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Protein modification by cyclopentenone prostaglandin addition: biological actions and therapeutic implications

Review Article

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Abbreviations: 15-deoxy-$\Delta^{12,14}$-PGJ_2, (15d-PGJ_2); activator protein 1, (AP-1); antioxidant response elements, (ARE); c-Jun NH2-terminal kinase (JNK); cyclooxygenase, (COX); cyclopentenone prostaglandins, (CyPG); extracellular signal regulated kinase, (ERK); glutathione-S-transferase, (GST); heat shock factor 1, (HSF1); hypoxia inducible factor, (HIF); IκB kinase, (IκK); inducible nitric oxide synthase, (iNOS); mitogen activated protein kinase, (MAPK); nitric oxide, (NO); nuclear factor of activated T cells, (NFAT); nuclear factor-kappaB, (NF-κB); peroxisome proliferator activated receptor, (PPAR); phospholipase A_2, (PLA_2); prostaglandin E synthase, (PGES); prostaglandins, (PG); reactive oxygen species, (ROS); reduced glutathione, (GSH); signal transducer and activator of transcription 1, (STAT-1); thioredoxin reductase, (TR); thioredoxin, (Trx)

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Summary

Cyclopentenone prostaglandins (cyPG) are naturally occurring eicosanoids which are generated by the spontaneous dehydration of other PG. The cyPG of the A series arise from the dehydration of PGE. The cyPG of the J series, such as 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2), arise from the dehydration of PGD_2. CyPG have been detected *in vivo* in body fluids and at sites of chronic and acute inflammation. However, the mechanisms underlying their generation, the levels attained *in vivo* and their pathophysiological significance are still not completely elucidated. CyPG display varied biological activities in cellular models including antiviral and antitumoral effects, induction of stress response and apoptosis. Anti-inflammatory and protective roles of 15d-PGJ_2 against several kinds of tissue injury have been recently evidenced in various animal models. This has led to propose the use of cyPG as therapeutic agents. The mechanism of action of cyPG may be multiple. Some cyPG, like 15d-PGJ_2, may act as a ligands of the nuclear receptor PPARγ. In addition, cyPG possess an α,β-unsaturated carbonyl group in the cyclopentane ring which may form adducts with cellular proteins or with glutathione, leading to a modulation of protein function and to changes in the cellular redox status. The modification of cellular proteins by addition of cyPG is arising as an important mechanism in the anti-inflammatory and anti-proliferative effects of these compounds. Several proteins have been identified which can be targets for this modification including several components of the pro-inflammatory transcription factors NF-κB and AP-1, proteins involved in the cellular response to oxidative or electrophile-induced stress, and H-Ras proteins. The identification of novel protein targets for modification by cyPG and the functional and structural characterization of this type of modification will contribute to the understanding of the mechanisms of action of cyPG and the assessment of their therapeutic potential.

I. Introduction

A. Prostaglandins: synthesis and actions

Prostaglandins (PG) are important lipidic mediators involved in the regulation of multiple biological processes, from the regulation of vascular tone, renal function and fertility to the regulation of gene expression in inflammation and cancer. Prostaglandins are produced from the essential fatty acid arachidonic acid. Arachidonic acid and the lipids synthesized from it are twenty carbon fatty acid derivatives, and for this reason they are also known as eicosanoids, from the Greek word “eicosi”, which means twenty. Upon cell activation by a variety of stimuli such as injury, cytokines or vasoactive peptides, among others, arachidonic acid is released from membrane phopholipids by the action of phospholipases (Dennis et

al, 1991), of which, cytosolic phospholipase A_2 (PLA_2) plays a major role (Funk, 2001) (**Figure 1**). Arachidonic acid is the substrate for key enzymes called cyclooxygenases (COX), which catalyze the rate-limiting step in the synthesis of PG and are located in the membranes of the endoplasmic reticulum and nucleus. COX are bifunctional enzymes that possess cyclooxygenase activity, which converts arachidonic acid into PGG_2, and peroxidase activity, which reduces PGG_2 to PGH_2. There are two main forms of COX with different patterns of expression and regulation. COX-1 is expressed under basal conditions in many tissues and cell types and it is believed to play a physiological role based on the synthesis of low, constitutive levels of PG. A variant transcript of COX-1 generated by alternative splicing (COX-3) has been cloned recently, but its significance is still not completely elucidated (Chandrasekharan et al, 2002). COX-2 is not normally present in most tissues and cell types but its expression is strongly induced in response to inflammatory mediators and tumor promoters. The induction of COX-2 is responsible for the increased synthesis of PG associated with inflammation and with some types of cancer, such as skin and colon cancer. PGH_2 is in turn the substrate for several synthases and isomerases which catalyze the synthesis of the different PGs and of thromboxane. The expression of these enzymes is subjected to a tissue-specific regulation which will determine the type of PG that will be produced in a given setting. Since several excellent reviews have recently addressed the current knowledge on prostanoid synthesis and biology (Funk, 2001; Straus and Glass, 2001), these subjects will be commented here briefly. PGE_2 is the major COX product detected in many cell types exposed to pro-inflammatory stimuli, including mesangial cells (Nakazato et al, 1991) and synovial fibroblasts (Martel-Pelletier et al, 2004) and appears to be the major PG that contributes to inflammation. There are several isoforms of PGE_2 synthase (PGES). The microsomal PGES-1 is upregulated in many tissues and cell types under inflammatory conditions. A coordinate induction of microsomal PGES and COX-2 in inflammatory settings contributes to the increase in PGE_2 generation (Funk, 2001). PGE_2 overproduction has been also related to the tumorigenic action of COX-2 in colon cancer, and mice in which PGE receptors have been genetically disrupted are protected from colon tumorigenesis (Sonoshita et al, 2001; Watanabe et al, 1999). PGD_2 is the major COX product detected in many rat tissues under normal situations (Ujihara et al, 1988) and it is very abundant in the brain and in mast cells.

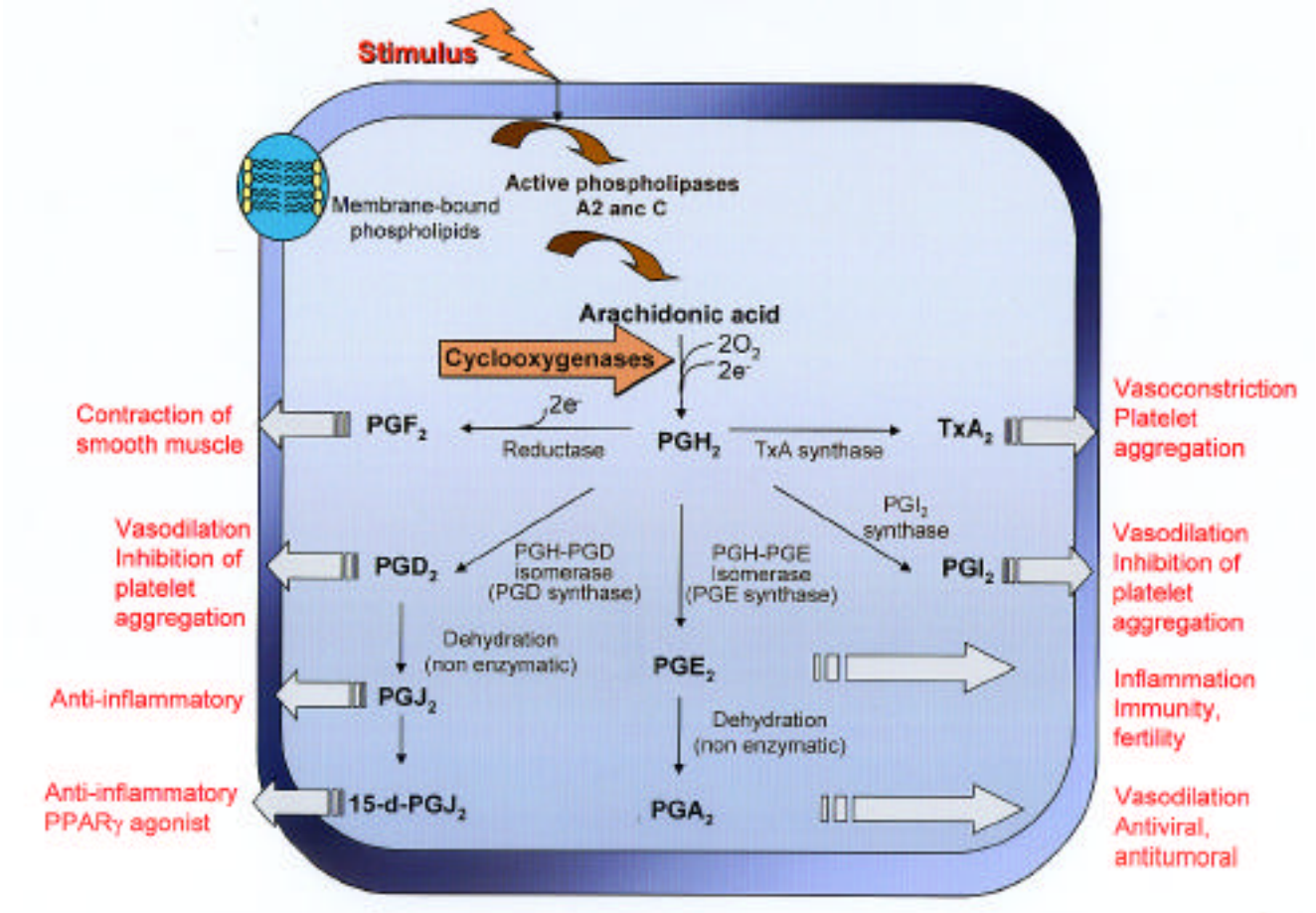

Figure 1. Scheme of the pathways of prostaglandin biosynthesis. Arachidonic acid released from membrane phospholipids upon cell stimulation is transformed by the action of cyclooxygenases into PGH_2, which is in turn converted into the various PG and thromboxane A_2 (TxA_2) by the action of PG and thromboxane synthases. The main biological actions of the different PG formed is shown. See text for details.

PGD_2 synthases belong to two classes that differ in amino acid sequence, tissue distribution and glutathione (GSH) dependency. The actions of PGD_2 include the regulation of sleep and ocular pressure, vasodilatation and inhibition of platelet aggregation (Eguchi et al, 1997) and references therein. PGI_2 is mainly synthesized in endothelial cells and induces vasodilatation and declumping of platelets.

Thromboxane A_2 is produced in platelets and macrophages and mediates vasoconstriction and platelet aggregation (Funk, 2001). As for PGF synthase, it is found in the uterus and PGF is involved in uterine smooth muscle contraction and parturition. In addition to the COX-mediated pathway for the synthesis of PG it has recently been reported that PGE_2 and D_2 can be generated non-enzymatically via the isoprostane pathway, which involves the free radical-catalyzed peroxidation of arachidonate in a manner independent of COX (Gao et al, 2003). This pathway has been proposed to contribute to the generation of approximately 30% of the PGD_2 detected in the urine of rats exposed to oxidant stress. Also, besides serving as substrates for the enzymatic reactions catalyzed by PG synthases, COX products can be subjected to non enzymatic transformations such as dehydration to yield a variety of PG. This is the case of PG with cyclopentenone structure (cyPG), some of which are shown in **Figure 2**. CyPG display varied biological effects, ranging from antiviral and antitumoral activity to the modulation of cell proliferation and inflammation. Most PG exert their actions through the interaction with G protein coupled receptors of the seven transmembrane helix type (Kobayashi and Narumiya, 2002), coupled to either Gi or Gs proteins, that transduce PG signals through the modulation of intracellular cAMP or calcium levels. However, cyPG display multiple mechanisms of action, some of which are receptor-independent and due to their ability to directly modify cellular proteins. This will be the main focus of this review.

B. Cyclopentenone prostaglandins

1. Generation and biological effects

CyPG are endogenous eicosanoids that arise from the spontaneous dehydration of their parent PG. Prostaglandins of the A series arise from the dehydration of PGE, while PG of the J series are produced by dehydration of PGD_2. CyPG have been detected in several biological fluids and tissues (see (Straus and Glass, 2001) for a detailed review). PGA_1 and PGA_2 were discovered in seminal plasma and kidney medulla, respectively, and were also identified as dehydration products of PGE derivatives *in vitro* (Granstrom et al, 1980). During the 1980s, several eicosanoids produced by the dehydration of PGD_2 were found to be generated *in vitro* or identified in biological fluids, such as 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2), which was found to arise from PGD_2 in

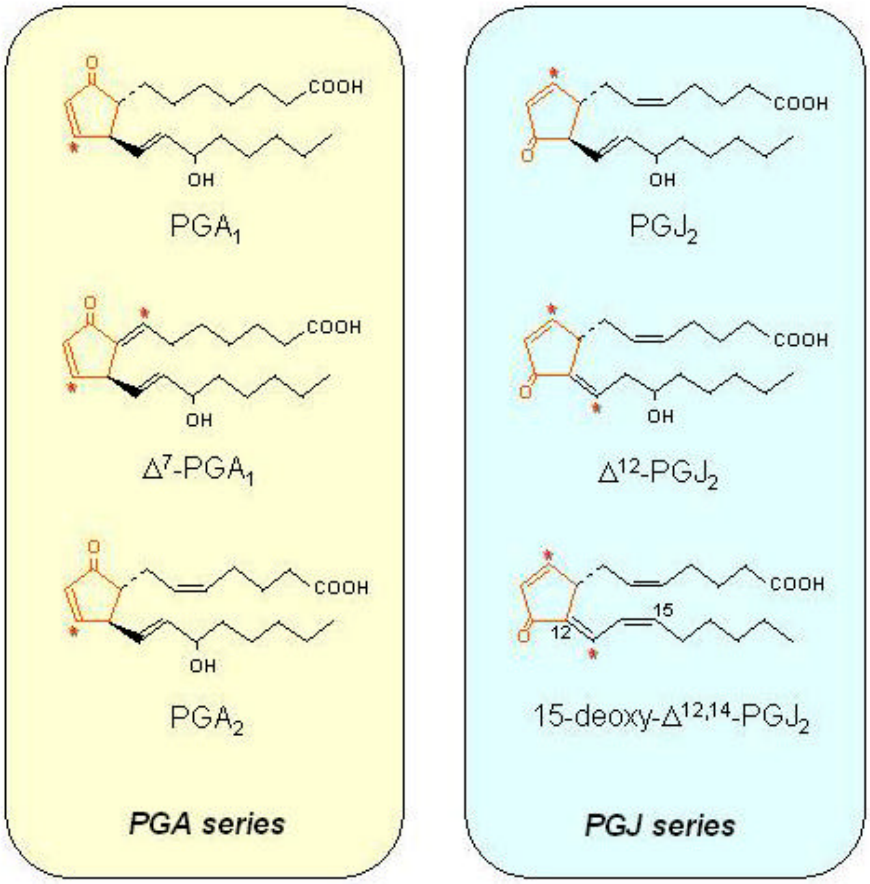

Figure 2. Structures of some prostaglandins of the A and J series. The cyclopentenone ring with the unsaturated carbonyl group is shown in orange and the electrophilic carbons are marked by red asterisks.

the presence of albumin (Fitzpatrick and Wynalda, 1983), and Δ^{12}-PGJ_2, which was found to be formed after incubation of PGD_2 with human plasma (Kikawa et al, 1984) and was detected in human urine (Hirata et al, 1988). The common characteristic of cyPG is the presence of an unsaturated α,β-carbonyl group in the cyclopentane ring (cyclopentenone). This structure confers a strong electrophilicity to one of the carbons in the cyclopentenone ring. Some cyPG possess a dienone structure with two electrophilic carbons (marked in **Figure 2** by asterisks). This particular structure was soon identified as an important requirement for the biological effects of cyPG.

Among the first biological or pharmacological actions of cyPG identified was their antitumoral activity. In a study by Kato and coworkers, several cyPG or cyPG derivatives were found to effectively increase the life span of Ehrlich ascites tumor-bearing mice (Kato et al, 1986). Subsequently, cyPG were found to inhibit the growth of several transformed cell lines (Ikai et al, 1987). The accumulated evidence indicates that cyPG may modulate cell proliferation in a concentration and cell-type dependent fashion. Inhibition of proliferation of tumoral cells has been often encountered in association with the induction of apoptosis (Kim et al, 1993). However, at low micromolar concentrations and in some cell types cyPG, and in particular 15d-PGJ_2, have been also shown to increase cell proliferation. This is the case of mesangial cells, NIH3T3 fibroblasts and colon cancer cells in which the expression of COX-2 has been blocked (Chinery et al, 1999; Rovin et al, 2002; Oliva et al, 2003). The pathophysiological significance of these effects of 15d-PGJ_2 is still not completely understood, but it has been proposed that cyPG could mediate some of the positive effects of COX-2 on cell proliferation in settings where these factors coexist, such as in chronic inflammation or colon tumorigenesis.

One of the most extensively studied effects of cyPG is the induction of a heat shock response, which has been evidenced in many cell types. The heat shock response plays a protective role against several kinds of stress, since it leads to the expression of cytoprotective proteins and molecular chaperones which prevent misfolded or damaged proteins from aggregation (Morimoto and Santoro, 1998). CyPG induce the activation of heat shock factor 1 (HSF1) and the expression of hsp70 (Amici et al, 1992), which is in turn associated with a cytoprotective effect during hypertermia and virus infection (Santoro, 2000). Induction of the heat shock response has been shown to elicit the expression of the inhibitory subunit of transcription factor NF-κB, IκBα (see below). PGA_1 also induces IκBα expression, thus providing a link between activation of HSF1 and inhibition of NF-κB which appears to occur coordinately in many experimental settings (Thomas et al, 1998). Induction of the heat shock response and/or inhibition of NF-κB appears to underlie the antiviral effects of cyPG. CyPG have been found to potently inhibit viral replication (Santoro, 1997). For instance, PGA_1 has been shown to inhibit the replication of the human immunodeficiency virus type-1 in cells by acting at several levels, including inhibition of viral mRNA transcription and viral protein synthesis (de Marco et al, 1998). CyPG have also been reported to inhibit the replication of other viruses such as influenza virus (Pica et al, 2000) or vesicular stomatitis virus (Pica et al, 1993) in several experimental models. Based on these findings a potential for the use of these compounds as antiviral agents has been proposed.

A great interest in the study of cyPG was raised when 15d-PGJ_2 was identified as a ligand for the transcription factor peroxisome proliferator activated receptor γ (PPARγ) (Forman et al, 1995; Kliewer et al, 1995)**,** which is involved in the control of lipid metabolism and adipocyte differentiation. Since at the time the endogenous ligand(s) for this factor were not known, this cyPG was considered the endogenous ligand for this receptor. This would imply a potential for 15d-PGJ_2 in the regulation of many pathophysiological processes related to lipid metabolism and vascular biology. It is now known that 15d-PGJ_2 may activate PPARα and γ and that several other cyPG could also activate these factors. The interest in PPAR agonists raised even more when these transcription factors were identified as negative regulators of inflammation. An anti-inflammatory role for PPARα was proposed in 1996 (Devchand et al, 1996). This was attributed to a negative feedback on inflammation produced by the induction of the expression of enzymes involved in the catabolism of pro-inflammatory lipidic mediators. Later, it was reported that PPARγ and its ligand 15d-PGJ_2 could reduce macrophage activation by inhibiting the expression of key inflammatory genes like iNOS (Ricote et al, 1998) and the release of pro-inflammatory cytokines by monocytes (Jiang et al, 1998). These findings spurred the research on the anti-inflammatory effects of these type of compounds. An anti-inflammatory role for COX-2 was proposed in the work by Gilroy et al. who detected a delayed production of PGJ_2 and its precursor PGD_2 at late times of inflammation in pleural exudates in rats, and proposed that these PG played a role in the resolution of inflammation (Gilroy et al, 1999). During the last several years numerous reports have described the negative effects of cyPG on the expression of pro-inflammatory genes, including monocyte chemoattractant protein 1 (Rovin et al, 2001) and matrix metalloproteinase-9 (Ricote et al, 1998), among others, in a wide variety of cellular models. Recently, a series of *in vivo* studies have shown that cyPG also display potent anti-inflammatory effects in intact animals. CyPG have been reported to contribute to the resolution of inflammation in a model of carrageenin-induced pleuresy in rats (Ianaro et al, 2001). This effect was associated with an inhibition of NF-κB. An inhibition of NF-κB activity appears to contribute also to the reduction of restenosis after balloon angioplasty in rats (Ianaro et al, 2003). These effects are related in part to the inhibition of the expression of pro-inflammatory genes such as iNOS. The beneficial effects of these compounds are not limited to inflammatory conditions, since a protective effect of cyPG has been also evidenced in several models of tissue injury, such as renal and intestinal ischemia reperfusion injury (Cuzzocrea et al, 2003b; Sivarajah et al, 2003) and ischemic acute renal failure (Chatterjee et al, 2004). 15d-

PGJ_2 has also been shown to attenuate the development of colon injury induced by dinitrobenzene sulphonic acid in the rat (Cuzzocrea et al, 2003a), reduce myocardial infarct size (Wayman et al, 2002), and reduce liver injury and multiple organ failure in endotoxic shock (Collin and Thiemermann, 2003; Collin et al, 2004). These observations point towards a potential use of cyPG or related compounds as therapeutic agents in pathological conditions with inflammatory or proliferative components like atherosclerosis, arthritis or glomerulonephritis.

Although there is a wide interest in these compounds as potential pharmacological tools, their potential pathophysiological role is far from being established. One of the reasons for this is the discrepancy between the concentrations of these compounds measured *in vivo* and those required to elicit the biological effects observed in cellular models or in intact animals. While the *in vivo* levels of cyPG are in the picomolar to nanomolar range, most of the biological actions described to date, including activation of PPARγ, have been observed using micromolar concentrations. This discrepancy has called into question the validity of the identification of 15d-PGJ_2 as an endogenous PPARγ ligand (Bell-Parikh et al, 2003). Several factors can complicate the estimates of cyPG in biological systems, among them, the possibility that cyPG may accumulate inside cells (Narumiya and Fukushima, 1986), thus reaching higher intracellular levels than those measured in the medium. Also, the high reactivity of the cyclopentenone moiety can result in the formation of adducts with several molecules and lead to underestimation of the generation of cyPG by methods which detect only the free forms. A recent report, using liquid chromatography-mass spectrometry for analysis of cyPG has not found alterations in 15d-PGJ_2 levels in various biological fluids during or following the induction of acute inflammatory response or in association with inflammatory or altered metabolic conditions, such as arthritis or obesity (Bell-Parikh et al, 2003). In contrast, procedures which detect cyPG by immunofluorescence using monoclonal antibodies that recognize the free form of the PG have allowed the observation of an increased generation of 15d-PGJ_2 in the foamy macrophages of human atherosclerotic plaques (Shibata et al, 2002), in macrophages of pleural exudates (Itoh et al, 2004) and in bacterial lipopolysaccharide (LPS)-stimulated Raw264.7 macrophages. In addition, an increase in 15d-PGJ_2 associated with increased COX-2 immunoreactivity has been reported in spinal cord sections of patients with sporadic amyotrophic lateral sclerosis (Kondo et al, 2002). Other determinations rely in the use of enzyme immunoassays that employ antibodies generated against the PG of interest. These assays may have some drawbacks since they may recognize both the free and the conjugated from of cyPG and may present cross-reactivity with other cyPG or with their parent PG. Using this type of assays the production of both PGD_2 and 15d-PGJ_2 has been observed in rat mammary gland in normal situations. However after administration of carcinogens, a switch towards the production on PGE_2 with a decrease in 15d-PGJ_2 has been measured (Badawi et al, 2004). Also, an increase in the generation of 15d-PGJ_2 by microglial cultures exposed to LPS has been detected by this technique (Bernardo et al, 2003). Although the importance of the pathways leading to the conversion of PGD_2 into cyPG of the J series *in vivo* is not established at present, it is clear that J_2 prostanoids are synthesized *in vivo*. Δ^{12}-PGJ_2 is a natural component of human body fluids and its synthesis is suppressed by treatment with COX-2 inhibitors (Hirata et al, 1988). On the other hand it is known that administration of PGD_2 *in vivo* leads to an increase in J prostanoids. In intact cells, transfection of expression vectors coding for enzymes implicated in biosynthesis of PGD_2, such as cytosolic PLA_2, COX-2 and PGD synthase has anti-inflammatory effects (Rossi et al, 2000). In summary, the physiological importance of cyPG generation is still under debate. While the elucidation of this important point awaits further studies it is clear that given the potent biological effects of cyPG *in vitro* and in animal models, the pharmacological properties and potential of these compounds deserves detailed investigation.

2. Mechanisms of action of cyPG

From the above exposed findings it can be inferred that the mechanism of action of cyPG may be complex and multiple. Some of the potential mechanisms involved in cyPG effects in cells are summarized in **Figure 3**. Some cyPG, including 15d-PGJ_2 and PGA_1 can behave as agonists of PPAR (α, β or γ) (Bishop-Bailey and Wray, 2003). The list of PPAR-regulated genes is very extensive and opens a broad spectrum of possibilities for the action of cyPG and in particular of 15d-PGJ_2 in the regulation of cellular function by direct or indirect mechanisms (Fajas et al, 2001; Blanquart et al, 2003). PPAR have been shown to interfere with cellular proliferation and activation pathways in various ways. Activated PPAR can interfere with the activity of other transcription factors, like NF-κB, AP-1, STAT-1 and NFAT, either by competing for limiting amounts of coactivators (Li et al, 2000), by modulating the expression of proteins involved in the regulation of transcription factor activity (Delerive et al, 2000), or by direct protein-protein interaction (Delerive et al, 1999; Chung et al, 2003). In addition to its role as PPAR agonist, 15d-PGJ_2 may interact with other proteins. The presence of binding sites for 15d-PGJ_2 in the plasma membranes of primary rat cortical neurons has been recently reported, thus suggesting the possibility of a membrane receptor involved in the effects of 15d-PGJ_2 in neuronal apoptosis (Yagami et al, 2003).

CyPG have also been found to modulate cellular redox status. This may occur through multiple mechanisms and in turn be at the basis of some of the effects of cyPG such as the induction of apoptosis (Kondo et al, 2001; Chen et al, 2002). Low micromolar concentrations of 15d-PGJ_2 have been shown to increase GSH concentrations and enhance GSH-dependent cytoprotection in human endothelial cells. This effect is mediated through the upregulation of glutamate-cysteine ligase, the rate-limiting enzyme of GSH synthesis (Levonen et al, 2001). However, cyPG have also been reported to induce potent oxidative stress in several experimental systems (Hortelano et al, 2000; Kondo et al,

2001; Nencioni et al, 2003). The source of reactive oxygen species (ROS) in cells treated with cyPG has not been characterized in detail, although mitochondria appear to play an important role (Kondo et al, 2001). In some cell types, such as macrophages, increased superoxide anion generation after treatment with 15d-PGJ_2 in the presence of high NO concentrations can give rise to the formation of cytotoxic peroxynitrite which causes mitochondrial dysfunction and apoptosis. This effect has been proposed to contribute to the resolution of inflammation (Hortelano et al, 2000). In neuroblastoma cells, an increase in ROS production after exposure to cyPG has been found in association with a depletion of antioxidant defenses, such as GSH and GSH-peroxidase, and with the production of protein-bound lipid peroxidation products (Kondo et al, 2001). Induction of oxidative stress has been proposed to be at the basis of other effects of cyPG like the increase in heme oxygenase expression (Alvarez-Maqueda et al, 2004), and the increase in the activity of mitochondrial complex I observed in endothelial cells (Ceaser et al, 2003). From a structural point of view it was early realized that the antitumoral effects of cyPG required the cyclopentenone moiety (Fukushima, 1983; Honn and Marnett, 1985), and structure-activity studies were conducted in order to find the most potent PG that could succeed as anticancer agents (Kato et al, 1986). The presence of the cyclopentenone moiety appears to be important also for the increased generation of ROS and apoptosis in several cell types (Shibata et al, 2003a). Due to the reactivity of the cyclopentenone moiety, cyPG may form covalent adducts with cellular thiols, such as GSH or cysteine through Michael addition (Noyori and Suzuki, 1993). Depletion of GSH due to reaction with cyPG is unlikely to contribute to oxidative stress, given the great excess of GSH over cyPG concentrations. This property, however, has some experimental implications. Some of the evidence which attribute a given effect of cyPG to the induction of oxidative stress are based on the use of thiol-containing antioxidants, such as GSH, N-acetyl-cysteine or DTT. The possibility should be considered that the inhibition of the effects of cyPG brought about by these antioxidants could be due to the formation of adducts that would reduce the concentration of the free PG. The potential adducts formed could display weaker biological activity than the free cyPG, as it has been observed for the antitumoral effect of the PGA_2-glutathione adduct (Honn and Marnett, 1985), or the inhibition of p50 DNA binding by GSH-pre-treated 15d-PGJ_2 (Cernuda-Morollón et al, 2001). The formation of adducts of cyPG and intracellular thiols such as GSH has been documented in several studies and it can occur spontaneously or by means of the catalytic activity of glutathione transferase (GST) (Atsmon et al, 1990b). This process is considered as one of the mechanisms involved in the detoxification of cyPG. In this context, it is known that the biological effects of cyPG depend on the intracellular GSH concentration, and some effects are potentiated by GSH depletion (Atsmon et al, 1990a; Kim et al, 1996). In consistence with this, overexpression of GST or of the transporters

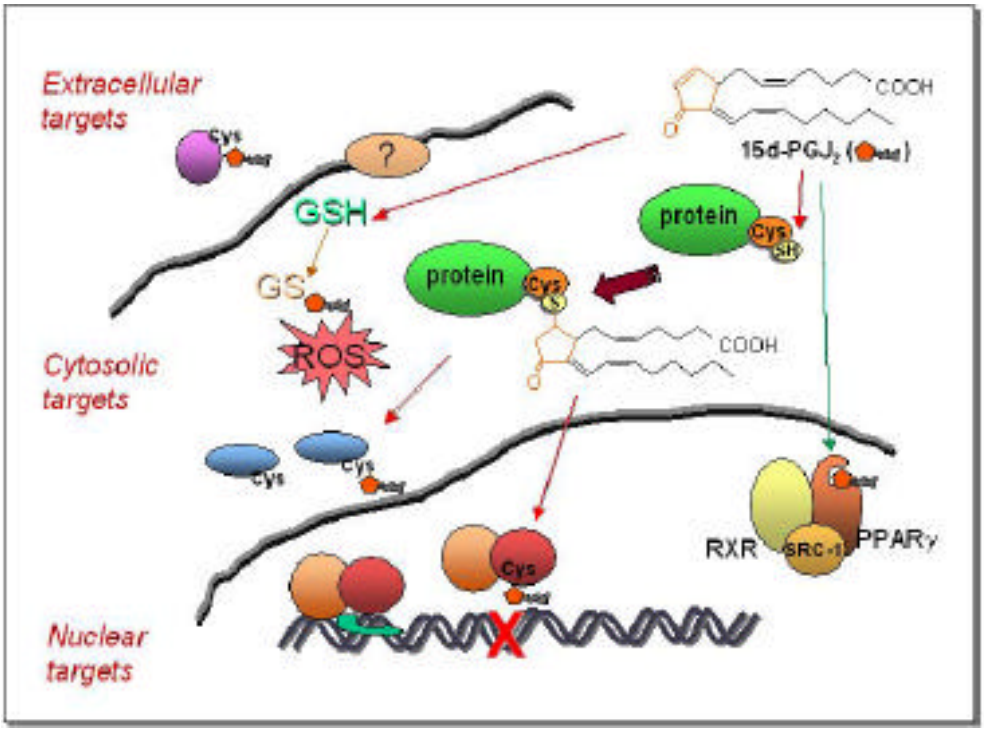

Figure 3. Potential mechanisms of action of cyPG. cyPG may exert their varied biological actions by multiple mechanisms. Some cyPG, like 15d-PGJ_2, schematized by the orange and grey icon, may act as a ligands of the nuclear receptor PPAR. In addition cyPG possess an α,β-unsaturated carbonyl group in the cyclopentane ring (in orange in the structure of 15d-PGJ_2) which may form adducts with cellular proteins or with glutathione (GSH) by Michael addition, leading to a modulation of protein function and to changes in the cellular redox status. Among the potential targets for modification by cyPG are cytosolic targets, like IKK or GSH, nuclear targets, like transcription factors, and possibly extracellular targets, like serum proteins. The presence of binding sites for cyPG in cell membranes has also been reported.

involved in elimination of GST conjugates, like the multidrug resistance proteins, reduce the biological activity of cyPG as measured by the activation of PPAR reporters or assessment of the cytotoxic effects (Paumi et al, 2003, 2004). In turn, different expression levels or activity of multidrug resistance proteins have been proposed to be involved in the differential sensitivity of tumor or immune cells towards the cytotoxic actions of cyPG (de Bittencourt Junior et al, 1998).

In addition to forming adducts with low molecular weight thiols, cyPG can react with the sulphydryl groups of cysteine residues by Michael addition and the resulting modification can have regulatory consequences for protein function. This type of protein modification, to which we refer as prostanylation, is schematized in **Figure 3**. In the past few years it is becoming more clear that the modification of cellular proteins by cyPG plays an important role in their biological effects. The characteristics and consequences of this modification are discussed below.

II. Protein modification by cyPG

As stated above, the fact that the cyclopentenone moiety of cyPG was required for many of the biological actions of these PG pointed towards the possibility that their ability to react with cellular thiols would play a major role in their mechanism of action. One of the first evidences of binding of cyPG to cellular proteins was obtained by incubating cells in the presence of a biotinylated analog of PGA_2, followed by SDS-PAGE and Western blot with streptavidin-alkaline phosphatase, and this binding correlated with the inhibition of cellular proliferation exerted by PGA_2 in K562 eritroleukemia cells (Parker, 1995). In a series of important studies Narumiya et al, (Narumiya and Fukushima, 1986; Narumiya et al, 1986, 1987), showed that radioactively labeled cyPG entered intact cells and accumulated in cell nuclei, where a portion of them could be found bound to an insoluble fraction. The proportion of cyPG associated with the insoluble fraction was higher for Δ^{12}-PGJ_2 than for PGA_2. The radioactive cyPG could be released by treatment with alkali and by digestion of the nuclear residues with proteases but not with DNases, which was consistent with the formation of covalent adducts between the cyPG and the proteins of chromatin and/or nuclear matrix. More recently, biotinylated analogs of 15d-PGJ_2 have been used to show the stable binding of 15d-PGJ_2 to cellular proteins, which is resistant to analysis by HPLC or SDS-PAGE (Cernuda-Morollón et al, 2001). Biotinylated analogs of cyPG can also be used to study the subcellular distribution of these compounds by means of fluorescence microscopy. An example of the pattern given by incubating HeLa cells in the presence of biotinylated 15d-PGJ_2 followed by detection with fluorescent streptavidin is shown in **Figure 4**. In this cell type, binding appears to be confined mainly to the nuclear compartment, whereas in other cell types such as macrophages or mesangial cells, both nuclear and cytoplasmic compartments are intensely labeled by biotinylated cyPG (Cernuda-Morollón et al,

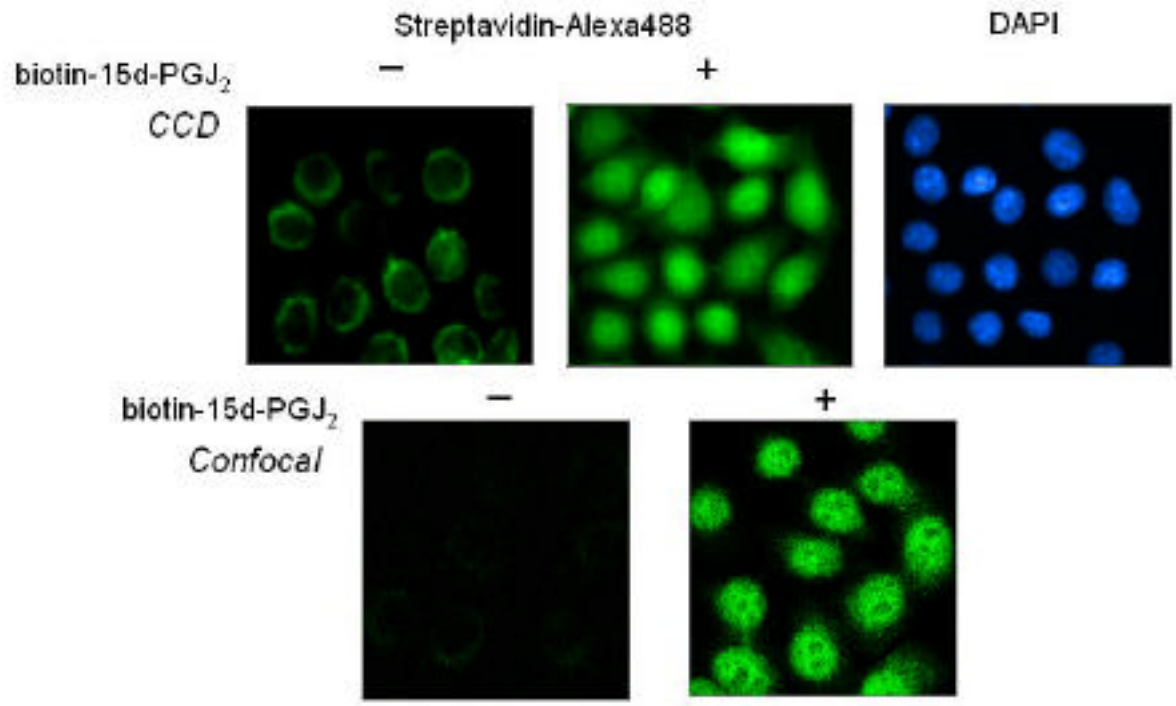

Figure 4. Subcellular distribution of biotinylated 15d-PGJ_2 in HeLa cells. HeLa cells were incubated in the absence or presence of a derivative of 15d-PGJ_2 biotinylated at the carboxyl group, so that the cyclopentenone moiety is free to react with cellular thiols. The sites for attachment of biotinylated 15d-PGJ_2 were visualized by fluorescence microscopy after cell permeabilization, fixation and incubation with streptavidin labeled with the fluorescent dye Alexa 488. Cell nuclei were stained with DAPI. Images were obtained with a CCD camera connected to a Zeiss fluorescence microscope or by confocal microscopy. The pattern observed in the absence of biotinylated 15d-PGJ_2 shows the subcellular distribution of endogenous biotinylated proteins. Cells incubated in the presence of biotinylated 15d-PGJ_2 show a marked nuclear staining indicative of the preferential nuclear distribution of 15d-PGJ_2 in this cell type.

unpublished observations). It should be noted, however, that, although biotinylated cyPG possess the reactive cyclopentenone moiety, their properties may not be identical to those of endogenous cyPG due to the presence of the biotin moiety.

Although the formation of adducts of cyPG with low molecular weight thiols is known to be reversible, protein prostanylation is considered an irreversible modification under physiological conditions (Noyori and Suzuki, 1993). The stability of the cyPG-protein adducts has been attributed to the decreased molecular motion in the polymeric biomatrices of cell nuclei, where cyPG are preferentially accumulated (Noyori and Suzuki, 1993). However, the stability of cyPG-protein adducts may depend also on cyPG structure. While the accumulation of Δ^{12}-PGJ_2 in cell nuclei and its cytotoxic effects could not be reversed by washing of cells in PG-free medium, the nuclear accumulation of PGA_2 decreased rapidly by washing and cell growth was partially restored (Narumiya et al, 1987). It has also been proposed that the stability of protein prostanylation could depend on the microenvironment surrounding the modified cysteine residue (Bickley et al, 2004). This could have important implications for determining which proteins would undergo stable protein prostanylation. The importance of protein prostanylation is being unveiled as the protein targets for this modification are identified. To date, about a dozen proteins have been reported to bind cyPG covalently (**Table 1**). Some of these proteins are involved in the modulation of inflammation, like some components of the NF-κB and AP-1 activation pathways, or in redox sensing. The structural and functional characterization of these targets will provide valuable information about the mechanism of action of cyPG and help define the factors involved in the selectivity and implications of protein prostanylation.

A. Interactions of cyPG with the NF-κB activation pathway

The functional importance of the interaction of cyPG with cellular proteins was suggested by several studies which showed that the effect of cyPG on specific signaling pathways was dependent on the presence of critical cysteine residues in protein components of these pathways. One such example with important implications for the anti-inflammatory effects of cyPG is the NF-κB activation pathway. NF-κB is a transcription factor constituted by dimers present in the cytoplasm of cells where it is kept inactive by binding to the inhibitory subunit IκB. Pro-inflammatory agents trigger a signaling pathway in which a kinase complex, known as IKK (IκB kinase) phosphorylates IκB. This phosphorylation directs the ubiquitination and degradation of this protein, and this allows the accumulation of the active NF-κB dimer in the cell nuclei, where it can bind to DNA and activate transcription (Baeuerle, 1998; Karin, 1999). Given the critical importance of the activity of NF-κB for the expression of many pro-inflammatory genes, this pathway has been the target for numerous studies in the search for anti-inflammatory agents. Since the first studies on the anti-inflammatory effects of PPAR agonists, it was found

Table 1. Protein targets for covalent modification by cyPG identified by various procedures

Protein	CyPG	Residue modified	Functional consequence	Reference
IKKα and β	15d-PGJ_2 PGA_1	Cys179	Inhibition	Rossi et al. 2000 Castrillo et al. 2000
NF-κB p65 subunit	15d-PGJ_2	Cys38	Inhibition	Strauss et al. 2000
NF-κB p50 subunit	15d-PGJ_2	Cys62	Inhibition	Cernuda-Morollón et al. 2001
Proteasome	15d-PGJ_2	Not identified	Inhibition	Shibata et al. 2003
H-Ras	15d-PGJ_2 PGA_1	Cys184 multiple	Activation	Oliva et al. 2003
Thioredoxin	15d-PGJ_2	Cys35 and Cys69	Inhibition	Shibata et al. 2003
Thioredoxin reductase	PGA_1	Not identified	Inhibition	Moos et al. 2003
Keap1	PGA_1	Not identified	Inhibition	Itoh et al. 2004 Levonen et al. 2004
c-Jun	15d-PGJ_2	Cys269	Inhibition	Pérez-Sala et al. 2003
c-Fos	15d-PGJ_2	Not identified	Inhibition	Pérez-Sala et al. 2003

that some of them, including 15d-PGJ_2, inhibited NF-κB activation and pro-inflammatory gene expression in cellular models of inflammation (Ricote et al, 1998). Although at first these effects were interpreted as a result of PPAR activation, several laboratories provided functional evidences pointing to a direct effect of cyPG on NF-κB signaling, by mechanisms independent from PPAR. A study by Castrillo et al, showed that treatment of murine macrophages with 15d-PGJ_2 inhibited the activation of NF-κB in response to treatment with LPS and IFN-γ (Castrillo et al, 2000). This effect was associated with a blockade of the decrease in IκBα and β levels induced by pro-inflammatory stimuli and with an inhibition of the phosphorylation of IκBα elicited by these agents. These authors showed that 15d-PGJ_2 was able to directly impair the function of activated IKK present in immunoprecipitates, thus reducing its ability to phosphorylate IκBα at serine 32, which is needed for the targeted degradation of IκB. This mechanism was also reported by Rossi et al. who addressed the structural determinants involved in this effect, and showed that only A- and J-type PGs inhibited IKK activity *in vitro* or in intact cells, while other arachidonic acid metabolites were ineffective (Rossi et al, 2000). Experiments using IKK mutants showed that the presence of a cysteine residue at position 179 in the activation loop of IKKβ was required for the inhibition, this suggesting the occurrence of an interaction between the cyclopentenone moiety of the cyPG and the sulphydryl group of this residue by Michael addition. The contribution of this mechanism to the inhibition of NF-κB depends on the cell system under study. Whereas in murine macrophages (Castrillo et al, 2000; Straus et al, 2000) and in rat mesangial cells (Cernuda-Morollón and Pérez-Sala, unpublished observations) cyPG impair IκBα phosphorylation and degradation, thus pointing to the involvement of the inhibition of IKK, in other experimental systems, such as HeLa cells, inhibition of NF-κB transcriptional activity and DNA binding occurs in spite of unaltered IKK activity, IκB degradation and nuclear translocation of NF-κB subunits (Straus et al, 2000; Cernuda-Morollón et al, 2001). In fact, 15d-PGJ_2, or the compound cyclopentenone, could inhibit the DNA binding of the recombinant subunit of NF-κB p65 *in vitro*, in a manner dependent on the presence of cysteine 38, located in the DNA binding domain (Straus et al, 2000). These observations pointed to the involvement of a direct interaction between the cyclopentenone moiety of the PG and cysteine residues of NF-κB subunits. This point was further substantiated by the demonstration that 15d-PGJ_2 could inhibit the DNA binding activity of the p50 subunit of NF-κB by interacting with cysteine 62 which is located in the DNA binding domain. This interaction resulted in a retardation in the retention time of the 15d-PGJ_2-treated recombinant protein in HPLC analysis and in an increment in the mass of the protein as detected by MALDI-TOF mass spectrometry analysis (Cernuda-Morollón et al, 2001). Moreover, incubation of intact cells with a biotinylated derivative of 15d-PGJ_2 resulted in the incorporation of biotin into p50 that could be evidenced by immunoprecipitation and avidin binding (Cernuda-Morollón et al, 2001). This type of approach allowed the demonstration of the covalent adduct between p50 and 15d-PGJ_2 and constituted the first identification of a protein target for this type of modification. Using a similar approach, adducts of biotinylated cyPG and both, IKKα and β have later been reported (Castrillo et al, 2003; Moos et al, 2003). The potential for interaction of cyPG with the NF-κB signaling pathway also extends to components of the protein degradation machinery. The regulated degradation of IκB by the proteasome is key for NF-κB activation. Several reports have identified inhibitory effects of 15d-PGJ_2 on proteasome activity. The ubiquitin isopeptidase activity of the proteasome is a target for cyPG of the J series (Mullally et al, 2001). The presence of the endocyclic double bond of 15d-PGJ_2 appears to be required for the effect of the PG on protein turnover, which suggested the involvement of the formation of Michael adducts in the mechanism of this effect. In fact, a 15d-PGJ_2-proteasome conjugate has been recently identified by using biotinylated 15d-PGJ_2 (Shibata et al, 2003b). In addition to the consequences on pro-inflammatory signaling, the effects of cyPG on the degradation machinery could have important implications for cell survival (Mullally et al, 2001). This has been envisaged as a promising feature for the development of proteasome inhibitors with applications in the treatment of inflammatory and proliferative diseases.

B. Interactions of cyPG with the thioredoxin-thioredoxin reductase system

CyPG may alter the cellular redox state by various mechanisms. One possible mechanism for this effect is the functional impairment of proteins involved in the control of cellular antioxidant defenses by direct or indirect interactions. Thioredoxin (Trx) is a low molecular weight protein which plays a key role in the maintenance of cellular redox balance. Trx plays an important role in the redox regulation of signal transduction and in the cell defense against oxidative stress. The activity of Trx depends on two critical cysteine residues located in the active site which undergo reversible oxidation and reduction. Protein substrates with disulfide bonds bind to Trx, a cysteine residue of which forms a mixed disulfide with the protein. Another cysteine residue attacks the mixed disulfide and releases the protein substrate in a reduced form. The oxidized Trx is then substrate for another enzyme, Trx reductase (TR), which regenerates the active form of Trx (Powis and Montfort, 2001). Among the protein substrates which may be regulated by Trx-TR cycling are key transcription factors involved in inflammation or in cell survival such as NF-κB, AP-1, p53 and HIF. Recently, both Trx and TR have been reported to be targets for modification by cyPG. In Trx, an adduct formed by one molecule of 15d-PGJ_2 and one molecule of Trx has been reported, although two cysteine residues, cysteines 35 and 69 in human Trx, have been identified by electrospray ionization-liquid chromatography/mass spectrometry/mass spectrometry as the potential targets for this modification. These cysteines have been proposed to be the direct sensors for electrophilic PG-induced changes

in cellular redox status (Shibata et al, 2003a). The modification of TR by cyPG results in the inactivation of this protein. This interaction has been shown by means of a biotinylated PGA_1 analog (Moos et al, 2003) and is associated with impairment of the activity of TR-dependent transcription factors, such as p53 and HIF. Interestingly, TR is a selenoprotein and the inhibitory effect of cyPG can be reduced by selenium supplementation. These results also provide a working hypothesis about the potential mechanisms by which cyPG and other electrophiles may contribute to carcinogenesis in some settings by impairing p53 function.

C. The modification of Keap1 as a mechanism of redox sensing

Cells possess critical defense mechanisms against oxidative stress and environmental electrophiles, including carcinogens and xenobiotics. This defense relies in the transcriptional regulation of antioxidant and cytoprotective enzymes. The transcriptional regulation of chemoprotective enzymes, is related to the presence of transcriptional enhancers in the promoters of these genes which are known as antioxidant response elements (ARE) or electrophile response elements. The transcription factor Nrf2 plays a major role in the regulation of genes encoding phase 2 detoxification and antioxidant enzymes through the interaction with ARE (Ishii et al, 2000, 2002). Nrf2 is kept inactive by association with a cytoplasmic inhibitor protein known as Keap1. This protein, does not simply sequesters Nrf2 in the cytoplasm but actively targets Nrf2 for ubiquitination and degradation by the proteasome under non-stimulated conditions (Zhang and Hannink, 2003). Upon stimulation, Nrf2 dissociates from Keap1, escaping Keap1-dependent degradation, and translocates to the nucleus where it forms dimers with other basic region-leucine zipper transcription factors and binds to the promoters of ARE-governed genes (Nguyen et al, 2003). Keap1 is a cysteine-rich protein. Human Keap1 has 27 cysteine residues, of which, cysteine 273 and cysteine 288 appear to be critical for Keap1-dependent repression and degradation of Nrf2. It has recently been shown that Keap1 is a target for modification by electrophilic lipid oxidation products, including cyPG. Thiol modification of Keap1 induced the dissociation of Nrf2 and activation of ARE (Levonen et al, 2004; Wakabayashi et al, 2004). The Keap1-cyPG adduct has been evidenced by using biotinylated analogs of both 15d-PGJ_2 and Δ^{12}-PGJ_2 (Itoh et al, 2004; Levonen et al, 2004), although the precise cysteine residues involved in the interaction with cyPG have not been identified. Activation of Nrf2 by cyPG has important implications in the induction of antioxidant and phase 2 enzymes and in the modulation of inflammation. 15d-PGJ_2 can stimulate GSH synthesis by transcriptional upregulation of the rate-limiting enzyme glutamate-cysteine ligase, by a mechanism dependent on the activation of ARE present in its promoter (Levonen et al, 2004). Also, cyPG have been shown to induce GST (Kawamoto et al, 2000), although the involvement of covalent protein modification in this effect has not been assessed. 15d-PGJ_2 activates Nrf2 in mouse peritoneal macrophages and triggers the expression of heme oxygenase 1 and peroxiredoxin 1. These changes have implications for the inflammatory response (Itoh et al, 2004). A negative correlation between the induction of heme oxygenase 1 by cyPG and the induction of pro-inflammatory genes like iNOS has been reported (Colville-Nash et al, 1998), and this effect has recently been claimed to be required for the anti-inflammatory effects of low concentrations of 15d-PGJ_2 (Lee et al, 2003). Thus, the modification of Keap1 by cyPG may provide links between the cellular antioxidant response and the modulation of inflammation.

D. Mechanisms involved in the regulation of AP-1 by cyPG

The transcription factor AP-1 plays a central role in the control of cell proliferation in response to mitogens, in the cellular response to stress and in inflammation (Karin, 1995). AP-1 is subjected to a complex regulation both at transcriptional and posttranscriptional levels. The active factor is composed of homo or heterodimers of the jun and fos family of proteins. AP-1 is a redox sensitive transcription factor. Its DNA binding activity requires that critical cysteine residues located in the DNA binding domains of jun and fos proteins are in a reduced state. Cysteine 269 of human c-Jun is located in close contact with DNA. Reactive oxygen and nitrogen species can modify this cysteine residue and inhibit DNA binding (Klatt et al, 1999a, 1999b). CyPG have been shown to inhibit AP-1 activity in several experimental systems (Ricote et al, 1998; Fahmi et al, 2001; Sawano et al, 2002). Multiple mechanisms may be involved in this inhibitory effect. We have recently shown that the cyPG 15d-PGJ_2 can directly bind to c-Jun and inhibit DNA binding (Pérez-Sala et al, 2003). The binding occurs by means of covalent attachment to cysteine 269 of c-Jun, as it has been evidenced by mass spectrometry analysis, and it takes place both *in vitro* and in intact cells, as shown by labeling with biotinylated 15d-PGJ_2. c-Fos can also be recovered in avidin pull-down assays of lysates of cells incubated with the biotinylated cyPG, thus suggesting that both components of the transcription factor may be targets for this modification. Interestingly, incubation of recombinant c-Jun with 15d-PGJ_2, but not with PGA_1 resulted in the formation of a dimer species. This is due to the formation of a bis-conjugate between one molecule of 15d-PGJ_2, which possesses two electrophilic carbons, and two monomers of c-Jun, and represents the first example of the induction of protein cross-linking by cyPG. In contrast, PGA_1, which possesses only one electrophilic center does not induce this phenomenon (Pérez-Sala et al, 2003). In addition to the direct modification of AP-1 proteins, cyPG may modulate AP-1 activity by acting at other levels, like the generation of cellular oxidative stress or the inactivation of TR or Trx, of which AP-1 is a substrate. It has been reported that cyPG may also exert both positive or negative effects on the activity of mitogen activated protein kinases (MAPK) involved in the regulation of AP-1 by transcriptional or posttranscriptional mechanisms, for instance, 15d-PGJ_2 has been reported to modulate ERK, JNK and p38 activities (Maggi et al, 2000; Sawano et al, 2002; Castrillo et al, 2003), although the mechanisms

involved have not been fully elucidated. Oxidation of reactive cysteine residues located in the active sites of tyrosine phosphatases and inactivation of phosphatase activity may contribute to the activation of tyrosine phosphorylation-dependent pathways (Lee et al, 1998). An inhibition of protein tyrosine phosphatase activity could result in a net increase in tyrosine phosphorylation which could have important implications in various signaling pathways, including MAPK activation. In addition, ERK activation could depend on the direct modification of H-Ras by cyPG, as it will be described below. An interference with AP-1 transcriptional activity could arise also from the activation of PPAR by cyPG which could directly interact with AP-1 proteins, or compete for co-activators, as it has been discussed above. Thus, the potential interactions of cyPG with AP-1-mediated signaling are multiple.

E. CyPG selectively activate H-Ras proteins

CyPG have been shown to modulate cellular proliferation in many cell types. However, the effects encountered appear to be very complex and cell-dependent. While in some cell types cyPG have been shown to inhibit cell proliferation and/or induce apoptosis, in others, a stimulation of cell proliferation has been observed. It is also possible to encounter biphasic effects of cyPG which in a dose-dependent manner can induce cell proliferation or apoptosis in the same cell type (Rovin et al, 2002). Several lines of evidence point towards a potential interaction of the cyPG 15d-PGJ_2 with the Ras-activated signaling pathways being implicated in the proliferative effects of this PG. Recently we have shown that treatment of mouse NIH-3T3 fibroblasts with 15d-PGJ_2 induces cell proliferation in association with Ras and ERK activation. The study of this phenomenon led us to identify a direct interaction of cyPG with H-Ras proteins, by which cyPG bind covalently and activate H-Ras (Oliva et al, 2003). Activation of the Ras pathway by this mechanism is associated with increased ERK and AKT phosphorylation and with an increase in cellular markers of proliferation. The effect of 15d-PGJ_2 is specific for H-Ras and it occurs through the covalent modification of cysteine 184 in human H-Ras. This cysteine residue is located near the carboxyl terminal end of the protein, and it is not present in N- or K-Ras (**Figure 5**). Mutation of this residue blocks both the attachment of 15d-PGJ_2 to H-Ras, and the stimulation of its activity. These observations constitute the identification of a novel mechanism for the activation of H-Ras-dependent signaling pathways. It will be interesting to explore whether other lipids with similar reactivity can also modify and activate H-Ras, and to assess the pathophysiological situations under which this type of mechanisms could operate. It could be hypothesized that this mechanism could contribute to the increased cell proliferation observed in some situations in which there is an increase in the generation of reactive prostaglandins or other reactive products of lipid peroxidation, as it may occur in chronic inflammation, tumorigenic process and neurodegenerative diseases.

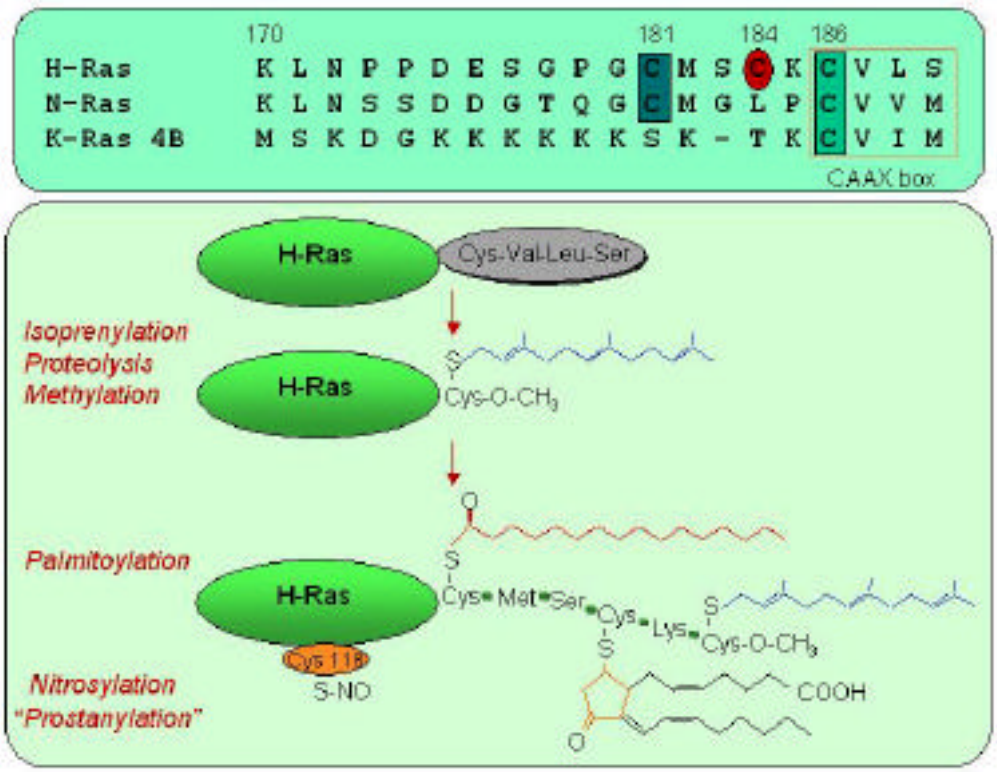

Figure 5. Posttranslational modifications of H-Ras proteins. The sequences of the three Ras proteins are shown on the top panel. The cysteine residues in the CAAX box are the site for isoprenylation. Cysteines 181 and 184 of H-Ras and 181 of N-Ras can be palmitoylated. Cysteine 184 of H-Ras has been identified as the site for attachment of 15d-PGJ_2. Cysteine 118 of H-Ras has been found to be the site for S-nitrosylation of this protein. The lower panel shows a scheme of these modifications with the farnesyl group depicted in blue, the palmitoyl group in red and the prostaglandin in black and orange as in **Figure 3**.

III. Future directions

Given the biological effects of cyPG and their potential interest as mediators of pathophysiological processes or tools for the development of therapeutic strategies, it is conceivable that during the next few years research on this field will be exciting. The elucidation of the pathophysiological role of cyPG would require more efforts to accurately measure the endogenous levels of cyPG which are generated under basal conditions and in disease. The fact that cyPG readily bind to thiols and may be conjugated to GSH by enzymatic and non-enzymatic mechanisms increases the difficulties for the accurate determination of these compounds. It should be important to assess the concentrations of the free and conjugated forms, and to explore the biological activity of the conjugates formed either intracellular o extracellularly. In this context, the evidence available thus far are conflicting. In an early report, exogenous administration of PGA_2-glutathione adduct displayed reduced potency as antitumoral agent than the parent PG (Honn and Marnett, 1985). In contrast, other works suggest that the formation of glutathione-related conjugates may be important for the cytotoxic action of cyPG in some cell types since GSH-depleted cells were insensitive to the cytotoxic effect of PGA_2 (Parker and Ankel, 1992). Interestingly, a recent report has shown that some conjugates of synthetic cyclopentenones with cysteine derivatives retain biological activity similar to that of the unconjugated parent compounds. While it has not been demonstrated that in this latter case the biological activity observed is the result of protein covalent modification, these observations open the possibility for the hypothesis that the conjugated forms of these compounds may behave as carriers for the active forms of cyPG (Bickley et al, 2004).

On the other hand, the characterization of the role of the different PG synthases involved in the formation of the precursor PG (PGES and PGDS), will shed light on the regulation of the generation of cyPG. It is interesting to note that in addition to cyPG, other endogenous lipids are generated which display a reactivity similar to that of cyPG. Inflammatory exudates contain a variety α,β-unsaturated aldehydes and ketones derived from eicosanoid metabolism and lipid peroxidation that could act by similar mechanisms to those identified for cyPG. One of the endogenous lipids with reactive α,β-unsaturated carbonyl groups is 4-hydroxy-nonenal, a reactive aldehyde that can be detected at low micromolar concentrations under control situations, but that can reach concentrations of 10 μM in tissues under conditions of oxidative stress (Esterbauer et al, 1990). There are also other cyclopentenone prostanoids formed *in vivo* like the cyclopentenone A_2/J_2- isoprostanes which have been detected esterified to lipids at approximately 125 ng/g in the livers of rats given CCl_4 (Chen et al, 1999). In addition, cyclopentenone eicosanoids with a reactivity similar to that of 15d-PGJ_2 have been found in normal brain tissue at relatively high levels (98 ng/g) (Fam et al, 2002). Therefore, from a pathophysiological point of view, cyPG could be only a small fraction of the lipids that may form covalent adducts with cysteine residues and be potentially involved in cellular apoptosis or induction of oxidative stress through this mechanism.

An important issue affecting both the potential pathophysiological role and therapeutic applications of protein prostanylation is its selectivity. From the available evidence it is becoming clear that protein modification by cyPG does not take place randomly but that it is a selective process that occurs at defined cysteine residues within given proteins. This view arises from several types of evidence. The monitorization of the modification of cellular proteins by biotinylated 15d-PGJ_2 using two dimensional electrophoresis has allowed the detection of approximately 30 modified proteins in human embryonic kidney cells (Levonen et al, 2004). By using this approach we have observed that a selective subset of cellular proteins is modified by biotinylated 15d-PGJ_2 in NIH-3T3 fibroblasts (Stamatakis et al, unpublished observations). The identification of the proteins susceptible to be modified by Michael addition of cyPG will provide a deeper insight into the factors that determine this selectivity and help to evaluate whether a potential "prostanylation motif" can be proposed. The selectivity of protein prostanylation can also be evidenced within a single protein. The case of Ras proteins is particularly interesting. Of the six cysteine residues present in H-Ras, only cysteine 184 seems to be the major site of attachment of 15d-PGJ_2, both in intact cells and *in vitro*. This cysteine residue is located near the carboxyl terminal end of the protein (**Figure 5**) and may be palmitoylated in cells. H-Ras possesses another particularly reactive cysteine residue, cysteine 118, which has been shown to be the site for S-nitrosylation, a modification which results in H-Ras activation. However, this cysteine residue is not critical for 15d-PGJ_2 attachment since the C118S mutant is fully modified and activated upon exposure to 15d-PGJ_2 (Oliva et al, 2003). In a recent study, Bickley et al, (2004) have raised an interesting hypothesis. According to these authors, the stability of the cyPG-protein adducts would be favored in an acidic environment. This would result in the stable modification of some proteins/residues and a much more labile binding of cyPG to others. This hypothesis raises another important issue in the biology/pharmacology of protein prostanylation, as it is its potential reversibility, a process which has not been explored and that would imply the possibility of a regulatory role for this type of posttranslational modification.

A novel effect of cyPG which will deserve future investigation is their role in the formation of protein cross-links. It was known that cyPG possessing two electrophilic carbons could form bis-adducts with low molecular weight thiols, such as GSH (Atsmon et al, 1990b). We have recently shown that 15d-PGJ_2 may induce protein cross-links in the case of the c-Jun protein (Pérez-Sala et al, 2003). The pathophysiological implications of this process have not been studied. Other reactive lipids possessing two or more electrophilic carbons include prostaglandins and isoprostanes of the J series and certain phospholipids, like some epoxycyclopentenone phospholipids which are components of oxidized low density lipoproteins (Subbanagounder et al, 2002) (**Figure 6**). These lipids

could participate in the cross-linking of proteins by mechanisms similar to those observed with 15d-PGJ_2. In addition, other lipid peroxidation products, such as 4-hydroxy-nonenal, malondialdehyde or reactive γ-ketoaldehydes of the isoketal or levuglandin type have been shown to induce protein cross-linking and aggregation under certain conditions (Davies et al, 2002). This has been proposed to lead to impaired protein degradation and to accumulation of damaged proteins, a phenomenon that is involved in the pathophysiology of inflammatory and neurodegenerative diseases. Therefore the extent and potential consequences of cyPG-induced protein cross-linking would present a high interest.

Given the beneficial effects of cyPG on several types of injury and inflammation, a potential for the therapeutic use of these compounds has been raised. In fact, several recent studies have addressed the effects of synthetic or natural cyclopentenones of other origins. There is a wide variety of compounds with unsaturated carbonyl groups that could present a reactivity similar to that of endogenous animal cyPG (**Figure 6**). Some of these compounds have been used in herbal medicine. In early studies, 4-hydroxy-2-cyclopentenone was found to be responsible for the anti-bacterial activity of an extract of leaves from Passiflora tetrandra (Perry et al, 1991). Also, the anti-inflammatory natural product parthenolide from the medicinal herb Feverfew, which possesses a sesquiterpene lactone structure, has been shown to directly bind and inhibit IKK (Kwok et al, 2001). Another example is the kaurane diterpene kamebakaurin, which can be obtained from plants traditionally used in oriental medicine to treat inflammation and cancer. This compound has recently been reported to inhibit NF-κB activity by modifying the p50 subunit, by a mechanism analogous to that described for cyPG (Lee et al, 2002). Therefore, plants may be an important source of cyclopentenone derivatives. In plants, compounds derived from the oxidative metabolism of polyunsaturated fatty acids are synthesized in response to external stimuli and cyclopentenone derivatives play an important role in plant defense responses (Howe, 2001). One of the compounds proposed to play such a role is the cyclopentenone 12-oxo-phytodienoate, which participates in the regulation of defense gene expression (Stintzi et al, 2001). Some of the cyclopentenone isoprostanes induced by reactive oxygen species in plants activate MAPK and induce the expression of GST (Thoma et al, 2003) by mechanisms not completely elucidated, but recent work suggests that the activity of the conserved electrophilic group is important for these effects (Farmer et al, 2003). In the light of the recent findings in animal cells, it is tempting to speculate that the mechanism of action of plant cyclopentenones could involve the direct modification of proteins.

Some of the cyclopentenones of natural origin are now being studied for their ability as anti-inflammatory or anti-proliferative agents, like the punaglandins, isolated from the coral Telesto riisei, which exhibit more potent anti-proliferative effects than the A- and J-series of cyPG (Verbitski et al, 2004). In addition, synthetic derivatives are tested in various biological assays, as neurotrophic compounds (Satoh et al, 2001), antiviral (Fitzmaurice et al, 2003), or anti-inflammatory agents (Bickley et al, 2004). The results obtained so far, encourage further efforts in the research into the potential therapeutic applications of these compounds.

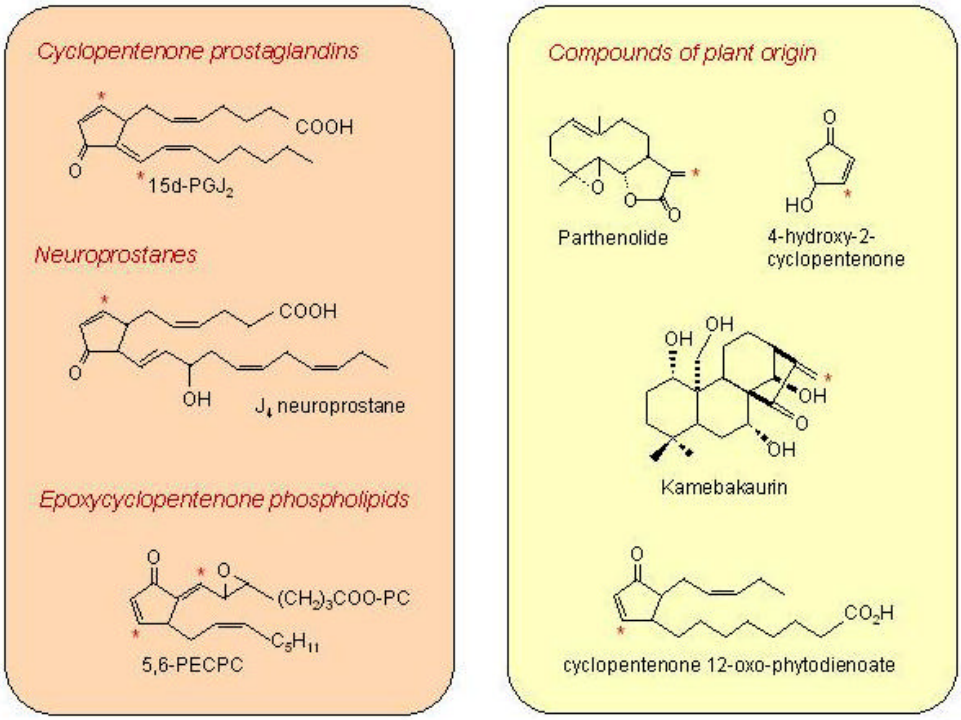

Figure 6. Structures of several compounds of animal or plant origin that present unsaturated carbonyl groups and may act by forming covalent adducts with proteins. Some of the electrophilic carbons potentially involved in the formation of these adducts are marked by red asterisks. PEPC, 1-palmitoyl-2-(epoxycyclopentenone)-*sn*-glycero-3-phosphorylcholine.

IV. Concluding remarks

Compounds with cyclopentenone structure display varied biological activities which may be of pathophysiological importance. The characterization of the regulation of cyPG generation and the levels attained will clarify their potential role in signal transduction. At pharmacological doses, cyPG exert beneficial effects in cellular and animal models of inflammation or injury. The covalent modification of cellular thiols plays an important role in the effects of cyPG. The identification of the proteins susceptible to be modified by Michael addition of cyPG will provide a deeper insight into the mechanisms of action of these prostanoids and could help identify novel targets for therapeutic intervention in antiviral, antitumoral or anti-inflammatory therapy.

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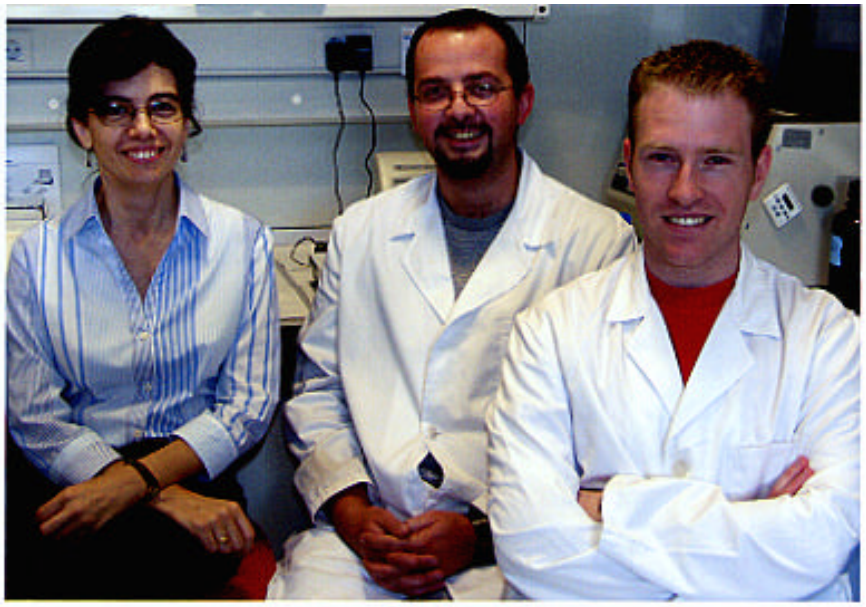

From left to right: Dolores Pérez-Sala, Konstantinos Stamatakis, Francisco J. Sánchez-Gómez