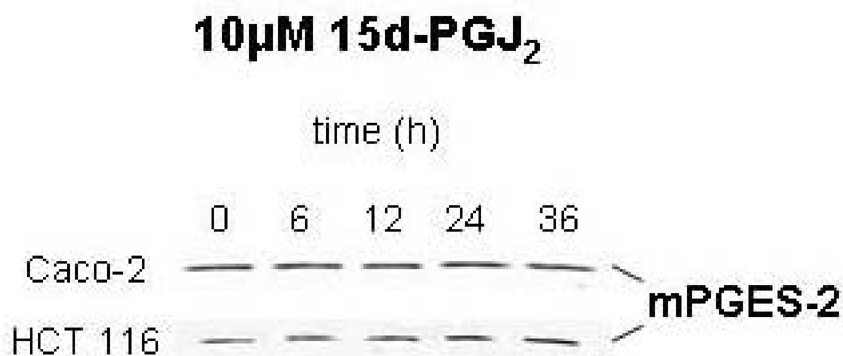


***Fig. 10. mPGES-2 protein distribution in the response to 15d-PGJ$_2$.*** *Western blot analysis of mPGES-2 protein expression in Caco-2 and HCT 116 cells incubated in the absence and presence of 10 µM 15d-PGJ$_2$ for the times indicated. In all lanes, 15 µg of protein from the cytosolic fraction of cells was analyzed. The results shown are representative of three separate experiments performed in duplicate.*

In contrast, no distinct mPGES-1 protein could be detected in either cell line. As shown in Fig. 11, treatment of cells with 10 µM 15d-PGJ$_2$ did not evoke any change in cPGES protein level.

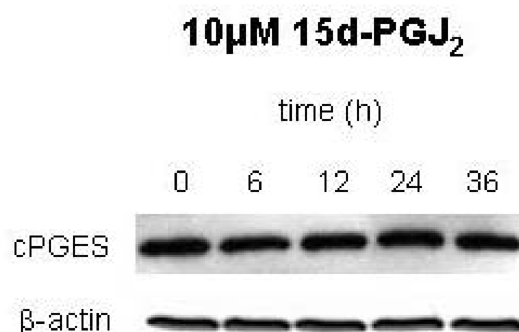


***Fig. 11. The effect of 15d-PGJ$_2$ on cPGES protein expression.*** *Western blot analysis of mPGES-2 protein expression in HCT 116 cells incubated in the absence and presence of 10 µM 15d-PGJ$_2$ for the times indicated. In all lanes, 15 µg of protein from the cytosolic fraction of cells was analyzed. The results shown are representative of two separate experiments performed in duplicate. Similar results were also obtained in Caco-2 cells (data not shown).*

### 4.1.3 15d-$PGJ_2$ reduces mPGES-2 enzyme activity

To confirm that down-regulation of mPGES-2 gene and protein expression leads to a decrease in $PGE_2$ production, Caco-2 and HCT 116 cells were treated with 10 µM 15d-$PGJ_2$ for 12 hours. Thereafter, cells were lysed, divided on fractions and PGES enzyme activity in both, cytosolic and microsomal fractions was determined. The amount of produced $PGE_2$ was 140 ng/min/mg protein in cytosolic fraction and about 20 ng/min/mg in microsomal fraction. The relative amount of activity as compared with control is depicted at Fig. 12.

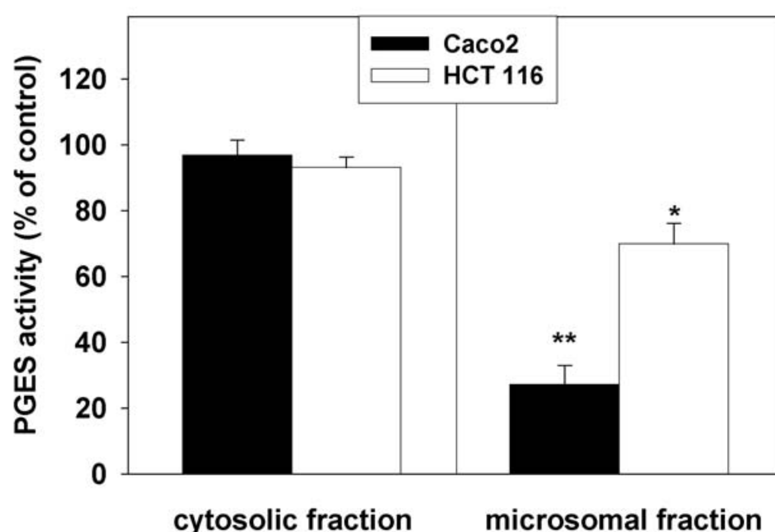


***Fig. 12. Dependence of PGES activity in the microsomal and cytosolic fraction by 15d-$PGJ_2$ in the colorectal cancer cell lines Caco-2 and HCT 116.*** *Caco-2 and HCT 116 cells were treated with 10 µM 15d-$PGJ_2$ for 12 h. Cytosolic and microsomal fractions were separated and enzymatic activity was performed as described in the experimental procedures. The relative amount of activity as compared with control in Caco-2 (black bars) and HCT 116 (white bars) is depicted. Mean ± SEM of three independent experiments performed in triplicate are plotted. The statistical significance of changes relative to unstimulated controls is expressed: *$P < 0.05$, **$P < 0.01$.*

In accordance to the down-regulation of the mPGES-2 on the gene and protein level, a substantial decrease in enzyme activity could be noticed upon treatment with 15d-$PGJ_2$. Enzymatic activity was only found to be reduced in the microsomal fraction, while in the cytosolic fraction the activity remained unchanged. The notably discrepancy in microsomal mPGES-2 activity reduction between both cell lines may be explained, most likely, by a

different sensitivity of HCT 116 and Caco-2 to 15d-$PGJ_2$, as already reflected in the gene expression studies.

## 4.2. 15d-$PGJ_2$ does not act via PPARγ or PGD receptors but may cause oxidative stress and covalent modification of proteins

### 4.2.1 Decreased expression of mPGES-2 in response to 15d-$PGJ_2$ treatment is PPARγ independent

Since 15d-$PGJ_2$ has long been established a natural ligand of PPARγ we examined whether another PPARγ agonist – the thiazolidinedione homolog MCC555 may also reduce mPGES-2 expression. Since the most efficient concentration of MCC555 necessary to activate PPARγ was described to be 50 µM (Reginato M. et al. 1998), this concentration was used to treat differentiated Caco-2 cells and HCT 116 cells. Cells were then cultured for various incubation periods (0-24 h). Total RNA was isolated from samples and RT-PCR was performed as described earlier. All values for mRNA were normalized to the corresponding mRNA amount for the housekeeping gene GAPDH. 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. The effect of MCC555 on mRNA expression of COX and PGES enzymes is summarized in Fig. 13A and B.

This observation suggested that the effect of 15d-$PGJ_2$ on mPGES-2 expression might be independent of PPARγ.

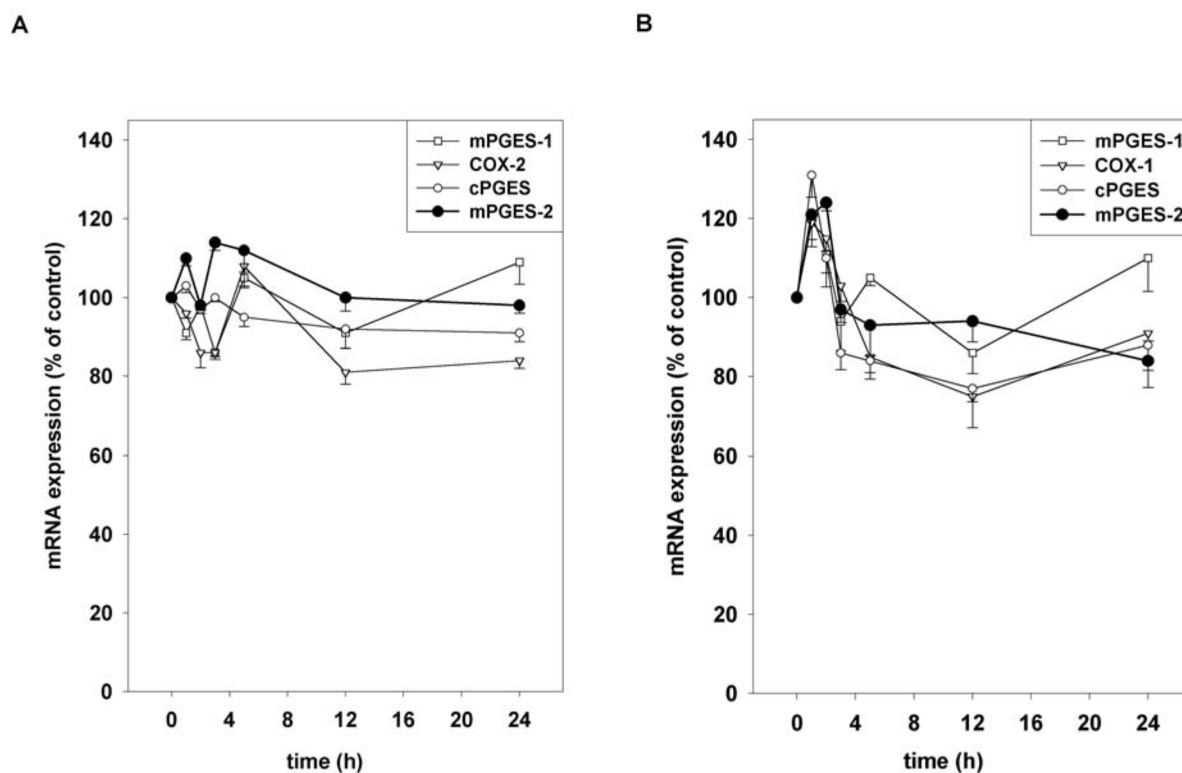


***Fig. 13. The effect of the thiazolidinedione homolog MCC555, a synthetic PPARγ agonist, on COX and PGES mRNA expression.*** *Caco-2 (A) and HCT 116 (B) cells were incubated with 50 μM MCC555 for indicated incubation periods. RT-PCR was performed on total RNA for COX-2, COX-1, mPGES-1, mPGES-2, cPGES, and GAPDH during the linear phase of amplification. All values for mRNA are normalized to the corresponding mRNA amount for GAPDH. The statistical significance of changes in mPGES-2 mRNA expression relative to untreated controls is $P < 0.001$.*

To further confirm this hypothesis, Caco-2 PPARγ dominant-negative mutant cells were subjected to 15d-$PGJ_2$ treatment. The same concentrations as for HCT 116 and Caco-2 cells in previous experiments (5, 10, 20, 40 μM) were used. Cells were incubated for 4 hours and mRNA expression of COX-2, mPGES-1, cPGES and mPGES-2 was analysed. As it shown in Fig. 14, the levels of cPGES, mPGES-1 and COX-2 remained unchanged following treatment with 15d-$PGJ_2$, while the expression of mPGES-2 mRNA was reduced to a similar extent when compared with the non-transfected Caco-2 cell line or HCT 116 cells. The 50% inhibition of mRNA expression was at 10 μM concentration of 15d-$PGJ_2$, similar to the results obtained with non-transfected Caco-2 cells.

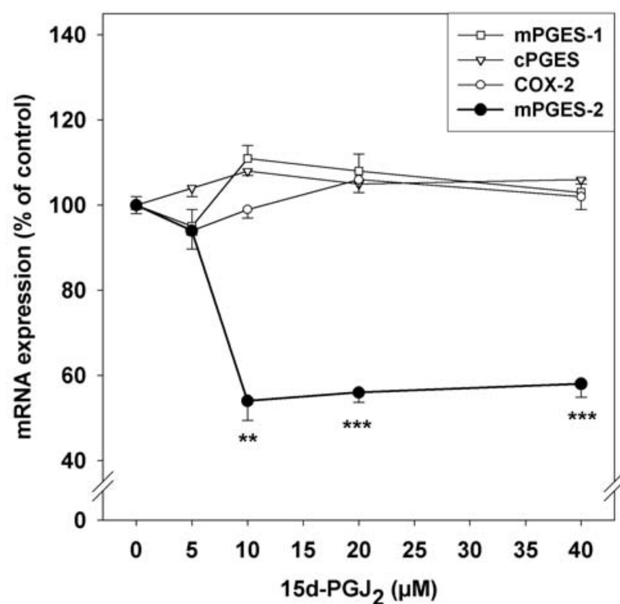


***Fig. 14. Inhibition of mPGES-2 mRNA expression by 15d-PGJ$_2$ in PPARγ dominant-negative mutant Caco-2 cells.*** *PPARγ dominant-negative mutant Caco-2 cells were treated for 4 h in the absence or presence of indicated concentrations of 15d-PGJ$_2$. RT-PCR was performed on total RNA for COX-2, mPGES-1, mPGES-2, cPGES, and GAPDH during the linear phase of amplification. All values for mRNA are normalized to the corresponding mRNA amount for GAPDH. The statistical significance of changes relative to untreated controls is expressed: **$P < 0.01$, ***$P < 0.001$.*

Additionally, the protein expression of mPGES-2 was determined. Caco-2 PPARγ dominant-negative mutant cells were incubated with 10 μM 15d-PGJ$_2$ for 12-36 h and protein amount in microsomal and cytosolic fractions were analysed by immunoblotting. The suppressive effect on protein expression was observed at 12-24 hours after treatment, as illustrated in Fig. 15, similar to the results obtained with Caco-2 non-transfected cells. At later time points, protein level gradually returned to baseline levels. In contrast, the concentration of mPGES-2 protein remained unchanged in cytosolic fraction.

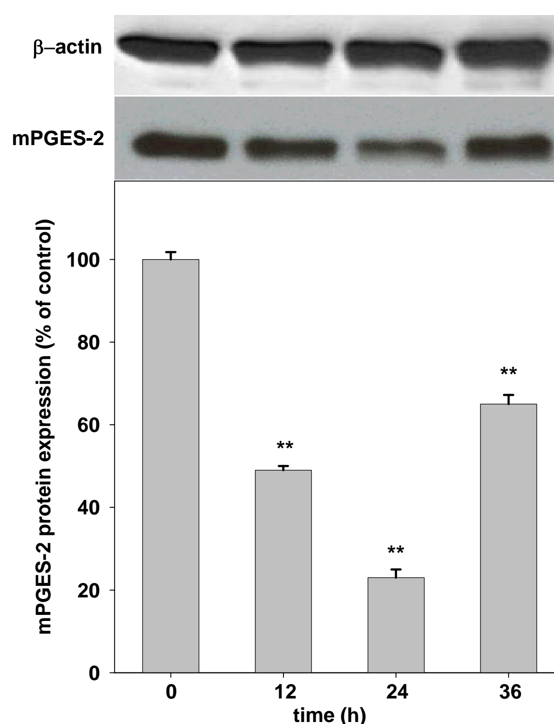


***Fig. 15. Inhibition of mPGES-2 protein expression by 15d-PGJ$_2$ in PPARγ dominant-negative mutant Caco-2 cells.*** *Immunoblot analysis of mPGES-2 protein expression in PPARγ dominant-negative mutant Caco-2 cells incubated in the absence and presence of 10 μM 15d-PGJ$_2$ for the times indicated. 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. The statistical significance of changes relative to untreated controls is expressed: **$P < 0.01$.*

PGES enzyme activity was also assayed in Caco-2 PPARγ dominant-negative mutant cells. The relative amount of $PGE_2$ produced by PGES after treatment with 10 μM 15d-PGJ$_2$ for 12 hours in microsomal and cytosolic fraction is shown in Fig. 16. Similar reduction of $PGE_2$ production could be detected if compared to Caco-2 non-transfected cells. A strong decrease of the enzyme activity in the microsomal fraction was observed, while in the cytosolic fraction the activity remained unchanged.

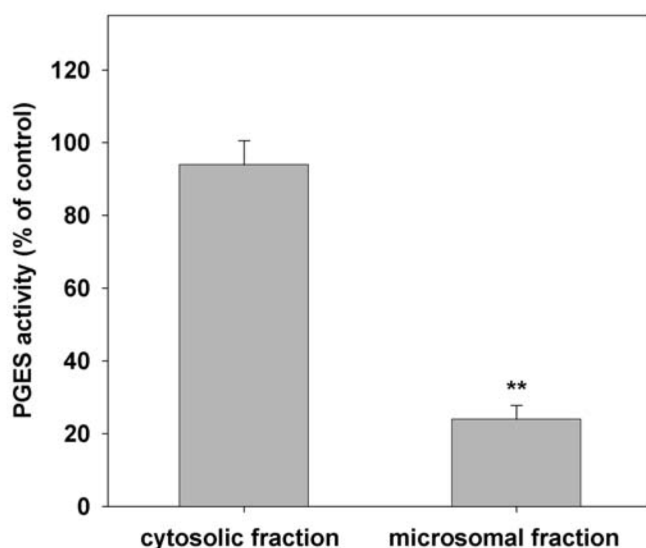


***Fig. 16. Dependence of PGES activity in the microsomal and cytosolic fraction by 15d-PGJ$_2$ in PPARγ dominant-negative mutant Caco-2 cells.*** *PPARγ dominant-negative mutant Caco-2 cells were treated with 10 µM 15d-PGJ$_2$ for 12 h. Cytosolic and microsomal fractions were separated and enzymatic activity was performed as described in the experimental procedures. The relative amount of activity as compared with control is depicted. Mean ± SEM of two independent experiments performed in triplicate are plotted. The statistical significance of changes relative to unstimulated controls is expressed: **P < 0.01.*

### 4.2.2 15d-PGJ$_2$ does not affect mPGES-2 expression via cell surface receptors

15d-PGJ$_2$ can exert its action on cell metabolism via binding to the PGD$_2$ receptors DP1 and CRTH2 (Powell W. 2003). DP1 activation by PGD$_2$ leads to the stimulation of adenylyl cyclase activity and an increase in intracellular cAMP levels, while CRTH2 activation by PGD$_2$ leads to an increase in intracellular calcium mobilization.

An activation of CRTH2 receptor by 15d-PGJ$_2$ have been examined measuring intracellular calcium levels, as illustrated in Fig. 17. Cells were loaded with the calcium sensitive fluorescent dye FURA-2AM and the effects of 15d-PGJ$_2$ on intracellular calcium levels in Caco-2, HCT 116 and Caco-2 PPARγ dominant-negative mutant cells, were compared with those of PGD$_2$ and another cyclopentenone prostaglandin PGA$_2$. Different concentrations of agonist (from 1 to 10 µM) were used. In repeated experiments neither 15d-

$PGJ_2$ and $PGA_2$ nor $PGD_2$ stimulated calcium mobilization. The cells did, however, respond normally to ATP, positive control.

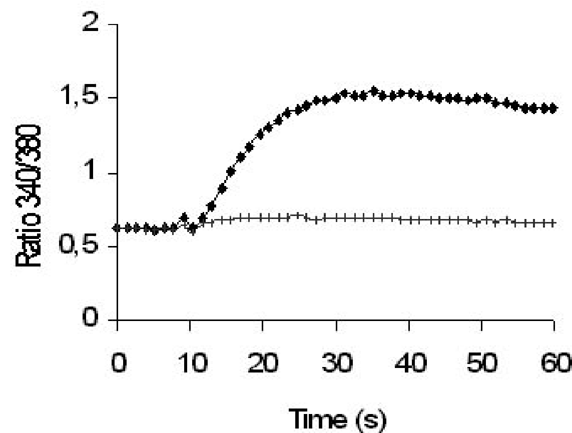


***Fig. 17. The regulation of mPGES-2 expression by 15d-PGJ$_2$ is not mediated via CRTH2 receptor activation.*** *Course of $[Ca^{2+}]_i$ in Fura-2/AM loaded HCT 116 cells. Cells were challenged with 10 µM 15d-PGJ$_2$ (+) or 10 nM ATP (♦), serving as a positive control. Representative data from three experimental observations are given. Similar results were also obtained with 15d-PGJ$_2$ in Caco-2 cells as well as with PGA$_2$ and PGD$_2$ in both, Caco-2 and HCT 116 cells (data not shown).*

Given that activation of the other $PGD_2$ receptor, DP1, leads to an increase in intracellular cAMP levels, the effect of increasing intracellular cAMP levels on mPGES-2 protein expression was tested. HCT116 cells were treated with 10 and 100 µM forskolin (Yang F. et al. 2004; Ostrom R. et al. 2001), which directly stimulates adenylyl cyclase and thus increases intracellular cAMP levels. Cells were cultured for 12-36 h, when the most intense protein expression inhibition by 15d-$PGJ_2$ was observed. However, Fig. 18 demonstrates that mPGES-2 protein expression was not affected following the treatment with various concentrations of forskolin.

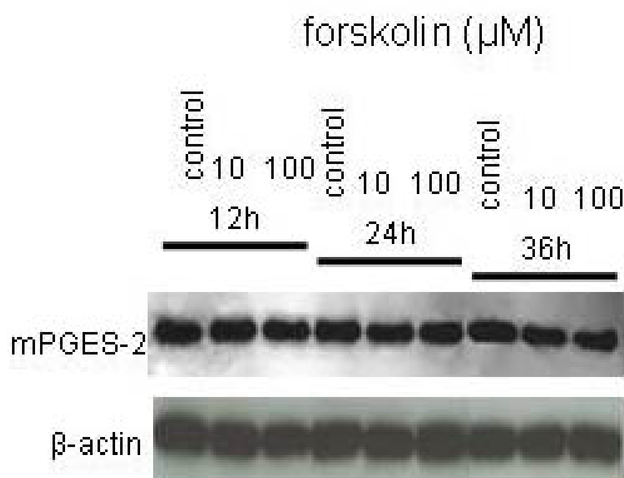


***Fig. 18. The regulation of mPGES-2 expression by 15d-PGJ$_2$ is not mediated via DP1 receptor activation.*** *Immunoblot analysis of mPGES-2 protein expression in HCT 116 cells in response to forskolin, which directly activates adenyl cyclase as an indication of DP1 receptor activation, for the various concentrations and times indicated. The results shown are representative of two independent experiments. Identical results were also observed in Caco-2 cells (data no shown).*

These experiments revealed that the effect of 15d-PGJ$_2$ on mPGES-2 expression is independent of PGD$_2$ receptor activation in HCT 116 and Caco-2 colon cancer cells.

#### 4.2.3 The role of cyclopentenone ring structure in the down-regulation of mPGES-2 expression

To verify or exclude a selective effect of 15d-PGJ$_2$ on mPGES-2, HCT 116, Caco-2 and Caco-2 dominant negative PPARγ mutant cells were treated with similar concentrations (1, 10, 20 μM) of another cyclopentenone prostaglandin, PGA$_2$. Cells were incubated for 12-36 hours and protein level in microsomal and cytosolic fractions was analysed by immunoblotting. PGA$_2$ displayed a similar inhibitory effect on mPGES-2 protein expression.

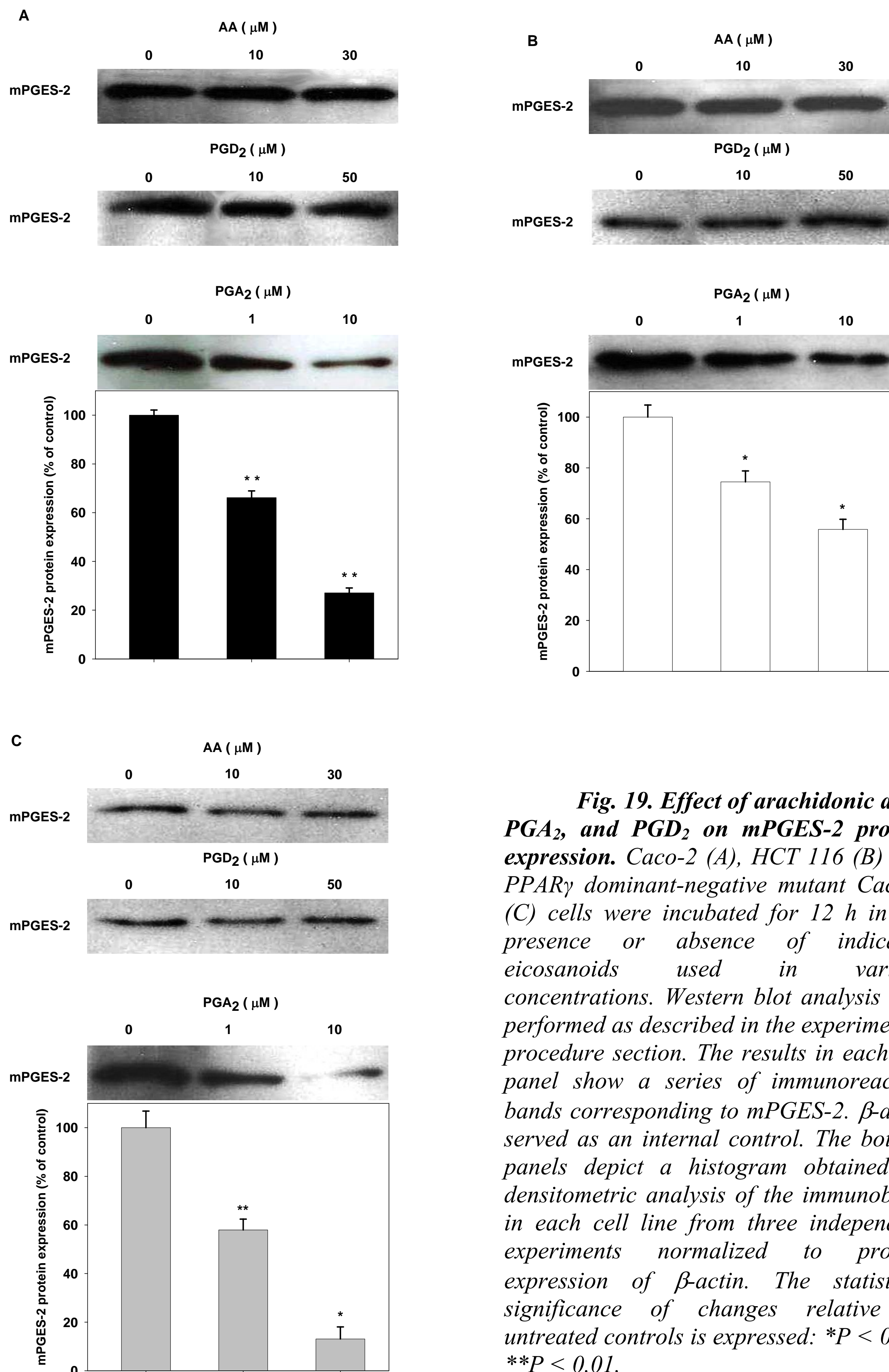


***Fig. 19. Effect of arachidonic acid, $PGA_2$, and $PGD_2$ on mPGES-2 protein expression.*** *Caco-2 (A), HCT 116 (B) and PPARγ dominant-negative mutant Caco-2 (C) cells were incubated for 12 h in the presence or absence of indicated eicosanoids used in various concentrations. Western blot analysis was performed as described in the experimental procedure section. The results in each top panel show a series of immunoreactive bands corresponding to mPGES-2. β-actin served as an internal control. The bottom panels depict a histogram obtained by densitometric analysis of the immunoblots in each cell line from three independent experiments normalized to protein expression of β-actin. The statistical significance of changes relative to untreated controls is expressed: *$P < 0.05$, **$P < 0.01$.*

The minimal effective concentration was found to be 1 μM in either, Caco-2 (Fig. 19A), HCT 116 (Fig. 19B) or Caco-2 dominant negative PPARγ mutant cells (Fig. 19C). The significant reduction in protein level was observed at 12 hours, whereas at 24 and 36 hours the protein concentration almost returned to baseline levels (Fig. 20). Concentrations above 20 μM led to cell death.

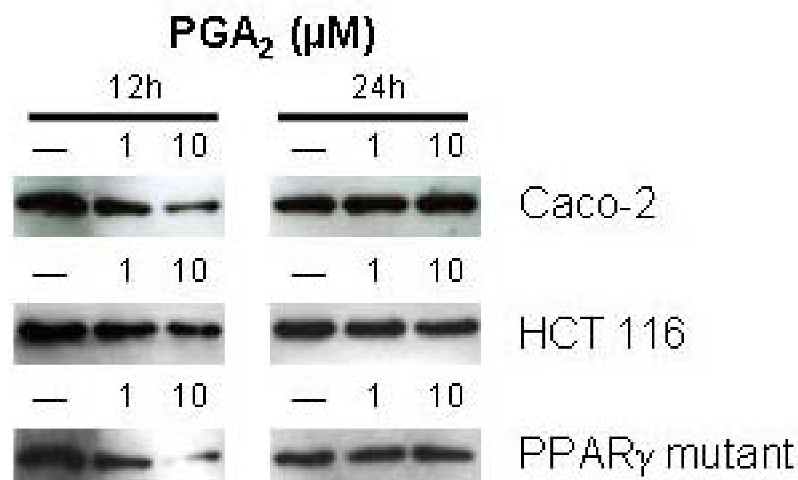


***Fig. 20. Effect of $PGA_2$ on mPGES-2 protein expression.*** *Caco-2, HCT 116 and PPARγ dominant-negative mutant Caco-2 cells were treated with different concentration of $PGA_2$ (1 and 10 μM) during 12-24 hours, as indicated. In all lanes, 15 μg of protein from the microsomal fraction of cells was analyzed. The results shown are representative of three separate experiments performed in duplicate.*

To ascertain whether the effect of prostaglandins on mPGES-2 expression is specific for cyclopentenones the cells were also treated with 10 and 50 μM $PGD_2$ and with 10 and 30 μM AA and incubated for the same period of time (12-36 h). In contrast to 15d-$PGJ_2$ and $PGA_2$, no change in mPGES-2 protein expression upon treatment with AA and $PGD_2$ could be observed (Fig. 19A, B, C).

Similar results were also obtained with leukotrienes (LT) in HCT 116 cells. Cells were treated with 10 nM $LTB_4$ or 100 nM $LTD_4$ for 12-36 hours. No changes in mPGES-2 protein concentration were observed, as presented in Fig. 21.

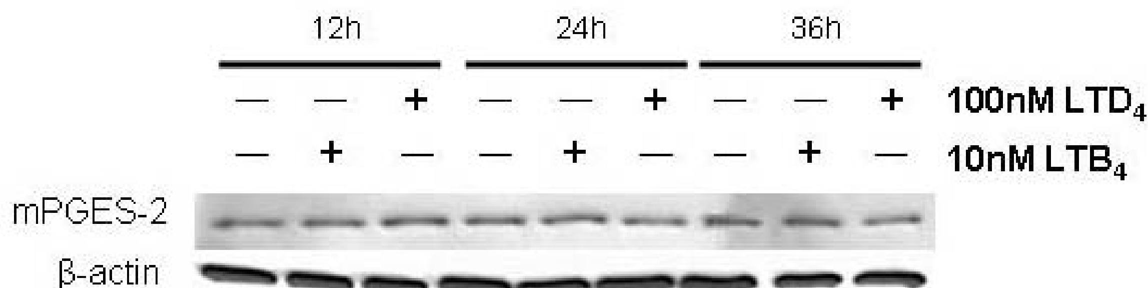


***Fig. 21. Effect of leukotrienes on mPGES-2 protein expression.*** *HCT 116 cells were treated with 10 nM $LTB_4$ and 100 nM $LTD_4$ during 12-36 hours, as indicated. In all lanes, 15 µg of protein from the microsomal fraction of cells was analyzed. The results shown are representative of three separate experiments performed in duplicate. Similar results were also obtained with $LTB_4$ and $LTD_4$ in Caco-2 cells.*

#### 4.2.4 Cyclopentenone prostaglandins induce oxidative stress

Cyclopentenone prostaglandins have been demonstrated to modulate gene expression via mechanisms involving oxidative stress; therefore the role of oxidative stress in mPGES-2 expression inhibitions was next studied. Caco-2 and HCT 116 cells were pretreated with or without antioxidants: 2 mM dithiothreitol (DTT) or 5-30 mM *N*-acetylcysteine (NAC), for 2 hours. Then cells were stimulated with 15d-$PGJ_2$ for the next 12 hours and protein level in microsomal and cytosolic fractions was analysed by immunoblotting. As shown in Fig. 22 and 23, 15d-$PGJ_2$ mPGES-2 protein expression inhibition was completely reversed by antioxidants and this effect was dose dependent. At concentrations of NAC higher than 5 mM even an upregulation of mPGES-2 protein could be observed.

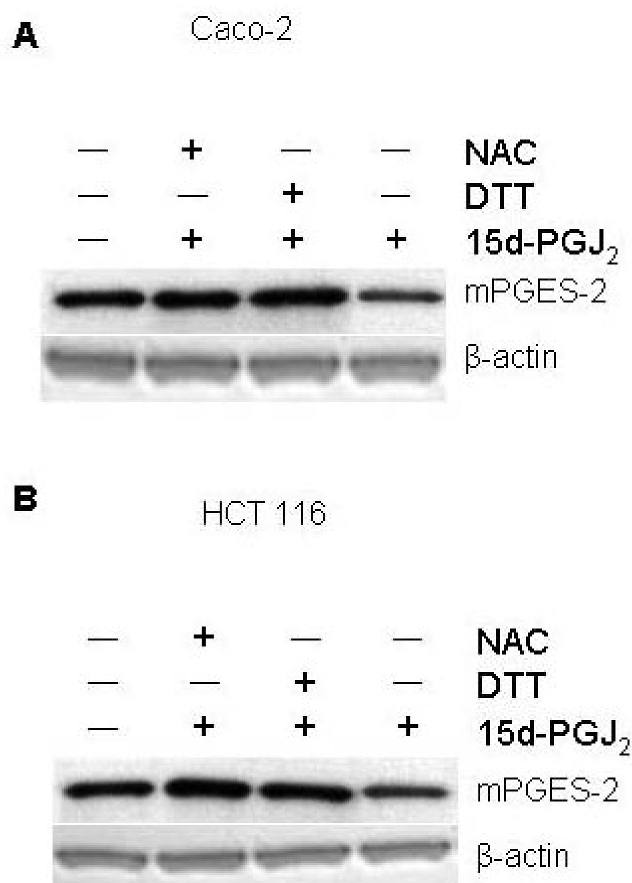


***Fig. 22. Influence of antioxidants on the inhibitory effects of 15d-PGJ$_2$ on mPGES-2 expression in colorectal cancer cells.*** *Caco-2 (upper panel) and HCT 116 (bottom panel) cells were pretreated for 2 h in the presence or absence of 30 mM NAC or 2 mM DTT. Then cells were stimulated with 10µM 15d-PGJ$_2$ for the next 12 h. Protein amount was analyzed using immunoblotting of 15 µg protein from the microsomal fraction of cells with β-actin serving as an internal control. The results shown are representative of three separate experiments.*

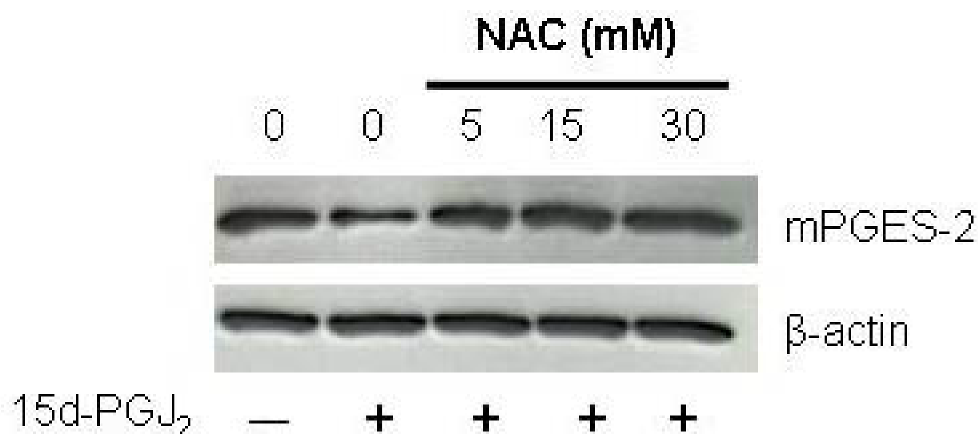


***Fig. 23. Influence of different concentration of antioxidant on the inhibitory effects of 15d-PGJ$_2$ on mPGES-2 expression in colorectal cancer cells.*** *HCT 116 cells were pretreated for 2 h in the presence or absence of 5, 15 and 30 mM NAC. Then cells were stimulated with 10 µM 15d-PGJ$_2$ for the next 12 h. Protein amount was analyzed using immunoblotting of 15 µg protein from the microsomal fraction of cells with β-actin serving as an internal control. The results shown are representative of three separate experiments.*

To further confirm the role of oxidative stress in mPGES-2 expression inhibition, HCT 116 and Caco-2 cells were subjected to 10 and 100 µM $H_2O_2$. Hydrogen peroxide is one of reactive oxygen species, which causes oxidative damage. Cells were incubated during 12-36 hours and protein level was analysed by immunoblotting. However, as presented in Fig. 24, oxidative stress induced by $H_2O_2$ did not affect mPGES-2 protein expression.

This may suggest that NAC or DTT bind 15d-PGJ$_2$, thereby preventing its impact on cellular target proteins or that exogenous hydrogen peroxide does not completely reproduce intracellular oxidative stress induced by 15d-PGJ$_2$.

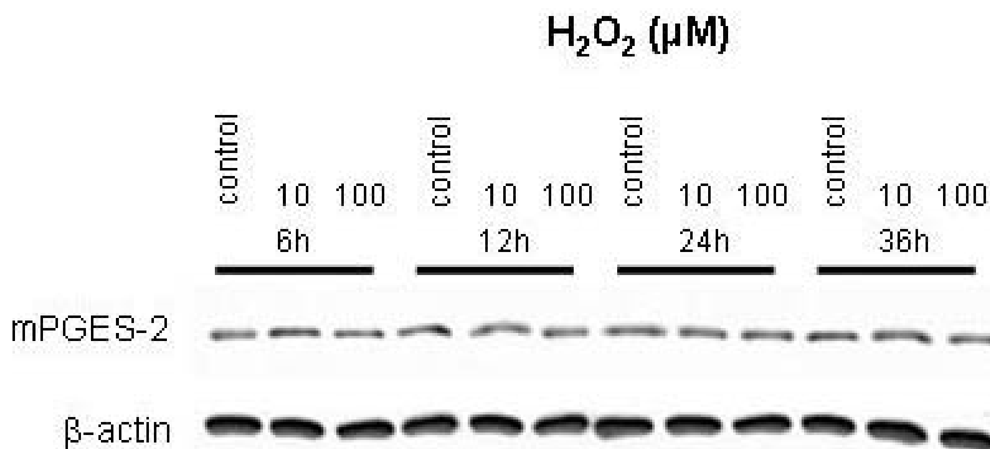


***Fig. 24. The effect of $H_2O_2$ induced oxidative stress on mPGES-2 expression in colorectal cancer cells.*** *HCT 116 cells were treated with different concentration of $H_2O_2$ (10 and 100 µM) during 6-36 hours, as indicated. In all lanes, 15 µg of protein from the microsomal fraction of cells was analyzed. The results shown are representative of two separate experiments performed in duplicate. Identical results were also observed in Caco-2 cells (data no shown).*

## 4.3 The role of mPGES-2 gene, protein and enzyme activity down-regulation in the colon cancer cell growth

### 4.3.1 The effect of 15d-$PGJ_2$ on cell growth

In order to investigate the role of mPGES-2 gene, protein and enzyme activity down-regulation by 15d-$PGJ_2$ in colon cancer cell growth, cell proliferation was analysed. HCT 116 cells were stimulated with 0.1, 1, 5, 10, 20 and 40 µM 15d-$PGJ_2$ in DMEM, containing 10% FBS. Since serum provides a broad spectrum of macromolecules including hormones and growth factors, which essentially positively affect cell growth; two controls were performed: cells grown in medium with or without FBS. 0,1 and 1 µM 15d-$PGJ_2$ had no significant effect on cell growth, whereas 5 µM 15d-$PGJ_2$ and 10 µM 15d-$PGJ_2$, demonstrated in Fig. 25, induced 20-30% and 40 – 67% reduction in cell amount at 24 – 48 h, respectively. 20 and 40 µM concentrations led to cell death after 24 hours of incubation.

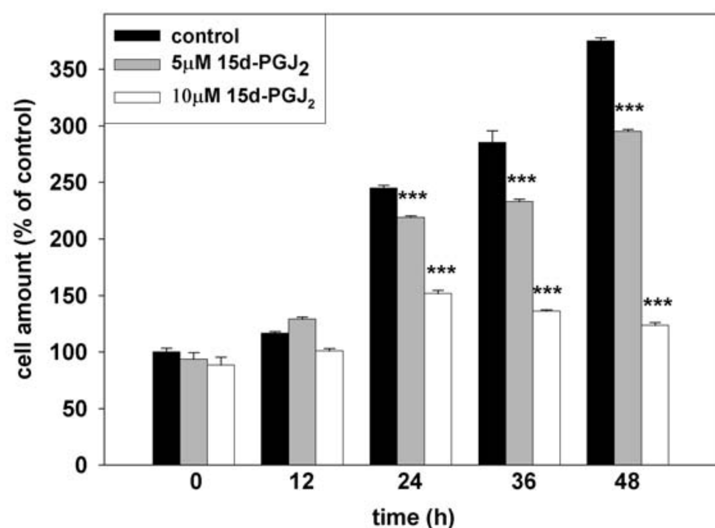


***Fig. 25. Anti-proliferative effect of 15d-PGJ$_2$ on colon cancer cell growth.*** *HCT 116 cells (2 × $10^4$/well) were placed in 96-well plates, grown in DMEM medium with 10% FBS until 40–50% confluent and then incubated in medium with or without 5 and 10 µM 15d-PGJ$_2$ for indicated time periods. After the end of experiment the number of viable cells was evaluated by staining with 0.5% crystal violet in 2% ethanol. The results were normalised to the amount of cells before stimulation. The statistical significance of changes relative to untreated controls is expressed: ***P < 0.001.*

The rate of proliferation after stimulation with 10 µM 15d-PGJ$_2$ was comparable to cells grown in serum free medium.

#### 4.3.2 The implication of PGE$_2$ formation in cell proliferation

As previously mentioned, both 15d-PGJ$_2$ and PGA$_2$ have been shown to decrease growth of cancer cells *in vitro*. Therefore the influence of PGA$_2$ on cell proliferation in the HCT 116 cell line as well as the potential involvement of the inhibition of mPGES-2 in the anti-proliferative effect of cyclopentenone prostaglandins was evaluated. HCT 116 cells were stimulated with 10 µM PGA$_2$ in DMEM, containing 10% FBS. As illustrated in Fig. 26A, exogenously added PGA$_2$ exerted a growth-inhibitory effect on HCT 116 cells, which resulted in a 20 – 30% reduction in cell amount at 24 – 48 h, respectively. At comparable concentrations the growth-inhibitory effect of PGA$_2$ was less potent than that of 15d-PGJ$_2$.

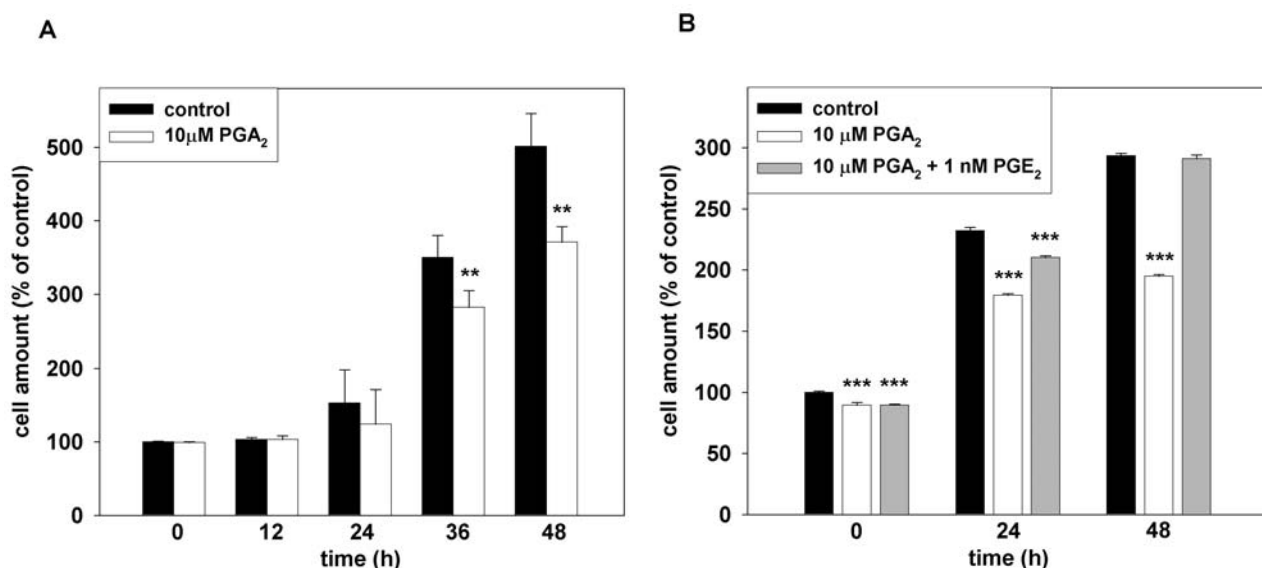


***Fig. 26. Anti-proliferative effect of $PGA_2$ on colon cancer cell growth.*** *(A) HCT 116 cells ($2 \times 10^4$/well) were placed in 96-well plates, grown until 40–50% confluent and then incubated in medium with or without 10 µM $PGA_2$ for indicated time periods. After the end of experiment the number of viable cells was evaluated by staining with 0.5% crystal violet in 2% ethanol. The results were normalised to the amount of cells before stimulation. (B) Undifferentiated HCT 116 cells ($2 \times 10^4$/well) were first treated with 10 µM $PGA_2$ for 24 h. Pretreated cells were then further stimulated with either 1 nM $PGE_2$ (grey bars) or 10 µM $PGA_2$ (white bars) and analysed at time points 0, 24 h and 48h (corresponding to 24, 48, and 72 h after the first addition of $PGA_2$). In parallel, control cells (black bars) that had not been pretreated with PGA2, were stimulated with vehicle alone. Cell count was performed as indicated above and plotted after normalisation to the amount of cells before stimulation. The statistical significance of changes relative to untreated controls is expressed: **$P < 0.01$, ***$P < 0.001$.*

As evident from Fig. 26B pre-treatment of HCT 116 with 10 µM $PGA_2$ for 24 h, and subsequent addition of 0.1-10 nM $PGE_2$ to the medium for up to 48 h completely abolished the anti-proliferative action of $PGA_2$, indicating, that down-regulation of mPGES-2 followed by reduced $PGE_2$ biosynthesis at least in part contributed to the anti-proliferative action of cyclopentenone prostaglandins in HCT 116.

# 5. Discussion

## 5.1 The role of mPGES-2 in colon cancer and its down-regulation by anti-tumour cyclopentenone prostaglandin, 15d-$PGJ_2$.

Colorectal cancer is ranked the third most common form of cancer worldwide in terms of incidence (9.4% of the world total) and mortality (7.9% of the total) (Brown et al. 2005). Interest in COX as a therapeutic target for colorectal cancer derives from epidemiologic studies that have consistently shown a 40–50% reduction in colon cancer incidence among chronic users of NSAIDs (Thun M. et al 1991; Sinicrope F et al. 2004) and the most important mechanism by which NSAIDs exert their anti-neoplastic effects is thought to be the inhibition of COX-2 activity. COX-2, but not COX-1, has also been shown to be overexpressed in an estimated 40% of human colorectal adenomas and 80% of adenocarcinomas compared to normal epithelial cells (Eberhart C. et al. 1994; Elder D. et al. 2002; Hao X. et al 1999). COX catalyze the conversion of AA to $PGG_2$ and $PGH_2$, which is subsequently enzymatically converted to a variety of PG. Studies on the roles of the various PG that act downstream of COX-2 during the development and progression of colorectal cancer clearly have established that $PGE_2$ is the major, if not only, PG implicated in colorectal cancer. Elevated levels of $PGE_2$ have been shown in colorectal cancer *in vivo* (Rigas B. et al. 1993; Pugh S. et al. 1994). The effects of exogenous $PGE_2$ on intestinal polyp formation in *Min* mice, which possess a germ line mutation in the *Apc* gene and are employed as a model of familial adenomatous polyposis in humans was also documented (Wilson J. et al. 2000; Hansen-Petrik M. et al. 2002). In addition, several *in vitro* reports have demonstrated that $PGE_2$ induces cell proliferation (Sheng H. et al. 2001), inhibits apoptosis (Sheng H. et al. 1998) as well as promotes motility and angiogenesis (Sheng H. et al. 2001; Fukuda R. et al. 2003).

Among the three known proteins capable to isomerise $PGH_2$ to $PGE_2$, mPGES-1 and mPGES-2 were found to be markedly increased in colorectal cancer tissues (Rees, B et al. 2003; Murakami M. et al. 2003). mPGES-1 and COX-2 are coexpressed in malignant and benign colorectal tumour cells and are involved in $PGE_2$ production and cell proliferation *in*

*vitro.* This effect was attributed to changes in the expression of a variety of genes related to proliferation and differentiation, transcriptional factors, genes related to cytoskeletal regulation and cell adhesion molecules (Kamei D. et al. 2003). However, until now no such information is available for mPGES-2.

As already pointed out, mPGES-1 and COX-2 enzymes are co-localized in the perinuclear envelope and functionally coupled. Coupling between COX-1 and mPGES-1 occurs only when a large amount of AA is supplied exogenously, or if burst activation of $cPLA_2$ takes place, endogenously. (Murakami M. et al. 2000). In contrast, mPGES-2 is synthesized as a Golgi membrane-associated protein. Thereafter, spontaneous cleavage of the mPGES-2 N-terminal hydrophobic domain leads to the formation of a truncated mature protein, which is distributed in the cytosol with local enrichment in the perinuclear region (Murakami M. et al. 2003). Because of such simultaneously localisation in the cytosol and perinuclear envelope, mPGES-2 can promote $PGE_2$ production via both, COX-2 and COX-1 proteins, which has been demonstrated by Murakami M et al (2003).

Several reports have also demonstrated that gene expression of mPGES-1 and COX-2 are co-regulated (Murakami M. et al. 2000; Yoshimatsu K. et al. 2001; Shinji Y et al 2005). However, in several cases COX-2 and mPGES-1 expression is not regulated by the same pathway (Rees B. et al. 2003; Devaux Y. et al. 2001; Masuko-Hongo K. et al. 2004) In contrast, expression of cPGES has been postulated to be linked with COX-1 (Tanioka T. et al. 2000). Coupling of COX-2 and mPGES-2 expression may also occur since decreased protein expression of mPGES-2 in $COX\text{-}2^{-/-}$ mice has been described (Bosetti F. et al. 2004). Based on these data PGES expression in cell lines with different COX-isoform phenotype: Caco-2 cells, which do not express COX-1 and HCT 116 cells that are not capable to produce COX-2 was examined to reveal a possible coregulation of PGES and COX enzymes.

Recent findings unravelled the role of the $15d\text{-}PGJ_2$ as a potent anti-tumoral agent. The role of apoptosis and growth inhibition caused by $15d\text{-}PGJ_2$ in a number of cell types, including endothelial cells, bone marrow-derived cells such as macrophages and lymphocytes and many tumour cells, has been evaluated. However, the apoptotic signalling pathways that are related to the $15d\text{-}PGJ_2$ are still poorly understood (Shimada T. et al. 2002; Vanaja D. et al. 2000, Emi M. et al. 2004). The inhibition of cell growth and induction of apoptosis by this compound in gastric cancer cells was accompanied by a decrease in COX-2 mRNA and protein expression as well as in $PGE_2$ production (Chen Y. et al. 2003). Changes in $PGE_2$

biosynthesis occurred prior to apoptosis, suggesting that down-regulation of COX-2 might be an upstream event of 15d-$PGJ_2$-induced cell growth inhibition and apoptosis. mPGES-1 activity was also shown to be inhibited by 15d-$PGJ_2$ (Quraishi O. et al. 2002). There is also some evidence, pointing out a link between PG biosynthesis and 15d-$PGJ_2$ in form of a feedback control of COX-2 by this PG metabolite (Inoue H. et al. 2000; Tsubouchi Y. et al. 2001). Additionally to these reports, 15d-$PGJ_2$ was found to regulate many transcriptional factors and signalling molecules related to cell cycle regulation and apoptosis including those controlled COX-2 and mPGES-1 expression. 15d-$PGJ_2$ modulates the expression and activity of p53 (Shan Z. et al. 2004), NF-κB (Rossi A. et al. 2000), JNK (Liu J. et al. 2003), PPARγ (Forman B. et al. 1995), H-RAS ( Oliva J. et al. 2003), Egr-1, Egr-3, HSP70 (Cippitelli M. et al. 2003). Together these data provide a hypothesis that 15d-$PGJ_2$ may be a potential regulator not only of COX but also PGES enzymes that could unravel a novel mechanism for the anti-proliferative effects of this compound.

In our study we sought to determine the potential implication of this cyclopentenone prostaglandin in $PGE_2$ mediated colorectal cancer promotion and PGES gene expression regulation. It was found that 15d-$PGJ_2$ selectively down-regulated mPGES-2 in the colorectal cancer cell lines Caco-2 and HCT 116 without affecting any other terminal PGES or COX. Inhibition of mPGES-2 mRNA expression was time- and dose-dependent and reduction in gene expression was followed by a time delayed decrease in mPGES-2 protein levels. The effect of 15d-$PGJ_2$ on mPGES-2 expression was transient.

To investigate the possible co-regulation of mPGES-2 with COX-2 or COX-1 colon cancer cells with different COX-isoform phenotype were treated with 15d-$PGJ_2$. Both cell lines displayed similar down-regulation of mPGES-2 gene and protein expression upon challenge with 15d-$PGJ_2$. COX-1 and COX-2 expression levels yet remained unaffected, indicating that the inhibitory effect of 15d-$PGJ_2$ on mPGES-2 is independent of COX-1 or COX-2 expression. mPGES-1 protein was not detected in our cell lines eliminating its role in metabolism and physiology of the cell lines analysed.

10 μM concentration of 15d-$PGJ_2$ induced reduction of mPGES-2 protein amount already at 6-12 hours, whereas 1 μM concentration was also efficient but on later time points, (Fig. 9, chapter 4). This suggests that little concentrations of 15d-$PGJ_2$ may reproduce the effect of high concentrations on mPGES-2 protein expression but longer incubation period are required. The reduction of mPGES-2 protein amount was observed only in the microsomal

fraction, whereas it remained unchanged in the cytosolic fraction (Fig. 10, chapter 4). Suggesting, that 15d-$PGJ_2$ does not involve cellular distribution of mPGES-2 or, most likely it could be explained by partial solubilisation of mPGES-2 during homogenisation, because this protein was described to be very sensitive to purification conditions (Watanabe K. et al. 1999). Down-regulation of mPGES-2 by 15d-$PGJ_2$ resulted in a distinct decrease in PGES activity in the microsomal fraction in both, Caco-2 and HCT 116 cells, whereas PGES activity in the cytosolic fraction remained unchanged. The relatively high level of $PGE_2$ in the cytosolic fraction may be explained by a contribution of cPGES to the cytosolic $PGE_2$ production. The protein concentration of cPGES also remained unchanged following the stimulation with 15d-$PGJ_2$ (Fig. 11, chapter 4).

Differentiated Caco-2 and undifferentiated HCT 116 cells displayed a different sensitivity to 15d-$PGJ_2$, which is expressed in a notably discrepancy in mPGES-2 protein level and activity reduction between both cell lines. This might be explained by the individual features of each cell line, which could represent different stages of carcinogenesis.

Changes in mPGES-2 mRNA and protein expression levels were not only accompanied by a significant reduction in PGES activity but also came along with diminished cell proliferation rates. The same antiproliferative effect was exerted by a parent compound, cyclopentenone prostaglandin, $PGA_2$. In contrast, exogenously added $PGE_2$ was able to completely reverse cyclopentenone prostaglandin action on cell growth. Increased proliferation by $PGE_2$ in colorectal cancer cells has already been demonstrated (Sheng H. et al. 2001; Mutoh M. et al. 2002). It was shown that $PGE_2$ mediates carcinogenic changes by acting on EP1 and EP4 receptors in the colon. EP2 receptor may also be involved in tumour progression, triggered by $PGE_2$. In contrast, EP3 does not appear to have influence in early stages of colon carcinogenesis (Mutoh M. et al. 2002; Sonoshita M. et al. 2001). Several mechanisms responsible for 15d-$PGJ_2$ induced growth inhibition have been described, including cell cycle arrest by inhibition of cyclin D1 and stimulation of p21 expression via PPARγ-dependent and –independent mechanisms (Miwa Y. et al. 2000; Wang C. et al. 2001; Kawakami S. et al. 2002), activation of the mitochondrial apoptosis pathway via inducing membranes depolarization and cytochrome *c* release (Nencioni A. et al. 2003) or by inhibition of NF-κB DNA-binding activity (Piva R. et al. 2005). Our findings strongly suggest that reduction of $PGE_2$ formation by down-regulation of mPGES-2, at least in part, may also contribute to a diminished growth of colon cancer cells in response to 15d-$PGJ_2$. In addition

to a current report by Murakami et al. (2003) demonstrating a markedly increased expression of mPGES-2 in colorectal cancer tissues, our data further support the hypothesis of an involvement of this enzyme in the development of colorectal cancer. Furthermore, the link between mPGES-2 and cancer might not be restricted to this tumour entity. Rees et al. (2003) showed that two gastric cancer cell lines (MKN-45 and MKN-74) did not express mPGES-1, but were still able to synthesize $PGE_2$. Because both, cPGES protein as well as cytosolic PGES activity could be detected, the authors assumed the possibility of an interaction of COX-2 with cPGES in gastric cancer cells. However, the expression of mPGES-2 was not evaluated in these *in vitro* models of gastric cancer. Moreover, Kino Y. et al (2005) studying COX and PGES expression in ovarian cancer have found that mPGES-1 was detected only in some cell lines and its presence did not correspond with $PGE_2$ production. Whereas cPGES and mPGES-2 were observed in all cell lines, and expression of both enzymes positively correlated with elevated $PGE_2$ production. Interestingly, that mPGES-2 and cPGES were expressed in concert with COX-1, but not COX-2 and selective COX-2 inhibitors (NS-398 and rofecoxib) did not inhibit $PGE_2$ production.

### 5.2 The regulation of mPGES-2 by cyclopentenone prostaglandins is independent of nuclear or membrane receptors activation, but may be mediated by covalent binding of the cyclopentenone ring to cysteine residues on signalling molecules or via a redox-dependent mechanism.

In order to find out the responsible mechanism for 15d-$PGJ_2$ induced mPGES-2 inhibition some of the known mechanisms attributed to the action of this cyclopentenone was investigated. 15d-$PGJ_2$ is an activating ligand for PPARγ (Forman B. et al. 1995, Kliewer S. et al. 1995) (Fig. 27). Although the affinity of 15d-$PGJ_2$ for PPARγ ($EC_{50}$ approximately 2 μM) is significantly lower than that of classical steroid hormones for their cognate intracellular receptors, it represents the natural ligand with the highest affinity for PPARγ yet identified (Straus D. et al. 2001).

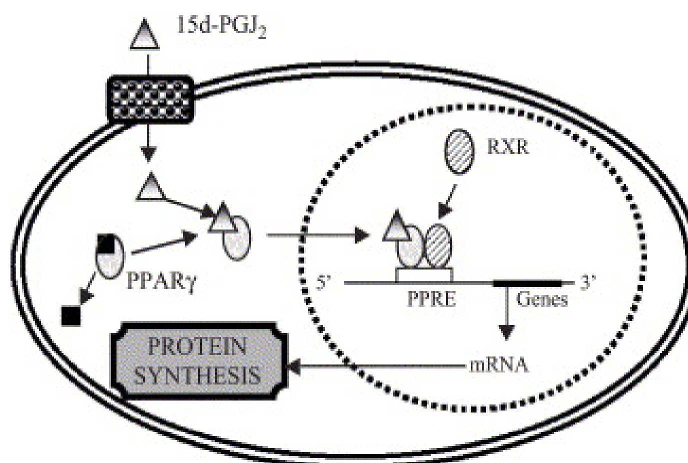


***Fig. 27. 15d-PGJ$_2$ regulation of PPARγ**. After entering the cell by an active transport, 15d-PGJ$_2$ binds to PPARγ, resulting in PPARγ translocation to the nucleus. 15d-PGJ$_2$ /PPARγ forms a heterodimer with the nuclear retinoid X receptor (RXR) to recognize PPAR-response elements (PPRE) in the promoter region of target genes and stimulate transcription.*

PPARs are members of the nuclear receptor superfamily and include three subtypes, PPARα, δ, and γ (Kersten S. et al. 2000). The PPARs form heterodimers with the retinoid X receptor and activate gene expression by binding to the peroxisome proliferator response element (PPRE) in DNA. PPARγ is the molecular target for thiazolidinedione antidiabetic drugs, which bind in a very large ligand binding pocket with the thiazolidinedione ring. The cyclopentenone ring of 15d-PGJ$_2$, presumable, occupies the same position as the thiazolidinedione ring (Nolte R. et al. 1998). The binding of ligands such as 15d-PGJ$_2$ to PPARγ results in activation of transcription of genes that have PPRE located in an enhancer or promoter element.

The role of PPARγ in tumour development remains the subject of intense debate: while extensive studies have documented the antiproliferative effects of PPARγ activation, equally convincing evidence describes its oncogenic actions using *in vivo* and *in vitro* systems (Nahle Z. 2004). Studies, using tumour cell lines derived from breast, colon, prostate carcinomas and liposarcoma as well as organ cultures have consistently reported growth arrest, differentiation or apoptosis upon ligandinduced PPARγ activation (Nahle Z. 2004;

Sarraf P. et al. 1998). Moreover, down-regulation of COX-2 expression in colon cancer cells via the activation of PPARγ was also demonstrated (Yang W. et al. 2001). There is also some evidence, that 15d-$PGJ_2$ may control COX-2 expression through PPARγ-dependent mechanism (Inoue H. et al. 2000). In the light of these findings its effect on mPGES-2 in Caco-2 PPARγ dominant negative cells was examined. However, regulation of mPGES-2 by 15d-$PGJ_2$ was not abolished in the absence of active PPARγ. In addition, the synthetic PPARγ agonist, MCC555, did not induce any changes in mPGES-2 expression in Caco-2 and HCT 116 cells, ruling out that down-regulation of mPGES-2 by 15d-$PGJ_2$ is under control of the PPARγ pathway.

There were several reports demonstrating that 15d-$PGJ_2$ can exert its action on cell metabolism via binding to cell membrane receptors (Fig. 28). The parent compound, $PGD_2$, acts through two such receptors: the DP1 receptor and the DP2 receptor, which is also known as the CRTH2 (Powell W. 2003). 15d-$PGJ_2$ activates the DP2 receptor on eosinophils with a potency ( [$EC_{50}$] 10 nM) nearly equal to that of $PGD_2$, the main ligand for this receptor (Monneret G. et al. 2002), whereas the $EC_{50}$ for DP1 is ~320 nM, much higher than that for $PGD_2$ (Wright D. et al. 1998).

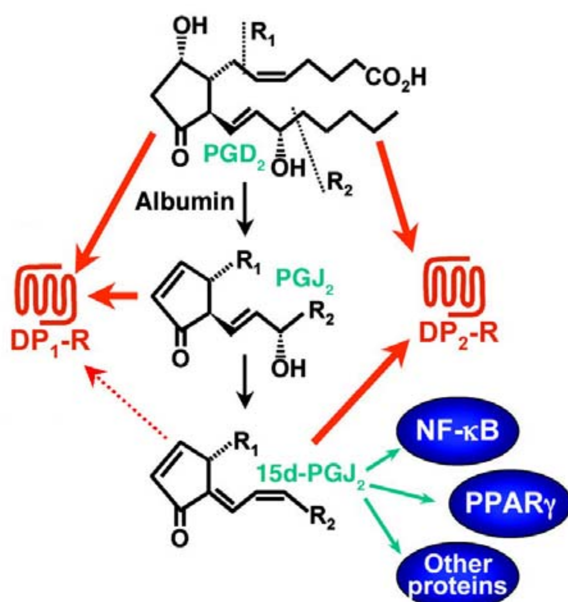


***Fig. 28. The effect of 15d-$PGJ_2$ on receptors and signalling molecules.*** *Some of the effects of 15d-$PGJ_2$ on intracellular proteins are mediated through its covalent binding to proteins. In addition to its effects on intracellular proteins, 15d-$PGJ_2$ also activates the DP2 and DP1 receptors. (Powell W. 2003)*

DP1 and CRTH2 are seven-transmembrane G protein–coupled receptors. DP1 activation by $PGD_2$ leads to the stimulation of adenylyl cyclase activity and an increase in intracellular cAMP levels (Boie Y. et al., 1995). In contrast, activation of CRTH2 by $PGD_2$ has been shown to lead to an increase in intracellular calcium mobilization in T helper type 2 cells, eosinophils, and basophiles. (Hirai H. et al., 2001). In order to determine a putative involvement of such cellular surface receptors in 15d-$PGJ_2$ mediated regulation of mPGES-2 expression either signalling pathway of the two receptors was scrutinized in our colorectal cancer *in vitro* models. However, 15d-$PGJ_2$ did not evoke any change in intracellular calcium and cAMP did not display any regulatory effect on mPGES-2 protein expression, thereby excluding the participation of the $PGD_2$ receptors DP1 and CRTH2 in 15d-$PGJ_2$ induced suppression of mPGES-2. $PGD_2$, a potent activator of CRTH2 receptor also did not induce any changes in intracellular calcium, suggesting that HCT 116 and Caco-2 colon cancer cells most likely do not express CRTH2.

These findings suggested that control of mPGES-2 by 15d-$PGJ_2$ might not be related to a mechanism specific for this cyclopentenone but more likely mediated by mechanisms inherent to cyclopentenone prostaglandins in general and may derive from their specific chemical properties. The cyclopentenone ring, which determines this eicosanoid family, has been suggested to determine the ability of these members to interact with cellular target proteins, alkylating exposed cysteine residues (Sanchez-Gomez F. et al. 2004). For example, 15d-$PGJ_2$ was found to directly inhibit NF-κB-dependent gene expression. In this case, two target protein cysteine residues were involved. One of these is located in the IκB kinase, which is required for NF-κB activation, and the other is located in the DNA-binding domains of NF-κB. This modification led to suppression of COX-2 expression, which is under transcriptional control of NF-κB and represent a PPARγ-independent mechanism for negative feedback regulation of one of the key enzymes of prostaglandin biosynthesis by cyclopentenone prostaglandins (Straus D. et al. 2000, Rossi A. et al. 2000; Tsubouchi Y. et al. 2001). In contrast to the inhibition of this transcription factor, which plays crucial roles in inflammation, immunity, cell proliferation and apoptosis, 15d-$PGJ_2$ could activate H-RAS, another well established signalling molecule in colorectal cancer, which may cause a several-fold increase in mPGES-1 promoter activity (Yoshimatsu K. et al. 2001). This effect was mediated by direct interaction of 15d-$PGJ_2$ with Cys-184 of H-Ras, which exists only in the H-Ras isoform, whereas N-Ras or K-Ras were not able to bind 15d-$PGJ_2$ ( Oliva J. et al. 2003).

Any member of cyclopentenone prostaglandins family may regulate signalling molecules through covalent modification of their cysteine residues. Based on these reports we reasoned that other cyclopentenone prostaglandin would display similar biological activities on mPGES-2 expression. Indeed, down-regulation of mPGES-2 by 15d-$PGJ_2$ was not unique and another cyclopentenone prostaglandin, $PGA_2$, could also mimic these biological actions. In contrast, other eicosanoids, which did not possess the cyclopentenone structure, such as AA and $PGD_2$ or leukotrienes did not affect mPGES-2 protein expression. These data indicate that the cyclopentenone ring structure of 15d-$PGJ_2$ may be involved in the regulation of mPGES-2 gene expression.

Various compounds within the cyclopentenone PG family possess potent anti-neoplastic activity. The cyclopentenone $PGA_2$ is a potent inhibitor of growth of cultured cells and exhibits antitumoural activity *in vivo* (Lin S. et al. 2000; Marini S. et al. 1990). It was noticed that $PGE_2$ may mediate the growth-inhibitory effect in human fibroblasts. Observed inhibition of cell growth was postulated to require $PGE_2$ dehydratation to the $PGA_2$. Thus, $PGA_2$ was discovered to display antiproliferative properties. (Ohno K. et al. 1986). Indeed, $PGA_2$ was also capable to reduce growth of cell lines analysed in this study. Concentrations of $PGA_2$ used before to induce cell arrest or apoptosis were in the range of 15-60 μM (Joubert A. et al. 2003; Lin S. et al. 2000), whereas in our experiments already 10 μM $PGA_2$ could reduce cell proliferation.

Finally, some of the biological activities specific for cyclopentenone prostaglandins was examined. Cyclopentenone prostaglandins were described as inducters of intracellular oxidative stress, the most active of which is 15d-$PGJ_2$ (Kondo M. et al. 2001). It was shown that this prostaglandin may increase intracellular reactive oxygen species (ROS) production (Nencioni A. et al. 2003) and pretreatment with the antioxidant NAC significantly inhibits the changes in intracellular redox status caused by cyclopentenones. The fact that cyclopentenone PGs are susceptible to nucleophilic addition reactions with thiols suggests that the action of cyclopentenone PGs is closely related to a direct reaction with GSH and/or other thiol compounds. However, reactive oxygen species (ROS) production was not only the result from GSH depletion alone, but also from the reduction of GSH peroxidase activity. GSH peroxidase contains a selenocysteine residue, which is essential for peroxidase activity. This selenocysteine residue resembles a cysteine residue in terms of chemical properties but with higher reactivity. Electrophiles, such as cyclopentenone PGs are likely to react with the

selenocysteine residue of GSH peroxidase, resulting in the depletion of GSH peroxidase activity (Kondo M. et al. 2001).

Oxidative stress is increasingly seen as a major upstream component in the signalling cascade involved in many cellular functions such as cell proliferation, inflammatory responses, stimulating adhesion molecule, and chemoattractant production. Transient fluctuations in ROS, generated during oxidative stress, serve important regulatory functions, but when present at high and/or sustained levels, ROS can cause severe damage to DNA, protein, and lipids. At the cellular level, oxidant injury elicits a wide spectrum of responses ranging from proliferation to growth arrest and to cell death. The particular outcome depends on the intracellular stress signalling pathways that are activated in response to the oxidative insult. These pathways exert their phenotypic influences largely through modulation of transcription factor activities that effect changes in the pattern of gene expression (Fig. 29) (Martindale J. et al. 2002).

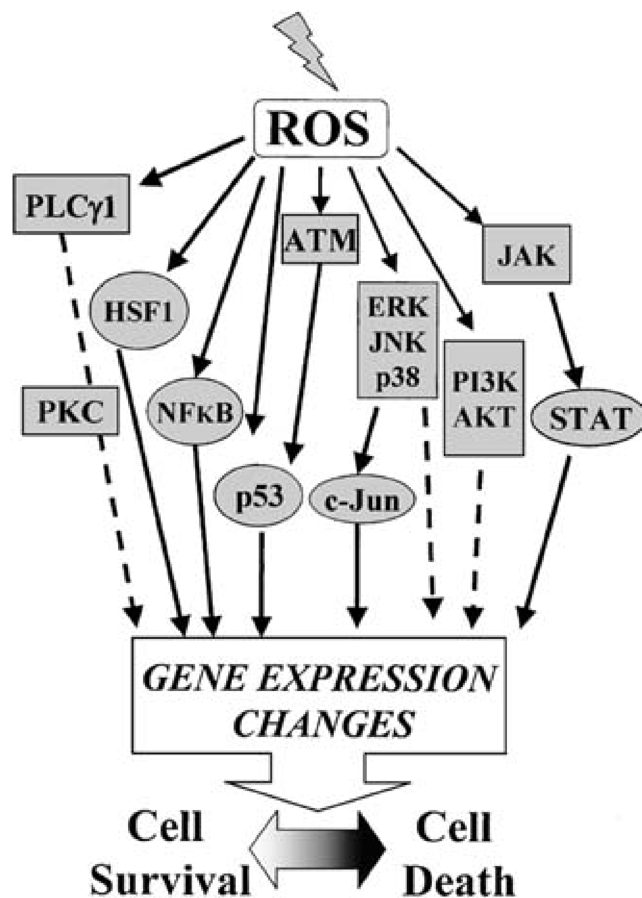


***Fig. 29. Oxidative stress activates numerous major signalling pathways.*** *Being highly reactive by nature, ROS can directly or indirectly modulate the functions of many enzymes (boxes) and transcription factors (ovals) through a multitude of signalling cascades as depicted here. Ultimately these signals result in changes in gene expression, which influence the ability of the cell to survive or die. The magnitude and duration of the stress as well as the cell type involved are important factors in determining which pathways are activated. ATM, ataxia-telangectasia mutated kinase; ERK, extracellular signal-regulated kinases; HSF1, heat shock transcription factor 1; JAK, Janus protein kinase; JNK, c-jun N-terminal kinases; NFκB, nuclear factor κB; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC-γ1, phospholipase C-γ1; STAT, signal transducers activators of transcription.*

A number of defence systems have evolved to combat the accumulation of ROS. These include various non-enzymatic molecules (e.g., glutathione, vitamins A, C, and E, and flavenoids) as well as enzymatic scavengers of ROS (e.g., superoxide dismutases (SOD), catalase, and glutathione peroxide). Antioxidants, such as NAC or DTT prevented the effect of 15d-$PGJ_2$ caused by induction of oxidative stress. Our data demonstrated that the antioxidants NAC and DTT were able to completely abolish the down-regulation of mPGES-2 protein expression by 15d-$PGJ_2$. However, when in additional experiments oxidative stress was induced by ROS directly, mPGES-2 expression was not affected. Since $H_2O_2$ may cause an oxidative stress and induce ROS production similar to 15d-$PGJ_2$ (Nencioni A. et al. 2003; Li L. et al. 2001) we thought that oxidative stress may not be involved in mPGES-2 expression regulation. Since all these antioxidants possess a thiol group, it is most likely, that NAC or DTT bind to 15d-$PGJ_2$, thereby preventing its impact on cellular target proteins.

Together, these findings provide new insights into the anti-proliferative properties of cylopentenone prostaglandins by the inhibition of mPGES-2 in colon carcinoma cells. Although the exact mode of action of 15d-$PGJ_2$ on mPGES-2 expression and the role of this enzyme in cancerogenesis remains to be determined, we speculate that mPGES-2 might be an interesting new target in the prevention of colorectal cancer. However, one important issue regarding many of the reported effects of cyclopentenone prostaglandins is whether they can be considered of physiological or pharmacological significance. Whereas the amount of 15d-$PGJ_2$ measured in several experimental models are in the nanomolar range (Gilroy D. et al. 1999), most of the biological effects of 15d-$PGJ_2$ and other cyclopentenone prostaglandins, including those described here, have been observed to occur at micromolar concentrations (Powell W. 2003). However, our experiments revealed that 1 μM concentration of 15d-$PGJ_2$ was also able to exert same effect as a 10 μM, but after the longer incubation period. That may suggest, nanomolar concentrations of this drug may be also efficient. Thus, in addition to defining the physiological role of 15d-$PGJ_2$, additional work is needed to establish the *in vivo* relevant doses of this cyclopentenone for the growth modulating.

## 6. Summary


Clinical, genetic, and biochemical evidence suggests that Prostaglandin (PG) $E_2$ is a significant contributor to the development and progression of colorectal cancer (Sheng H. et al. 2001; Sheng H. et al. 1998; Ben-Av P. et al. 1995). In addition, increased amount of $PGE_2$, has been detected in colorectal adenomas and cancers (Yoshimatsu K. et al. 2001).

Until now three prostaglandin E synthases in mammals have been identified: cytosolic PGES (cPGES), membrane associated PGES (mPGES-1) and membrane-associated PGES (mPGES-2) (Tanioka T. et al. 2000; Jakobsson PJ. et al. 1999; Tanikawa N. et al. 2002). Among these three known proteins capable to isomerise $PGH_2$ to $PGE_2$, mPGES-1 and mPGES-2 were markedly increased in colorectal cancer tissues (Rees B. et al. 2003; Murakami M. 2003). While recent data already demonstrated that mPGES-1, in concert with Cyclooxygenase-2 (COX-2) (Murakami M. et al. 2000), can be associated with cellular transformation and cancer development, until now no such information is available for mPGES-2. Thus, there is a particular interest in the identification of a possible role of mPGES-2 in the development of colorectal cancer.

Cyclopentenone prostaglandins are members of prostaglandins family contained a cyclopentenone ring structure, which is characterized by the presence of a chemically reactive α,β-unsaturated carbonyl. Various members of the cyclopentenone prostaglandin family have anti-neoplastic, anti-inflammatory and anti-viral activities (Straus D. et al. 2001). 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$), cyclopentenone prostaglandin, was described as a key regulator of negative feedback of the COX pathway in an inflammatory setting and as a potent anti-tumoral agent (Inoue H. et al. 2000; Tsubouchi Y. et al. 2001; Straus D. et al. 2001). We sought to determine its potential implication on $PGE_2$ mediated colorectal cancer promotion.

The effect of 15d-$PGJ_2$ on gene and protein expression of cytosolic PGES, mPGES-1, -2 and COX-1, COX-2 as well as on PGES activity, HCT 116 and differentiated Caco-2 cells was investigated. While the expression of mPGES-2 mRNA was down-regulated by 15d-$PGJ_2$ in both cell lines tested, levels of cPGES, mPGES-1, COX-1 (expressed in HCT 116) and COX-2 (expressed in Caco-2) remained unchanged following treatment with this

cyclopentenone, indicating that the inhibitory effect of 15d-$PGJ_2$ on mPGES-2 is independent of COX-1 or COX-2 expression.

The down-regulation of mPGES-2 gene expression was followed by a time-delayed transient reduction of this enzyme on the protein level. In accordance to the down-regulation of this PGES on the mRNA and protein level, a substantial decrease in enzyme activity could also be noticed. Together with $PGA_2$, another cyclopentenone, 15d-$PGJ_2$ also displayed a growth-inhibitory effect in HCT 116 cells, which was counteracted by $PGE_2$.

In order to work out the responsible mechanism for 15d-$PGJ_2$ induced mPGES-2 inhibition we investigated some of the known mechanisms attributed to the action of this cyclopentenone. Since 15d-$PGJ_2$ is an established natural ligand of peroxisome proliferator-activated receptor γ (PPARγ) (Forman B. et al. 1995, Kliewer S. et al. 1995) we examined the effect of another PPARγ agonist – the thiazolidinedione homolog MCC 555 on mPGES-2 mRNA expression. No changes in gene expression of mPGES-2 or any of the other enzymes examined (COX-1, COX-2, mPGES-1 and cPGES) after treatment Caco-2 and HCT 116 cell lines with this compound were observed. This was further confirmed by subjecting Caco-2 cells, transfected with a mutant receptor to inhibit wild type PPARγ action, to 15d-$PGJ_2$ treatment. Gene, protein expression and enzyme activity of mPGES-2 were reduced to a similar extent when compared with the non-transfected Caco-2 cell line or HCT 116 cells, ruling out that down-regulation of mPGES-2 by 15d-$PGJ_2$ is under control of the PPARγ pathway.

15d-$PGJ_2$ can also exert its action on cell metabolism via binding to the $PGD_2$ receptors DP1 and Chemoattractant receptor–homologous molecule expressed on TH2 cells (CRTH2) (Monneret G. et al. 2002; Powell W. 2003). DP1 activation leads to the stimulation of adenylyl cyclase activity followed by an increase in intracellular cAMP levels (Boie Y. et al. 1995). CRTH2 activation enhances intracellular calcium mobilization (Hirai H. et al. 2001). In order to determine a putative involvement of such cellular surface receptors in 15d-$PGJ_2$ mediated regulation of mPGES-2 expression we therefore investigated whether treatment of colon cancers cells with 15d-$PGJ_2$ might induce a calcium release or incubation with cAMP would affect mPGES-2 expression. However, 15d-$PGJ_2$ did not evoke any changes in intracellular calcium and cAMP display any regulatory effect on mPGES-2 protein expression, thereby excluding that 15d-$PGJ_2$ exerts its action by binding to G-protein coupled receptors.

In addition, 15d-$PGJ_2$ can exert its effect through the mechanisms specific for all cyclopentenone, such as oxidative stress induction (Nencioni A. et al. 2003; Kondo M. et al. 2001). In order to investigate whether oxidative stress is involved in 15d-$PGJ_2$ mPGES-2 expression inhibitions, Caco-2 and HCT 116 cells were pretreated with or without two different antioxidants prior to stimulation with 15d-$PGJ_2$. The 15d-$PGJ_2$ inhibitory effect on mPGES-2 protein expression was completely reversed by both antioxidants, Dithiothreitol (DTT) and *N*-acetylcysteine (NAC). However, in additional experiments oxidative stress induced by $H_2O_2$ did not affect mPGES-2 expression. Thus, it appears likely, that NAC or DTT bind to 15d-$PGJ_2$, thereby preventing its impact on cellular target proteins.

Finally, biological activity of cyclopentenone prostaglandins can derive solely from their specific chemical properties. The cyclopentenone ring determines the ability of these compounds to interact with cellular target proteins (Straus D. et al. 2001). Therefore we treated Caco-2 and HCT 116 cells with $PGA_2$, another cyclopentenone prostaglandin. $PGA_2$ displayed similar biological actions, whereas eicosanoids not containing the cyclopentenone structure, such as $PGD_2$ or leukotrienes did not affect mPGES-2 protein expression.

Together, our findings provide new insights into the anti-proliferative properties of cyclopentenone prostaglandins through the inhibition of mPGES-2 in colon carcinoma cells and expose mPGES-2 as a new potential drug target in the treatment of colorectal cancer.

# 7. Zusammenfassung

Das kolorektale Karzinom ist weltweit das dritthäufigste Karzinom. Schon lange bekannt ist die antikarzinogene Wirkung von sog. nichtsteroidale Antiphlogistika (NSAIDs), (nicht-)selektiven Inhibitoren der Cyclooxygenase-2 (COX-2), in der Entstehung des kolorektalen Karzinoms. Eine vergleichbare Wirkung geht von neu entwickelten COX-2-Inhibitoren aus, die eine selektive COX-2-Hemmung hervorrufen (Brown J. et al. 2005). Zahlreiche Untersuchungen von COX-2 nachgeschalteten Prostaglandinen (PG) unterstreichen die herausragende Bedeutung von $PGE_2$ in der Ätiopathogenese des kolorektalen Karzinoms. So ist die Konzentration von $PGE_2$ in kolorektalen Adenomen und Karzinomen regelhaft gesteigert (Yoshimatsu K. et al. 2001). $PGE_2$ führt darüber hinaus *in vitro* zu einer Induktion der Angiogenese *in vitro* induzieren und Reduktion der Apoptoserate (Ben-Av P. et al. 1995). Ferner steigert $PGE_2$ Proliferation und Motilität kolorektaler Tumozellen (Sheng H. et al. 1998; Sheng H. et al. 2001).

Die Biosynthese von $PGE_2$ aus Arachidonsäure (AA) erfolgt über verschiedene enzymatische Reaktionen, an denen Phospholipase $A_2$ ($PLA_2$), COX sowie die terminale $PGE_2$ Synthasen(PGES) beteiligt sind. Die am besten untersuchte PGES ist ein perinukleäres membrangebundenes Enzym, mPGES-1, welche zur MAPEG-Proteinfamilie (MAPEG = membrane associated proteins in eicosanoid and glutathione metabolism) gehört (Jakobsson PJ. et al. 1999). Die Expression von mPGES-1 wird durch proinflammatorische Stimuli gesteigert und durch Glucocorticosteroide reduziert. Die Expression dieses Enzyms ist dabei zumeist an die der COX-2 gekoppelt (Murakami M. et al. 2000). Eine Induktion der Expression von mPGES-1 findet sich bei einer Reihe von physiologischen und pathophysiologischen Zuständen wie Entzündung, Fieber oder auch der Tumorgenese (Murakami M. et al. 2004; Rees B. et al. 2003). Im Gegensatz zu mPGES-1 wird die cytosolische Form von PGES (cPGES) in einer Vielzahl von Zelltypen konstitutiv und ubiquitär exprimiert. Diese Enzym scheint vorwiegend an die durch COX-1-vermittelte $PGE_2$-Bildung gekoppelt zu sein (Tanioka T. et al. 2000). Vor wenigen Jahren wurde ein zweites membran-assoziiertes PGES (mPGES-2) identifiziert, das für seine katalytische Aktivität im Gegensatz zu mPGES-1 nicht zwangsläufig Glutathion (GSH) als Kofaktor benötigt. Auch die mPGES-2 wird 2 in einer Vielzahl von Zellen und Geweben konstitutiv

exprimiert. Anders als für mPGES-1 konnte eine Induktion dieses Enzyms im Rahmen inflammatorischer Prozesse (Rheumatoide Arthritis, Myokardinfarkt) nicht nachgewiesen werden; dagegen werden beide mikrosomale PGES beim humanen kolorektalen Karzinom überexprimiert (Tanikawa N. et al. 2002; Murakami M. et al. 2003).

Cyclopentenon-Prostaglandine sind durch eine Cyclopentenon-Ringstruktur mit chemisch reaktionsfähiger α,β-ungesätigten Carbonyl-Gruppe charakterisiert. Verschiedene Vertreter dieser Prostaglandin-Familie weisen antineoplastische, antiinflammatorische und antivirale Aktivitäten auf (Ohno K. et al. 1986; Kato T. et al. 1986; Straus D. et al. 2001). 15-deoxy-$\Delta^{12,14}$-Prostaglandin $J_2$ (15d-$PGJ_2$) ist ein Cyclopentenon-Protaglandin der J-Klasse, einem Derivat von $PGD_2$. $PGA_2$, ein Vertreter der A-Klasse, entsteht dagegen durch Dehydration von $PGE_2$. Eine Regulation von COX-2 durch 15d-$PGJ_2$ im Rahmen inflammatorischer Vorgänge wurde von mehreren Autoren beschrieben. Dieser negative Feedback-Mechanismus scheint an der Resolution von Entzündungsprozessen beteiligt zu sein und durch eine Hemmung der NF-κB-Aktivität vermittelt zu werden. Darüber hinaus hemmt dieser $PGD_2$-Metabolit mPGES-1 *in vitro* (Quraishi O. et al. 2002). In der vorliegenden Arbeit sollte die mögliche Wirkung von 15d-$PGJ_2$ auf die $PGE_2$-vermittelte Entstehung des kolorektalen Karzinoms untersucht werden.

Die Wirkung von 15d-$PGJ_2$ auf die Gen- und Proteinexpression von cPGES, mPGES-1 und mPGES-2 sowie COX-1 und COX-2, wie auch die PGES-Aktivität wurde in den beiden kolorektalen Tumorzelllinine HCT 116 und Caco-2 untersucht. Dabei zeigte sich eine selektive Hemmung der Expression von mPGES-2 durch 15d-$PGJ_2$ an beiden Zelllinine ohne Effekt auf andere, an der PGES beteiligten Gene. Die Hemmung der mRNA-Expression von mPGES-2 war zeit- und dosisabhängig. Parallel zur Reduktion der mRNA-Expression fand sich eine zeitlich verzögerte Abnahme der Proteinkonzentration von mPGES-2. Mehrere Studien haben in der Vergangenheit gezeigt, daß die Genexpression von mPGES-1 und COX-2 einer Ko-Regulation unteliegt (Murakami M. et al. 2000), so dass eine Kopplung der Expression von COX-2 mit mPGES-2 durchaus denkbar wäre. Eine verringerte mPGES-2-Proteinexpression wurde in COX-$2^{-/-}$-Mäusen beschrieben (Bosetti F. et al. 2004). Andere Autoren fanden jedoch keinen Zusammenhang bezüglich der Expression von mPGES-1 und COX-2 (Devaux Y. et al. 2001; Masuko-Hongo K. et al. 2004). Darüber hinaus wurde eine $PGE_2$-Bildung durch mPGES-2 aus $PGH_2$ mit Hilfe von COX-1 und COX-2 demonstriert

(Murakami M. et al. 2003). Dagegen wird für cPGES eine funktionelle Kopplung mit COX-1 postuliert (Tanioka T. et al. 2000).

Es gibt Hinweise auf eine Verbindung der der PG-Biosynthese mit 15d-$PGJ_2$ im Sinne einer Rückkopplungkontrolle von COX-2 durch diesen PG-Metabolit (Inoue H. et al. 2000; Tsubouchi Y. et al. 2001) Um eine vergleichbare Rückkopplungkontrolle von mPGES-2, unter 15d-$PGJ_2$ Behandlung zu untersuchen, wurde die Expression von COX in Zelllinien mit verschiedenen Phänotypen bezüglich der beiden COX-Isoformen analysiert: die Caco-2-Zelllinie exprimiert keine COX-1, während HCT 116-Zellen zur Expression von COX-2 nicht in der Lage sind. Beide Zelllinien wiesen eine vergleichbare Hemmung der Gen- und Proteinexpression von mPGES-2 als Folge der 15d-$PGJ_2$-Behandlung auf. Das Expressionsniveau von COX-1 und COX-2 beider kolorektaler Tumorzelllinien änderte sich nicht, so daß die Hemmwirkung von 15d-$PGJ_2$ auf mPGES-2 keiner Kopplung mit der Expression von COX-1 oder COX-2 unteliegt.

Übereinstimmend mit der transienten Hemmung der Expression von mPGES-2 auf mRNA und Proteinebene konnte eine signifikante Abnahme der enzymatischen Aktivität beobachtet werden. Darüber hinaus wiesen sowohl 15d-$PGJ_2$ wie auch $PGA_2$ einen wachstumshemmenden Effekt in HCT 116-Zellen auf, der den durch $PGE_2$ hervorgerufenen proliferativen Stimulus entgegenwirkt. Neben dieser Wirkung sind für die 15d-$PGJ_2$-induzierte Wachstumshemmung weitere Mechanismen, wie z.B. Zellzyklusarrest oder Aktivierung von mitochondrialen apoptotischen Signalwegen bekannt (Miwa Y. et al. 2000; Nencioni A. et al. 2003) Die hier präsentierten Ergebnisse legen nahe, daß die Reduktion der $PGE_2$-Bildung über eine Hemmung von mPGES-2 zumindest partiell an dem reduzierten Wachstum von HCT 116-Zellen als Antwort auf 15d-$PGJ_2$ beteiligt ist. Zusätzlich zu einer kürzlich erschienenen Publikation, bei der eine Überexpression von mPGES-2 in kolorektalem Karzinomgewebe nachgewiesen werden konnte (Murakami M. et al. 2003), stützen unsere Ergebnisse die Hypothese, daß dieses Enzyms bei der Entwicklung des kolorektalem Karzinom beteiligt ist.

Um den Mechanismus der 15d-$PGJ_2$-induzierten mPGES-2-Hemmung verstehen zu können, wurden einige bekannte Reaktionsmechanismen dieses Cyclopentenon untersucht. 15d-$PGJ_2$ ist ein natürlicher Ligand des Peroxisome Proliferator-Activated Receptor $\gamma$ (PPAR$\gamma$) (Forman B. et al. 1995, Kliewer S. et al. 1995). Die Bindung von Liganden an PPAR$\gamma$, wie zum Beispiel 15d-$PGJ_2$, führt anschließend zur Aktivierung der Transkription

von Genen, die PPAR-response-Elemente (PPRE) in der Enhancer- oder Promotersequenz aufweisen. Studien an Tumorzelllinien aus Liposarkomen, Brust-, Kolon- und Prostatakarzinomen, aber auch an Organzellkulturen, haben übereinstimmend PPARγ-vermittelten Wachstumsarrest, Differenzierung und Apoptose gezeigt (Nahle Z. 2004; Sarraf P. et al. 1998). Zudem wurde beim Kolonkarzinom auch eine erniedrigte COX-2-Expression durch PPARγ-Aktivierung demonstriert (Yang W. et al. 2001). Von einigen Autoren wird eine Kontrolle der COX-2-Expression durch PPARγ-abhängige Mechanismen postuliert (Inoue H. et al. 2000). Angesichts dieser Ergebnisse wurde die Wirkung eines weiteren PPARγ-Agonisten, des Thiazolidinedionhomologs MCC555, auf die mPGES-2-mRNA-Expression untersucht. Nach Behandlung von Caco-2- und HCT 116-Zellen mit MCC555 fand sich jedoch keine Veränderungen in der Genexpression von mPGES-2 wie auch COX-1, COX-2, mPGES-1 und cPGES. Dieses Ergebnis wurde durch die Behandlung von Caco-2-Zellen, bei welchen die PPARγ-Aktivität durch eine dominant negative Rezeptor gehemmt wurde, bestätigt. Die Gen- und die Proteinexpression, sowie die enzymatische Aktivität von mPGES-2 waren im gleichen Maße reduziert wie in nicht-transfizierten Caco-2-Zellen oder HCT 116-Zellen. Eine Kontrolle der Regulation von mPGES-2 durch 15d-$PGJ_2$ über den PPARγ-Signaltransduktionsweg kann somit ausgeschlossen werden.

Als weitere Möglichkeit der Vermittlung 15d-$PGJ_2$–spezifischer Zellefekte ist die Bindung an $PGD_2$-Rezeptoren, wie dem DP1 und dem CRTH2 Rezeptor (= Chemoattractant receptor–homologous molecule expressed on TH2 cells), beschrieben (Monneret G. et al. 2002; Powell W. 2003). Die Aktivierung von DP1 führt über eine Stimulierung der Adenylcyclase-Aktivität, mit nachfolgender Steigerung der intrazellulären cAMP-Konzentration (Boie Y. et al. 1995). Die Aktivierung von CRTH2 verstärkt die intrazellulare Kalziumfreisetzung (Hirai H. et al. 2001). Um eine mögliche Einbindung dieser Oberflächenrezeptoren an der 15d-$PGJ_2$-vermittelten Regulation der mPGES-2-Expression zu überprüfen, wurde eine mögliche Kalziumfreisetzung von Kolonkarzinomzellen nach Behandlung mit 15d-$PGJ_2$ untersucht sowie die Expression von mPGES-2 nach cAMP-Inkubation überprüft. 15d-$PGJ_2$ zeigte jedoch weder Effekte auf die intrazelluläre Kalziumkonzentration, noch übte cAMP keine regulatorische Wirkung auf die mPGES-2-Expression aus. Somit konnte eine Beteiligung dieser Rezeptoren an der Regulation von mPGES-2 durch 15d-$PGJ_2$ ausgeschlossen werden. Zusammengefaßt deuten diese Ergebnisse darauf hin, daß die 15d-$PGJ_2$-vermittelte Kontrolle von mPGES-2 möglicherweise nicht auf einem Mechanismus beruht, der spezifisch für dieses Cyclopentenon-Protaglandin ist,

sondern vielmehr auf einem generellen Wirkprinzip von Cyclopentenon-Prostaglandinen beruht. So wird ein Teil der biologische Aktivität von Cyclopentenon-Prostaglandinen über oxidativen Stress ausgelöst. Dieser basiert auf einer gesteigerten intrazellulären Produktion von reaktiven Sauerstoffspezies mit teilweiser Reduktion des GSH-Gehalts. Vorbehandlung mit N-Acetylcystein (NAC) verhindert signifikant die Cyclopentenon-Prostaglandin-abhängigen Veränderungen des intrazellulären Redox-Status (Nencioni A. et al. 2003; Kondo M. et al. 2001; Grau R. et al. 2004). Oxidativer Stress kann über einen drastischen Rückgang der mitochondrialen Energieproduktion Apoptose induzieren. Außerdem führt die Bildung von reaktiven Sauerstoffspezies zur Aktivierung verschiedener Signalmoleküle wie beispielsweise NF-κB, p53, Proteinkinase C, MAPK etc., die zur einer veränderten Genexpression führen (Martindale J. et al. 2002). Um eine potentielle Beteiligung von oxidativem Stress an der durch 15d-$PGJ_2$ vermittelten Expressionshemmung von mPGES-2 zu untersuchen, wurden Caco-2-Zellen und HCT 116-Zellen vor der 15d-$PGJ_2$-Stimulation mit verschiedenen Antioxidantien behandelt. Die Hemmwirkung von 15d-$PGJ_2$ auf die mPGES-2-Proteinexpression konnte sowohl mit Dithiothreitol (DTT) wie auch NAC komplett aufgehoben werden. Dagegen zeigte der durch $H_2O_2$ induzierte oxidative Stress jedoch keine Auswirkung auf die mPGES-2-Expression. Es ist daher denkbar, daß die Antioxidantien NAC oder DTT direkt an 15d-$PGJ_2$ binden und so dessen Wirkung auf zelluläre Zielproteine unterbinden.

Letztlich sind Cyclopentenon-Prostaglandine aufgrund der chemischen Eigenschaften ihrer typischen Ringstruktur zur direkten Interaktion mit zellulären Zielproteinen in der Lage. So hemmt bsp. 15d-$PGJ_2$ direkt die NF-κB-abhängige Genexpression durch eine kovalente Modifikation von Cysteinen in der IκB-Kinase und in den DNA-bindenden Domänen der NF-κB-Untereinheiten (Straus D. et al. 2000), andererseits wird H-Ras durch eine kovalente Modifikation aktiviert (Oliva J. et al. 2003). Cyclopentenon-Prostaglandine modulieren ferner die Expression und Aktivität von p53 (Shan Z. et al. 2004), JNK (Liu J. et al. 2003), Egr-1, Egr-3, HSP70 (Cippitelli M. et al. 2003) und weiterer, an Zellzyklusregulation und Apoptose beteiligter, Transkriptionsfaktoren und Signalmoleküle. In der Tat war die Regulation von mPGES-2 nicht auf 15d-$PGJ_2$ begrenzt. Auch $PGA_2$, ein weiteres Cyclopentenon-Prostaglandin, übte eine der von 15d-$PGJ_2$ vergleichbare biologische Aktivität auf die Expression von mPGES-2 in den kolorektalen Tumorzelllinien HCT 116 und Caco-2 aus. Andere Eikosanoide, wie $PGD_2$ oder Leukotriene, die über jeweils keine Cyclopentenon-

Struktur verfügen, oder aber auch Arachidonsäure, Präkursor der Eikosanoide, zeigten keine regulatorischen Effekte auf Expression von mPGES-2.

Mit den hier vorgestellten Resultaten einer Hemmung der Expression von mPGES-2 und damit auch der PGES-Aktivität in kolorektalen Tumorzelllinien wird das Spektrum der antiproliferativen Mechanismen von Cyclopentenon-Prostaglandine erweitert. Die prinzipielle Bedeutung dieser Eikosanoide in der Therapie des kolorektalen Karzinoms bleibt jedoch offen. Darüber hinaus wurde mit der PGES-2 ein neues interessantes Zielprotein in der Theraopie des kolorektalen Karzinoms etabliert, welches sich in der Zukunft als potentiell wertvolles Drug-Target erweisen könnte.

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# 9. Appendix

**Table I**

Sequence of oligonucleotides and PCR conditions

| Gene | Primer sequence | Annealing temperature (ºC) | No. of cycles |
|---|---|---|---|
| G3PDH | Forward:<br>5’-GCACCGTCAA GGCTGAGAAC-3’<br>Reverse:<br>5’-CCACCACCCTGTTGCTGTAG-3’ | 45 | 24 |
| mPGES-2 | Forward:<br>5’-CCTGCAGCTGACCCTGTACCAGTA-3’<br>Reverse:<br>5’-CCCACTTGTCAGCAGCCTCATAGA-3’ | 51 | 31 |
| mPGES-1 | Forward:<br>5’-GCACGCTGCTGGTCATCAAGATGTA-3’<br>Reverse:<br>5’-CCGCTTCCCAGAGGATCTGCAGA-3’ | 49.5 | 38 |
| cPGES | Forward:<br>5’-GCAAAGTGGTACGATCGAAGGGACTAT-3’<br>Reverse:<br>5’-CCCAGTCTTTCCAATTATTGAAGTCGA-3’ | 48 | 33 |
| COX-2 | Forward:<br>5’-CCCTTCTGCCTGACACCTTTCAAATT-3’<br>Reverse:<br>5’-GCTCTGGATCTGGAACACTGAATGAAGT-3’ | 48 | 35 |
| COX-1 | Forward:<br>5’-GTGGGCTCCCAGGAGTACAGCTAC-3’<br>Reverse:<br>5’-GCAATCTGGCGAGAGAAGGCATC-3’ | 48 | 37 |

## 10. Curriculum vitae

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Theme: "Regulation of Microsomal Prostaglandin $E_2$ Synthase by Cyclopentenone Prostaglandins in Colon Cancer Cells".

**Jul. 2000-Apr. 2002** Research assistant in the Condensed Matter Laboratory of Joint Institute for Nuclear Research (Dubna, Russia) and Group of Nucleoprotein Physics at the Institute of Protein Research, RAS (Pushchino, Russia). Supervisor: Prof. Igor Serdyuk.

Theme: "Studying of molecular dynamics of psychrophile, mesophile and thermophile cells and ribosomes".

**Sep. 1999-Apr. 2000** Student of Advanced Courses in Molecular Biology at the Institute of Protein Research, Pushchino, Russia.

**Jun. 2000** Defended diploma paper in the Department of Biochemistry, Kharkov State University, Ukraine and in the Laboratory of Nucleoprotein Physics, Institute of Protein Research, Pushchino, Russia. Supervisors: Prof. Igor Serdyuk and Dr. Natalia Bulankina.

Theme: "Preparation of ribosomal complexes to 3' domain 30S subunit and fragment corresponding to 890-1155 nucleotides".

**Sep. 1995-Jun. 2000** Undergraduate student in the Department of Biochemistry, Kharkov State University, Ukraine.

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‡These authors contributed equally to this publication.

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