MODIFICATION OF CYSTEINE RESIDUES BY CYCLOPENTENONE PROSTAGLANDINS: INTERPLAY WITH REDOX REGULATION OF PROTEIN FUNCTION

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Short title: cyPG-redox interactions: proteomic studies

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Abstract

Cyclopentenone prostaglandins (cyPG) are endogenous lipid mediators involved in the resolution of inflammation and the regulation of cell proliferation and cellular redox status. Upon exogenous administration they have shown beneficial effects in models of inflammation and tissue injury, as well as potential antitumoral actions, which have raised a considerable interest in their study for the development of therapeutic tools. Due to their electrophilic nature, the best known mechanism of action of these mediators is the covalent modification of proteins at cysteine residues through Michael addition. Identification of cyPG targets through proteomic approaches, including MS/MS analysis to pinpoint the modified residues, is proving critical to characterize their mechanisms of action. Among the targets of cyPG are proinflammatory transcription factors, proteins involved in cell defense, such as the regulator of the antioxidant response Keap1 and detoxifying enzymes like GST, and key signaling proteins like Ras proteins. Moreover, cyPG may interact with redox-active small molecules, such as glutathione and hydrogen sulfide. Much has been learned about cyPG in the past few years and this knowledge has also contributed to clarify both pharmacological actions and signaling mechanisms of these and other electrophilic lipids. Given the fact that many cyPG targets are involved in or are targets for redox regulation, there is a complex interplay with redox-induced modifications. Here we address the modification of protein cysteine residues by cyPG elucidated by proteomic studies, paying special attention to the interplay with redox signaling.

Keywords

Electrophilic lipids, cyclopentenone prostaglandins, Ras proteins, palmitoylation, redox regulation.

I. INTRODUCTION

Cyclopentenone prostaglandins (cyPG) are reactive lipid species that are produced within the cell and that, upon exogenous administration, have displayed anti-viral, anti-tumoral and anti-inflammatory effects. Therefore, their use as potential therapeutic agents has placed the spotlight on their involvement in pathophysiological processes and their molecular mechanisms of action (Stamatakis, Sánchez-Gómez & Pérez-Sala, 2004; Straus & Glass, 2001). cyPG are electrophiles that can react with nucleophilic residues of proteins such as cysteine residues, hence eliciting conformational changes in these proteins or modifying their binding properties. Interestingly, cyPG structure determines the ability to bind to specific cysteine residues even within the same protein, giving rise to a molecular selectivity that is reflected in

the cellular processes affected by cyPG action (Garzón et al., 2011; Renedo et al., 2007). For instance, cyPG anti-inflammatory effects are achieved through covalent modification and inhibition of certain proteins in the NF-κB pathway, while the cellular redox status and stress response can be regulated by cyPG binding to redox sensitive proteins (Díez-Dacal & Pérez-Sala, 2010; Kim & Surh, 2006). It is thus important to pinpoint specific molecular mechanisms of cyPG action and study their role in pathophysiology.

A. cyPG Formation

cyPG are generated from membrane fatty acids, especially arachidonic acid which is a precursor for other electrophilic lipids *via* enzymatic and non-enzymatic pathways (Fig. 1). Arachidonic acid is first cyclooxygenated and peroxidated by cyclooxygenases (COX) to give rise to PGH_2, which is further transformed by different prostaglandin synthases to generate PGE_2 and PGD_2, among others (Funk, 2001) (see Figure 1). Formation of these PGs has also been reported to occur through the isoprostane pathway in which peroxidation of arachidonic acid occurs by non-enzymatic mechanisms generating E/D-ring isoprostanes, which can suffer epimerization to yield compounds identical to COX-derived PGE_2 and PGD_2 (Gao et al., 2003). cyPG of the A series are produced by dehydration of PGE_2 while J series cyPG arise from dehydration of PGD_2 (Narumiya & Fukushima, 1985; Ohno et al., 1986; Shibata et al., 2002). Upon dehydration, PGD_2 gives rise to PGJ_2, which can be further dehydrated to yield 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) or it can be derived to Δ^{12}-PGJ_2 in an albumin-dependent process (Shibata, et al., 2002). Dehydration of PGE_2 in turn yields PGA_2, whereas PGA_1 arises by non-enzymatic dehydration of PGE_1, which may derive from dihomo-γ-linolenic acid by means of COX enzymes (Levin et al., 2002; Ziboh, Miller & Cho, 2000).

B. Reactivity

The common feature of this wide variety of cyPG is the presence of an α,β-unsaturated carbonyl group in the cyclopentane ring conferring a high electrophilicity to the carbon(s) in the -β position by which they can suffer the attack of nucleophilic groups, such as thiol or amino groups in amino acids, and form Michael adducts (Fig. 1). For cysteine residues the reactive species is the thiolate anion. Ionization of cysteine thiols is highly dependent on the structure of the surrounding area since the properties of surrounding residues can affect the pKa of cysteines (Mossner, Iwai & Glockshuber, 2000). In general, cysteines with a low pKa would be more reactive, with those located in a low pH environment favouring the formation

of more stable cyPG adducts (Bickley et al., 2004). Steric hindrances or exposure to the cellular milieu can also influence the formation of adducts. In addition, the presence of distinctive structural features in cyPG confers selectivity to their binding to cellular proteins to elicit their effects.

cyPG belong to an extended family of lipid electrophiles, which are generated in low amounts under basal conditions but increase upon oxidative stress (Ceaser et al., 2004; Koenitzer & Freeman, 2010). In situations associated with increased generation of reactive oxygen or nitrogen species, as in inflammation, unsaturated lipids prone to oxidation, like the polyunsaturated fatty acids present in biological membranes, in addition to their enzymatic transformations, can suffer various non-enzymatic oxidations, giving rise to highly varied structures including the reactive aldehyde 4-hydroxynonenal or nitro-fatty acids. Many of these reactive species may coexist in the cell at a given time and exert biological actions due to alkylation of biological molecules. One general problem of the work with these compounds is their detection and quantitation, hampered in part by their high reactivity. Controversy has accompanied the study of both cyPG and nitro fatty acids since the concentrations used in most biochemical or pharmacological studies are in the micromolar range whereas concentrations measured in biological systems are lower, in the picomolar to nanomolar range depending on the reports (Garzón et al., 2011; Schopfer, Cipollina & Freeman, 2011; Tsikas et al., 2009). Conversely, the stability of cyPG binding to proteins has fostered their use in proteomic studies. The wealth of information gathered through these studies is paving the way to unveil the mechanisms of action and targets of other electrophilic mediators recently identified.

C. Biological Actions of cyPG

The biological effects of cyPG binding to proteins involved in numerous cellular processes have been studied both in vitro and in vivo, highlighting the large number of pathways that can be targeted by these compounds. One of the most widely studied roles of cyPG is their regulation of inflammation. For instance, 15d-PGJ_2 blocks the induction of several genes such as iNOS, COX-2 or ICAM-1 that are upregulated by proinflammatory signals, reduces inflammatory cytokine release and inhibits monocyte trafficking *via* cell adhesion molecules (Gilroy et al., 2004). The anti-inflammatory potential of cyPG relies heavily on their ability to inhibit important proinflammatory transcription factors such as NF-κB and AP-1 and/or to activate transcription factors of the PPAR family or Nrf2. Though these compounds are generally regarded as anti-inflammatory mediators, cyPG have also been shown to exert

proinflammatory effects in certain settings, as in the activation of eosinophils and Th2 lymphocytes (Gazi et al., 2005; Monneret et al., 2002). A dual effect of cyPG has also been observed regarding cellular redox status and the electrophilic response. On one hand, oxidative stress may be induced by cyPG at mitochondria or by acting on other ROS producers such as NADPH oxidase, giving rise to potential protein oxidation (Landar et al., 2006c; Martinez, Perez-Castillo & Santos, 2005; Shin et al., 2009). On the other hand, cyPG activation of the transcription factor Nrf2 induces the expression of antioxidant response-related genes. Interestingly, 15d-PGJ_2 has been observed to exert biphasic effects on cellular GSH content and on cytokine-elicited induction of proinflammatory genes, depending on the levels present of this cyPG and in correlation with the activation of an antioxidant response (Levonen et al., 2001; Martínez et al., 2012).

The biphasic nature of cyPG is also reflected by their role in proliferation, tumorigenesis and apoptosis. Initially, cyPG were described as anti-proliferative and pro-apoptotic agents in cancer cells and animal models. Their anti-proliferative behaviour was related to multiple antineoplasic mechanisms such as blocking cell cycle progression, inducing oxidative stress or activating the mitochondrial apoptotic pathway (Kamagata et al., 2007; Kim et al., 1993; Kondo et al., 2001; Nencioni et al., 2003). However, some studies have reported an induction of mouse skin tumorigenesis mediated by 15d-PGJ_2 (Millán et al., 2006) as well as an inhibitory role on the tumor suppressor transcription factor p53, potentially promoting tumor growth (Kim et al., 2010). It has also been observed that cyPG have anti-viral effects by inducing stress responses and the expression of heat shock proteins (e.g. Hsp70), by inhibiting viral replication factors such as NF-κB and through direct binding to viral proteins (Kalantari et al., 2009; Santoro, 1997). The biological effects of cyPG depend heavily on their concentration, cellular context, and specific target proteins in cells or tissues. Therefore, as described above, cyPG can act as dual mediators similarly to other redox regulators like NO, modifying numerous cellular processes.

D. Mechanisms of Action

cyPG are able to exert their effects by targeting a plethora of proteins, though the nature of the interaction can vary. Most cyPG targets have been identified by proteomic studies performed in conditions under which labile interactions are lost. Therefore, covalent protein-cyPG adducts have been more widely studied and many target cysteines have been identified (though histidine residues can also bind cyPG covalently- see (Yamaguchi et al., 2010)). Depending on the chemical properties of the cyPG, i.e. one (single enones) or two (dienones)

electrophilic carbons, these reactive lipids will bind to free cysteine residues in specific protein regions that sterically allow cyPG access. In fact, some cyPG with two electrophilic moieties have been shown by protein fingerprinting to bind simultaneously to two cysteine residues of the same or different proteins (Oeste et al., 2011; Pérez-Sala, Cernuda-Morollón & Cañada, 2003). 15d-PGJ_2 binds covalently to transcription factors such as PPARγ or NF-κB (Cernuda-Morollón et al., 2001; Shiraki et al., 2005), cytoskeletal proteins including actin or vimentin (Stamatakis, Sánchez-Gómez & Pérez-Sala, 2006) and redox-regulated proteins like H-Ras, Keap1 or GSTP1-1 (Levonen et al., 2004; Oliva et al., 2003; Sánchez-Gómez et al., 2007), as it will be discussed further below. However, cyPG also interact non-covalently with proteins such as PG membrane receptors or soluble carrier proteins like albumin (Gazi, et al., 2005; Yamaguchi, et al., 2010). DP2, one of the receptors for PGD_2, is a G-protein-coupled receptor expressed in several leukocytes that participates in chemotaxis and allergic inflammation (see (Sandig, Pease & Sabroe, 2007) for review). The cyPG 15d-PGJ_2 and Δ^{12}-PGJ_2 are ligands for this receptor (Sawyer et al., 2002), though the effects produced by DP2 activation vary in different models and warrant further studies. Interestingly, both covalent and non-covalent modes of interaction of cyPG with other proteins, including GSTP1-1 (Paumi et al., 2004; Sánchez-Gómez, et al., 2007) and PGES1 (Prage et al., 2012) have been detected, showing the multivalent binding nature of these electrophiles.

E. Therapeutic Potential

Many exogenous and endogenous stimuli elicit inflammatory responses, so cytoprotective mechanisms are essential to maintain cellular homeostasis. Various endogenous electrophiles have been identified as mediators of protective pathways, like the antioxidant response or the resolution of inflammation, thus presenting therapeutic opportunities (Gilroy et al., 1999; Itoh et al., 2004; Rajakariar et al., 2007). In this context, 15d-PGJ_2 has been shown to ameliorate several pathological processes. Through the inhibition of NF-κB, this cyPG shows protective properties against ischemic acute renal failure in rats (Chatterjee et al., 2004). Inhibition of NF-κB together with AP-1 and activation of PPARγ are possible mechanisms of protection against multiple-organ failure and systemic inflammation caused by endotoxic shock (Dugo et al., 2004; Zingarelli et al., 2003). 15d-PGJ_2 also shows protective effects against gastrointestinal damage by reducing the production of proinflammatory cytokines and iNOS expression (Cuzzocrea et al., 2003). Cardiovascular function may also be preserved through cyPG action, such that a cyPG-liposome formulation known as “LipoCardium” has been shown to reduce vascular lesions derived from atherosclerosis in mice (Homem de Bittencourt et al., 2007).

Regarding tumorigenesis, cyPG have shown pro-apoptotic and/or anti-proliferative effects in tumor cells and other in vivo models (Kato et al., 1986; Sasaki et al., 1999). However, this process is highly dependent on the experimental model, for there are instances in which cyPG may potentiate tumor growth (Kim & Surh, 2008; Millán, et al., 2006). A similar dual effect of cyPG has been observed in the nervous system, where both neuroprotective and neurotoxic effects can be elicited by prostaglandins of the A series (Qin et al., 2001; Yan et al., 2005; Zhang et al., 2008). For example, the mouse model for multiple sclerosis (experimental autoimmune encephalomyelitis) showed improvement after treatment with 15d-PGJ_2 (Diab et al., 2002) and this cyPG also inhibited beta-amyloid-derived proinflammatory responses and neurotoxicity (Combs et al., 2000). However, 15d-PGJ_2 may give rise to protein aggregation resulting in neurodegenerative symptoms in mice (Pierre, Lemmens & Figueiredo-Pereira, 2009). Therefore, though cyPG have been studied as chemopreventive and protective agents against chronic inflammatory pathologies, their systemic effects are varied and context-dependent, so further studies are needed to establish their therapeutic potential.

In this context, proteomic approaches provide valuable tools for the comprehension of cyPG-elicited cellular processes. Both, identification of cellular targets for covalent modification and assessment of protein expression changes or other posttranslational modifications elicited by treatment with these compounds may provide important clues on the pathways modulated by cyPG and the mechanisms underlying their beneficial or deleterious effects.

II. PROTEIN TARGETS OF CYCLOPENTENONE PROSTAGLANDINS

The modification of protein targets by cyPG has been shown to occur largely through Michael addition at cysteine residues. Reactive cysteines targeted by cyPG are often involved in redox regulation, either because they are susceptible to modification by oxidation or electrophilic compounds or because they actively participate in redox catalysis (Kansanen, Kivela & Levonen, 2009; Shibata et al., 2003). Indeed, redox-related proteins often contain nucleophilic cysteines with particularly low pKa. In fact, in the cyPG field, some targets have been identified through a hypothesis-driven approach, exploring the potential modification of proteins previously known to undergo cysteine oxidation through various mechanisms, like thiolation or formation of sulfenic acid, as in the case of c-Jun (Pérez-Sala, Cernuda-Morollón & Cañada, 2003). Conversely, in some cases, the identification of novel protein targets for modification by cyPG has led to unveil their role in redox regulation. Many targets of cyPG identified through the use of proteomic approaches have been listed in recent reviews (Garzón, et al., 2011). In

this work we will focus on those targets playing an important role in or being regulated by cellular redox status.

Cysteine residues targeted by cyPG are also generally susceptible to modification induced by other agents. Some cyPG targets have been shown to be nitrosylated, glutathionylated or be involved in disulphide formation under conditions of oxidative stress. In some cases the functional consequences of these various modifications are similar, as in the case of the cysteine residues present in the DNA binding domains of NF-κB p50 or c-Jun, for which modification by various agents, including cyPG, results in inhibition of DNA binding (Cernuda-Morollón, et al., 2001; Pérez-Sala, Cernuda-Morollón & Cañada, 2003; Pineda-Molina et al., 2001). However, in other cases the structure of the modifying agent appears to be important for the functional outcome. There are several examples of this in the literature, including transcription factor PPARγ. It has been described that several electrophilic lipids can bind to a cysteine residue present in the ligand binding domain of PPARγ. However, not all of them produce an effective activation (Waku et al., 2009; Zorrilla, Garzón & Pérez-Sala, 2010). Similarly, as it will be discussed below, H-Ras proteins possess several cysteine residues that can also be targeted by various oxidative modifications, including glutathionylation and addition of various electrophiles with diverse functional consequences. Another example of the structure-function relationships of electrophilic or oxidative modifications is provided by enzymes of the aldo-keto reductase (AKR) family. Various members of this family possess a cysteine residue close to the active site, which is important for function and for the interaction of inhibitors. In the case of aldose reductase or AKR1B1, modification of this residue has been reported to lead to functional consequences depending on the nature of the modification, thus, whereas alkylation by iodoacetatic acid (Liu et al., 1993), acrolein or S-nitrosylation (Srivastava et al., 2003) have been reported to increase AKR1B1 catalytic activity, modification by HNE (Del Corso et al., 1998) or glutathionylation (Srivastava, et al., 2003) appear to cause the inactivation of the enzyme. We have observed that cyPG, in particular PGA_1, binds covalently to Cys299 in AKR1B10, potentially docking at the active site, and this causes inactivation of the enzyme. Similarly, other electrophilic compounds with cyclopentenone structure, including cyclopentenone isoprostanes inactivate the enzyme. However, the compound cyclopentenone, which does not possess the lateral chains of the PG does not inhibit enzymatic activity (Díez-Dacal et al., 2011). Therefore, the size and/or the orientation of the interacting molecules may be important for the impairment of catalytic activity. Interestingly, as for other enzymes with metabolic activity towards compounds containing α,β-unsaturated carbonyls, such as glutathione transferases (see below), a reciprocal relationship

may exist between various Michael acceptors, including cyPG, and AKR enzymes. Whereas cyPG or HNE can inhibit various AKR, some enzymes of this family have been shown to metabolize these molecules and/or cyPG precursors, thus reducing their bioavailability and biological effects (Byrns et al., 2010; Shen et al., 2011).

A. Ras Proteins

Though ROS production and subsequent protein oxidation was commonly regarded as a toxic cellular event, numerous studies have now shown that many proteins can regulate and be regulated by redox switches and pathways. Such is the case of Ras proteins, which contain cysteine residues that can undergo oxidative modifications which will affect signal transduction. Ras proteins possess a common cysteine residue, Cys118, located at the GTP binding site. It was observed early on that Ras is activated in cells treated with redox modulating agents and as such is a key transmitter of redox stress signals (Lander et al., 1997). S-Glutathionylated and S-nitrosylated forms of H-Ras are detected in vitro and in cells when the protein is exposed to thiol oxidants such as hydrogen peroxide or S-nitrosoglutathione, respectively (Mallis, Buss & Thomas, 2001). The structural and functional consequences of nitrosylation of Cys118 have been studied in detail proposing a radical-based mechanism that stimulates guanine nucleotide exchange (Heo et al., 2005). Moreover, oxidative posttranslational modifications of cysteine thiols of Ras associated with redox modulation of activity has been studied in vitro using isotope-coded affinity tags and mass spectrometry (MS) (Sethuraman et al., 2007). Of the three Ras proteins, H-Ras differs from other Ras proteins (N- or K-Ras) especially at its C-terminus, which contains three cysteine residues (Cys181, Cys184 and Cys186), of which, Cys186 is the isoprenylation site whereas Cys181 and Cys184 are sites for palmitoylation (Hancock et al., 1989) and suffer lipidation cycles involved in protein localization and activation (Pérez-Sala, 2007; Rocks et al., 2005). Interestingly, in cells, glutathionylation of H-Ras has been reported to exert different functional consequences depending on the target cysteine: whereas glutathionylation of Cys118 has been reported to increase Ras activity due to nucleotide exchange (Clavreul et al., 2006a) glutathionylation of the C-terminal cysteines has been shown to interfere with palmitoylation altering Ras subcellular localization and favoring apoptosis (Burgoyne et al., 2012). In contrast, guanylation of Cys184 correlates with H-Ras activation at the plasma membrane (Nishida et al., 2012). Redox regulation of Ras has been proposed to play a role in pathophysiology, including atherosclerosis, in association with oxLDL generation (Clavreul et al., 2006b) and metabolic stress (Burgoyne, et al., 2012). Other proteins of the Ras superfamily may also be subject to

redox regulation depending on the modified agent and the targeted cysteine(s) (Heo et al., 2006).

Ras proteins are also selectively modified by lipid electrophiles with different structures, such as cyPG, that elicit conformational changes which in some cases lead to activation of these small GTPases (Oliva et al., 2003; Renedo et al., 2007). cyPG bind covalently to Ras proteins both in vitro and in cells in a highly selective manner that depends on cyPG and protein structure. The electrophile moiety of particular cyPG, i.e. single enones or dienones, produce distinct binding patterns according to the Ras isoform they modify (Renedo et al., 2007). Using MALDI-TOF MS it was observed that single enone cyPG (e.g. PGA_1 and PGA_2) formed multiple adducts with H-Ras and bound mostly to the cysteine residue in the GTP-binding domain (Cys118). In contrast, modification by 15d-PGJ_2 mapped specifically at the C-terminal region of the protein (Oliva et al., 2003). Combination of proteomic and mutagenesis studies identified Cys184 as the residue required for 15d-PGJ_2 binding. Further studies showed that 15d-PGJ_2 and Δ^{12}-PGJ_2, being bifunctional PG, could simultaneously bind to Cys181 and Cys184, inducing intra-molecular cross-linking (Garzón et al., 2011; Oeste et al., 2011). Considering the differences amongst Ras proteins, this selective modification gives rise to specific activation patterns of H-, K- and N-Ras according to the cyPG structure (Renedo et al., 2007). Interestingly, contrary to the redistribution elicited by glutathionylation of Cys181 or Cys184, cyPG modification of H-Ras at its C-terminus is associated with activation at the plasma membrane. Remarkably, modification of Cys181 and Cys184 by other cysteine cross-linking agents with cyclic structure such as phenylarsine oxide or dibromobimane is associated with H-Ras activation at endomembranes (Oeste et al., 2011). Some of the structure-function relationships of the modification of H-Ras C-terminal cysteines by various species are summarized in Fig. 2. Therefore, oxidative or cyPG modifications of Ras proteins present yet another level of complexity for the regulation of these crucial cell signalling components.

B. The Keap1-Nrf2 System

The regulation of the stability of transcription factor Nrf2 by its partner Keap1 is one of the best examples of regulation of a biological function through modification of cysteine residues. Keap1 is a cysteine-rich protein which interacts with Nrf2 regulating its degradation and thus its cellular levels and function. Several models have been proposed to explain the complex regulation of the Keap-Nrf2 system. According to recent interpretations, Nrf2 is bound to Keap1, which in turn functions as an adaptor for the ubiquitin ligase complex Cul3. Under basal conditions, this interaction results in the constitutive ubiquitination and degradation of Nrf2.

Under conditions of oxidative or electrophilic stress, modification of cysteine residues in Keap1 induce conformational changes which disrupt Nrf2 ubiquitination either by a partial detachment of the Nrf2-Keap1 complex or by dissociation of the Cul3-Keap1 interaction. This results in stabilization of Nrf2 and increased binding to the antioxidant response elements in the promoters of its target genes including GSTP1-1, γGCS, several members of the aldo-keto reductase (AKR) family, hemooxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (see (Hayes et al., 2010; Zhang, 2010) for review).

Keap1 possesses 27 cysteine residues, which appear to be selectively modified by several electrophilic agents or oxidative stress. Various experimental approaches have been employed to elucidate the chemical mechanisms controlling Keap1 function. In vivo models with Keap1 mutants have mapped Cys151, Cys273 and Cys288 as functionally important residues. In addition, proteomic analyses have been performed with purified Keap1 from various origins. Different electrophiles appear to target diverse cysteine residues for which a classification in several categories has been proposed (Kobayashi et al., 2009).

cyPG bind to Keap1 and activate Nrf2. Activation of Nrf2 by cyPG appears to play an important role in cyPG effects, since it has been reported that Nrf2 is required for the anti-inflammatory actions of 15d-PGJ_2 in some experimental systems (Itoh et al., 2004; Lin et al., 2012). Although the mechanisms involved are not completely elucidated, the Nrf2-dependent induction of γGCS leading to GSH synthesis has been reported to mediate 15d-PGJ_2 anti-inflammatory actions through the glutathionylation and inhibition of proinflammatory proteins like NF-κB (Lin et al., 2012). Moreover, the Nrf2-dependent induction of HO-1, which itself exerts anti-inflammatory actions, has been used as a readout for the biological effects of 15d-PGJ_2 (Oh et al., 2008). Using purified mouse Keap1 and MS analysis, adducts of 15d-PGJ_2 with Cys273 and 288 and of PGA_2 with Cys273, 297 and 489 have been reported (Kobayashi et al., 2009). However, other cysteine residues may be involved in binding of cyPG to Keap1 (Kansanen, Kivela & Levonen, 2009). Similarly, it is not yet clear which residues are those functionally important for cyPG effects. It seems clear that Cys151 is dispensable for Keap1 modulation of Nrf2 levels in response to 15d-PGJ_2 (Takaya et al., 2012). In zebrafish embryo, a Cys288Ser mutant has been shown to retain function and the ability to respond to 15d-PGJ_2. Therefore, further studies are needed to elucidate the nature of cyPG-Keap1 interactions.

C. GSTP1-1

Interactions of cyPG with enzymes of the GSH biotransformation system, particularly with glutathione S-transferases (GSTs), have long been recognized. GSTs are involved in the

detoxification of numerous endogenous and exogenous electrophiles through their conjugation with GSH, thus favoring their export from cells. In the case of GSTP1-1, an isoform related to tumorigenesis and chemoresistance, the interactions with cyPG may be multiple because cyPG may be both substrates and inhibitors of this enzyme. Both covalent and non-covalent modes of inhibition have been reported (Paumi et al., 2004; Sánchez-Gómez et al., 2007) (Fig. 3). The covalent modification of GSTP1-1 by 15d-PGJ_2, observed by MALDI-TOF MS, leads to enzyme inactivation, which appears to be irreversible under physiological conditions (Sánchez-Gómez et al., 2007). Interestingly, the nature of the interaction of cyPG with GSTP1-1 appears to depend on the structure of the PG. cyPG with dienone structure induce an irreversible oligomerization of GSTP1-1, both in vitro and in intact cells, which requires the presence of Cys101 (Sánchez-Gómez et al., 2010). These observations are important because Cys101 is present in the human but not in the murine enzyme, in which many studies on the response of GST to electrophiles have been performed. However, the residues involved in the oligomerization have not been identified by MS and the available evidence comes from the use of mutants. Interestingly, the pattern and structural features of 15d-PGJ_2-induced oligomerization shows similarities with that induced by treatment with H_2O_2, but not by other oxidants like HNE or diamide. However, only dienone cyPG-induced oligomerization was resistant under reducing conditions. A summary of the potential interactions of cyPG with GSTP1-1 is shown in Fig. 3.

D. Other Redox-Regulated Proteins

Another major sensor of cellular stress, including oxidative stress, is the heat shock pathway. Primary players in the heat shock response are the heat shock factors (HSF), transcription factors which control the expression of diverse cell defense genes including heat shock proteins (Hsp) like Hsp70, Hsp90 and the small Hsp (Westerheide & Morimoto, 2005). In a simplified view, under resting conditions, HSF1 is in an inactive monomeric form bound to Hsp90. However, upon stimulation, HSF1 dissociates from Hsp90 and forms trimers which bind to the response elements on the promoters of the target genes (Zhang et al., 2011). HSF is activated by thermal stress, redox changes and electrophiles (Ianaro et al., 2003; Morimoto & Santoro, 1998). Cysteine modification of HSF or of its negative regulators may lead to HSF activation. Thus, many compounds can activate both HSF and Nrf2-dependent responses. In an elegant recent study, Zhang et al. have dissociated both pathways by means of MEFs from HSF, Nrf2 or Keap1 KO mice, concluding that induction of Hsp70 by activators of the Keap1/Nrf2 pathway is independent from Nrf2 (Zhang et al., 2011). Interestingly, cyPG have been long

known to activate HSF and induce the expression of Hsp (Amici et al., 1992). Although HSF has not appeared among the identified cyPG targets, Hsp90 has been found in proteomic studies using biotinylated cyPG combined with either 2D electrophoresis and MALDI-TOF MS (Stamatakis, Sánchez-Gómez & Pérez-Sala, 2006) or avidin chromatography and LC-MS/MS (Garzón et al., 2010; Gharbi et al., 2007). Covalent modification of Hsp90 by PGA_1-B has also been demonstrated in vitro (Garzón et al., 2010), although the residues involved in the modification have not been elucidated. Modification of Hsp90 could disrupt the interaction with HSF, resulting in its activation. In addition, several heat shock proteins, including Hsp70, 71 and 60 have been found retained on avidin after treatment of murine fibroblasts or mesenteric vessels with biotinylated cyPG and identified by LC-MS/MS (Charles et al., 2011; Garzón et al., 2010). Taken together these results indicate that cyPG may interact with the heat shock response pathway at several levels, which may contribute to the protective and anti-inflammatory effects of these compounds (Ianaro et al., 2003).

Other cyPG targets sustaining the interplay with redox signaling are shown in Table 1.

III. *INTERACTIONS OF cyPG WITH REDOX-ACTIVE SMALL MOLECULES*

In addition to binding to proteins, cyPG, like other electrophilic molecules can interact with other redox mediators or with thiol-containing molecules like glutathione (GSH). This may influence the fate of cyPG and their ability to interact with proteins. cyPG have been shown to form adducts with GSH, both enzymatically and non-enzymatically. A single adduct resulting from the in vitro reaction of 15d-PGJ_2 with GSH was detected by electrospray MS and characterized by NMR showing to involve C9 of the PG (Paumi et al., 2003). A similar adduct was also formed in cells. In an earlier study, a single adduct of 9-deoxy-Δ^9,Δ^{12}(*E*)-prostaglandin D_2 was observed by fast atom bombardment MS to form with GSH through C9 in vitro, and a GSH adduct was detected in vivo after administration of radioactive cyPG to rats. In this case, a bis-adduct was also observed under prolonged incubations of GSH with excess PG (Atsmon et al., 1990), although it is not known whether bis-adducts can be formed in cells. CyPG-GSH adducts may be exported from cells through MRP transporters (Paumi, et al., 2003), for which cyPG adduct formation is considered as a mechanism for detoxification and attenuation of cyPG effects (Díez-Dacal & Pérez-Sala, 2012). However, it is not yet clear whether CyPG-GSH adducts may display biological activity. In fact, certain cyPG-cysteine adducts have been reported to inhibit NF-κB activity (Bickley et al., 2004). CyPG-GSH adducts are reversible and display different stability depending on the cyPG structure, with dienone cyPG-GSH adducts being less stable, this having consequences on the availability of cyPG for protein modification

(Suzuki et al., 1997). Thus, it has also been hypothesized that some soluble thiol-cyPG adducts could protect these electrophilic molecules from nucleophiles until they reach a protein environment where they would establish more stable adducts (Bickley et al., 2004). Interestingly, the interactions between cyPG and GSH are reciprocal, since cyPG may also influence GSH availability and metabolism. Recently, 15d-PGJ_2 has been proposed to deplete intracellular GSH through a cycle involving cellular export of the GSH-15d-PGJ_2 conjugate, hydrolysis of the conjugate and re-entry of the cyPG (Song et al., 2011). Interestingly, cyPG may also influence GSH levels by controlling GSH synthesizing enzymes through Nrf2-mediated mechanisms (Levonen et al., 2001).

15d-PGJ_2 may react intracellularly with other small molecule thiols or related species. Recently, hydrogen sulfide anion (HS^-) generated through the enzymatic action of cystathionine-β-synthase and cystathionine-γ-lyase has been reported to react with various electrophiles, thus contributing to modulate their reactivity and biological action (Nishida et al., 2012). The resulting electrophile-SH adduct can have various fates, including undergoing dehydration, oxidation and subsequent desulfhydration or dimerization. In the case of 15d-PGJ_2 nucleophilic addition or substitution of HS^- at the electrophilic carbon gave rise to an unstable sulfhydrated product, 15d-PGJ_2-SH, that was rapidly converted to sulfonated 15d-PGJ_2-SO_3H, which has been characterized by LC-MS (Nishida et al., 2012).

These multiple reactions of 15d-PGJ_2 with low molecular weight thiols should be taken into account when detecting or quantitating levels of 15d-PGJ_2 and potentially other cyPG in biological systems.

IV. PROTEIN MODIFICATIONS INDUCED BY cyPG TREATMENT

Exposure of cells to cyPG may cause alterations in GSH metabolism and induce oxidative stress. Therefore, other protein modifications, due to the action of ROS or to the secondary formation of electrophiles can occur in cells upon treatment with cyPG. Interestingly, MS analysis of proteins treated in vitro with cyPG shows a higher occurrence of oxidative modifications in several proteins, including GSTP1-1 and H-Ras (Sánchez-Gómez et al., 2007) (Oliva et al., 2003). The influence of the presence of the PG in these modifications requires further study. In cells, several oxidative modifications have been observed. The occurrence of S-thiolation in cells treated with 15d-PGJ_2 has been previously demonstrated, and several target proteins for this modification, including α-enolase, heat shock proteins and GSTP1-1, have been identified through 2D-electrophoresis and MALDI-TOF MS (Ishii & Uchida, 2004). Treatment with 15d-PGJ_2 also induced protein carbonylation, being the component of the

proteasome S6 ATPase the major target identified by biotin-LC-hydrazide derivatization followed by 2D-electrophoresis and MALDI-TOF MS of tryptic digests (Ishii et al., 2005). Similarly, by using biotinylated iodoacetamide we showed that treatment with 15d-PGJ_2 reduced the free thiol content of cellular proteins (Sánchez-Gómez et al., 2007). In the case of cellular GSTP1-1 these modifications included both reversible and irreversible oligomerization and induction of a reversible intramolecular disulphide bond (Sánchez-Gómez et al., 2010). In addition, increased glutathionylation has been shown to occur in 15d-PGJ_2-treated cells (Lin et al., 2012). Therefore, the contribution of the various modifications induced directly or indirectly by cyPG treatment to the biological effects of these compounds deserves further study. In the context of redox and electrophile signaling it is important to consider that there is a wide interplay which affects the modification of several redox-active targets. Both direct and indirect modifications may affect proteins involved in redox control, including thioredoxin (Trx) and Trx reductase, or in the generation of inflammatory mediators, like transcription factors such as NF-κB and AP-1, which control COX-2 expression, and thus, the synthesis of cyPG precursors. Moreover, the levels and duration of the exposure to cyPG may determine the accumulation of adducts in cells leading to an intensification of the biological effect (Oh et al., 2008). Therefore, for any given target, a mixture of molecular entities resulting from its modification by various reactive species may coexist in a cell under cyPG treatment and oxidative stress conditions. These various entities may not share completely overlapping functions, thus resulting in diverse biological outcomes. As an example, a scheme showing some of the species of GSTP1-1 which have been detected in cells treated with 15d-PGJ_2 is shown in Fig. 3.

V. PROTEOMIC APPROACHES FOR IDENTIFICATION OF cyPG TARGETS

Early studies on the biological effects of cyPG revealed the importance of the reactive cyclopentenone moiety for their antiproliferative and antitumoral activity. In studies with radioactive compounds it was observed that cyPG accumulated inside cells binding irreversibly to some cellular components, in particular, nuclei (Narumiya et al., 1987). The use of biotinylated analogs of cyPG which are cell-permeable greatly facilitated the study of the fate of these compounds showing their binding to proteins, which is stable under denaturing conditions (Cernuda-Morollón et al., 2001; Parker, 1995). Since these early studies, the binding of cyPG to proteins has been repeatedly demonstrated both through cellular and in vitro approaches.

A. Proteomic Studies In Cells Or Cell Fractions

Biotinylated cyPG analogs have constituted essential tools for the identification of cyPG targets from cellular models. Figure 4 depicts a classical work flow for the isolation, identification and characterization of cyPG protein targets. Early studies used targeted or hypothesis-driven approaches to explore the involvement of particular proteins in the effects of cyPG. These studies employed whole cell approaches followed by immunoprecipitation of the hypothetical targets to assess the presence of a bound biotin moiety (Cernuda-Morollón et al., 2001; Pérez-Sala, Cernuda-Morollón & Cañada, 2003; Shibata et al., 2003). Complementary approaches included pull-down on avidin beads to confirm the presence of the protein of interest in the retained fraction. These strategies allowed confirming the modification of various proteins in intact cells, including the p50 subunit of NF-κB, transcription factor c-Jun and Trx (Cernuda-Morollón et al., 2001; Pérez-Sala, Cernuda-Morollón & Cañada, 2003; Shibata et al., 2003). Whole cell extracts have been used also for 2D-electrophoresis in duplicate gels used for biotin detection and protein staining, respectively, followed by spot excision and protein identification by peptide mass fingerprint by MALDI-TOF MS or MALDI-TOF-TOF MS/MS (Aldini et al., 2007; Stamatakis, Sánchez-Gómez & Pérez-Sala, 2006).

cyPG display a subcellular distribution which depends on the structure of the PG and of the cell type. Factors like GSH concentration or GST activity may affect the preferential distribution of cyPG into cytosolic and nuclear compartments (Straus et al., 2000). Evidence of a preferential subcellular localization of these compounds has been obtained by monitoring their biological effects or by the use of tagged analogs coupled to fluorescent detection in microscopy applications or in cell fractionation studies. Biotinylated cyPG-labeled proteins show a distinct distribution between soluble and particulate or nuclear and cytosolic fractions that suggests the presence of specific targets (Garzón et al., 2010; Gharbi et al., 2007; Straus et al., 2000).

Soluble and particulate cellular fractions have been used to identify targets of PGA_1 through proteomic approaches involving chromatography on avidin, separation of the retained proteins in SDS-PAGE gels and LC-MS/MS analysis of extracts from protein bands subjected to trypsin digestion (Garzón et al., 2010; Gharbi et al., 2007). Avidin chromatography provides an enrichment step for the targets that facilitates their identification. In addition, other strategies have been used, including immunodepletion of interfering abundant proteins in order to reveal the proteins of interest (Díez-Dacal et al., 2011). In cytosol-mitochondria fractionations, 15d-PGJ_2-B has been shown to bind preferentially to mitochondrial targets in MDA-MB231 cells (Diers et al., 2010). Treatment of isolated mitochondria with 15d-PGJ_2-B and subsequent

enrichment on avidin beads followed by 2D-electrophoresis and peptide mass fingerprinting by MALDI-TOF MS, allowed the identification 17 potential targets for 15d-PGJ_2 in this organelle, including glutamate dehydrogenase, ATP synthase and catalase, which may be involved in the induction of permeability transition and cytochrome C release elicited by this cyPG (Landar et al., 2006b). Importantly, some of the targets identified in mitochondria like catalase or aldehyde dehydrogenase could be involved in the increase in ROS generation and in the alterations of cellular redox status elicited by cyPG, contributing to their cytotoxic effects (Landar, et al., 2006b). A remarkable approach has been the preparation of a 15d-PGJ_2 analog specifically targeted to mitochondria through the conjugation with the lipophilic cation triphenylphosphonium (TPP). Here, 2D-electrophoresis followed by detection with anti-TPP antibody allowed the detection of the modified proteins. Using this approach, 53 and 22 spots were found to be unique for 15d-PGJ_2-B or TPP detection, respectively, with 10 spots being positive for both (Diers et al., 2010). More recently, fluorescent derivatives of arachidonic acid have been used for detection of proteins modified by arachidonic acid-derived electrophilic lipids either by fluorescence microscopy or SDS-PAGE in rat ventricular myocytes and isolated mitochondria (Cummins et al., 2012), which will enable the identification of targets.

In considering the presence of cyPG targets in defined subcellular fractions, it should be noted that cyPG can also alter the subcellular distribution of some of its targets through direct or indirect mechanisms, which can influence the results of the proteomic studies. In certain cell types, cyPG promote the cytoplasmic retention of transcription factor NF-κB by directly inhibiting IKK, and thus, IκBα phosphorylation and degradation (Straus et al., 2000). The appearance of protein aggregates in dopaminergic neurons upon in vivo infusion of 15d-PGJ_2 has also been reported (Pierre, Lemmens & Figueiredo-Pereira, 2009). TDP-43 is a nuclear protein involved in the neurodegenerative disorder known as TDP-43 proteinopathy (amyotrophic lateral sclerosis and frontotemporal lobar degeneration with ubiquitin-positive oclusions), where TDP-43 is abnormally accumulated in the neuronal cytoplasm. 15d-PGJ_2 has been shown to induce alterations in TDP-43 subcellular localization by promoting its cytoplasmic localization (Zhang et al., 2010). These effects should be taken into account when exploring the effects and targets of cyPG.

Other strategies used to detect 15d-PGJ_2 in specific compartments include the use of antibodies, with which an increased signal has been evidenced in the spinal motor neurons in amyotrophic lateral sclerosis (Kondo et al., 2002), and in the cytoplasm of foamy macrophages in human atherosclerotic plaques (Shibata et al., 2002). However, since this antibody apparently detects free 15d-PGJ_2, it is not applicable in proteomic studies. Later on, other antibodies have been developed against cyPG, including Δ^{12}-PGJ_2 and 15d-PGJ_2, for use in

ELISA (Syeda et al., 2012a; Syeda et al., 2012b). However, to the best of our knowledge they have not been used in proteomic studies to date.

B. Proteomic In Vitro Approaches Using Peptides And Proteins

Due to the difficulties in characterizing the sites of cyPG addition in proteins from complex biological samples, most proteomic studies to date combine the detection of adducts in cells by using tagged cyPG with the analysis of purified proteins or peptides modified in vitro with cyPG in order to identify the sites of modification. Thus recombinant or purified proteins have been used to confirm and characterize cyPG addition in the case of actin, tubulin, vimentin, H-ras proteins or Keap-1, among others (Aldini et al., 2007; Cocca et al., 2009; Gharbi et al., 2007; Kobayashi et al., 2009; Renedo et al., 2007) (for detailed information on the approaches used to characterize cyPG-protein interactions by proteomic methods, please see (Garzón et al., 2011)). Although this approach is useful it poses some limitations. Protein modification in vitro and in the cellular context may not display the same selectivity. For instance, cyPG may covalently bind to either Cys47 or Cys101 of GSTP1-1 under in vitro conditions, as observed by MALDI-TOF MS (Sánchez-Gómez et al., 2007). In contrast, in intact cells there is a clear selectivity for Cys101, as evidenced by the use of GSTP1-1 mutants and biotinylated cyPG analogs (Sánchez-Gómez et al., 2010). We have previously shown that the cellular context, and in particular the presence of GSH is responsible for the differential modification of proteins by 15d-PGJ_2-B and PGA_1-B (Gayarre et al., 2005). Moreover, GSH depletion may favor the incorporation of PGA_1-B into selective proteins, like the tumor marker AKR1B10 (Díez-Dacal et al., 2011). In addition, in the cellular context, steric factors arising from interactions with other proteins or the presence of ligands or substrates may affect the accessibility of residues targeted by cyPG. In the case of GSTP1-1 for instance, incubation in the presence of non-hydrolysable GSH analogs reduces the interaction with cyPG, thus pointing to the influence of conformational or steric factors. In contrast, in the case of Ras proteins, a similar selectivity of cyPG-induced modification has been observed in vitro by MS using recombinant proteins, and in cells through the use of mutants. Therefore, it would be important to assess whether the cellular context also influences the selective modification of other cellular proteins by electrophiles and/or to confirm the sites of modification identified in vitro through studies in intact cells.

For some of the targets identified, as in the case of IKKβ or CRM1 (Hilliard et al., 2010; Vunta et al., 2007), after evidencing binding of cyPG to the full-length protein, the site of addition has been proposed on the basis of their binding to isolated peptides containing the

residues of interest. Strategies using purified peptides do not fully take into account factors related to the protein and the cellular context that may influence the site of modification, and the results obtained require confirmation using the full-length protein, i.e., through the use of site-specific mutants. Still, they may provide some valuable information on specific regions of the targets. For instance, in the case of H-Ras, as detailed above, the use of a peptide from the hypervariable region of the protein containing the two palmitoylation cysteines was a valuable tool to show the ability of cyPG with dienone structure to induce an intra-peptide cross-linking that also takes place in the full-length protein (Oeste et al., 2011).

VI. TECHNICAL ISSUES AND STRATEGIES FOR THE PROTEOMIC DETECTION OF cyPG-PROTEIN ADDUCTS

In spite of the numerous proteomic studies published in this field, to the best or our knowledge, no studies have reported the detection of the adducts formed in cells by endogenous or exogenously added cyPG by MS methods, nor the identification of the modification sites. All published studies detecting the cyPG-peptide adducts have been performed using purified proteins or peptides incubated with the cyPG in vitro, as detailed above. In most of these studies, MALDI-TOF(TOF) or various forms of LC coupled to ESI MS/MS have been used and most analysis have been performed in the positive mode, detecting adducts of 15d-PGJ_2 with proteins formed by Michael addition with increments equal to the mass of the cyPG (+316.5, +317.5 in the protonated form) (Hilliard et al., 2010; Kim et al., 2007; Liu et al., 2011; Pérez-Sala, Cernuda-Morollón & Cañada, 2003; Renedo et al., 2007; Shibata et al., 2003). In a study on 15d-PGJ_2-modified microtubules, in addition to the ion at *m/z* 317.4 Da, corresponding to the monoisotopic, protonated 15d-PGJ_2 molecule, an increment of +299.4, corresponding to one PG molecule with the loss of one H_2O molecule was observed and used for precursor ion scanning (Cocca et al., 2009).

The difficulties to observe cyPG-protein adducts in samples from cells may be due, among other reasons, to the low abundance of the modified species or the use of non-optimal conditions for LC separation of the modified peptides or for MS analysis. In addition, the potential occurrence of protein or peptide cross-links or of additional modifications may hamper the identification of the modified peptides. For these reasons, optimization of the MS analysis conditions plus a detailed characterization of adducts formed is needed for detection of the modified peptides from biological samples.

It should be also taken into account that certain buffer components may interfere with adduct formation or detection. Buffers may contain DTT or HS^-. Both molecules can react with

cyPG, thus reducing the amount of reactive or detectable cyPG. In addition, when DTT is present during the incubation of peptides or proteins with cyPG in vitro, the formation of peptide-DTT-cyPG or peptide-cyPG-DTT adducts, in the case of cyPG dienones, may theoretically occur. Indeed, after incubation of H-Ras with cyPG in vitro in the presence of DTT, peptides are detected compatible with the formation of H-Ras-DTT-cyPG adducts, an example of which is shown in Fig. 5. Moreover, although cyPG-protein adducts are considered stable under physiological conditions, high concentrations of thiol-containing compounds could affect their stability.

Unlike the scarce information on cyPG-protein adducts formed in cells, adducts of other electrophilic lipids with cellular proteins have been detected through various methods. Using exogenous biotinylated HNE, site-specific protein adducts have been identified through enrichment on avidin followed by LC-MS/MS, affecting residues like Cys328 in vimentin or His246 in aldolase A (Chavez et al., 2010). Similarly, GAPDH has been found to be nitro-alkylated by nitro fatty acids in vivo in red blood cells from healthy donors, by means of LC nanospray linear ion trap MS/MS (LTQ), the alkylation occurring at the catalytically active Cys149 and His303 (Batthyany et al., 2006). In mitochondria isolated from old rats, derivatization of carbonyls with an aldo-keto reactive probe followed by enrichment on avidin and LC-MS/MS allowed the identification of various modification sites in mitochondrial proteins. Interestingly, some of the target sites appeared modified by diverse species (Chavez et al., 2011).

The combination of cyPG with other cysteine reagents can also provide complementary information on their interaction with thiol groups in proteins. In the case of H-Ras, pre-incubation of the C-terminal peptide containing Cys181 and Cys184 with dienone cyPG led to the incorporation of a single cyPG molecule, as detected by MALDI-TOF MS, and completely blocked the incorporation of iodoacetamide. In contrast, single enone cyPG allowed the simultaneous incorporation of the PG and one carbamidomethyl moiety, whereas two carbamidomethyl moieties were observed in the control peptide. These results helped to confirm the formation of an intramolecular cross-link by dienone cyPG (Oeste et al., 2011). Conversely, once the target cysteine residue for cyPG in a given protein has been identified, tagged cyPG have been used as probes for the interaction of other species with the target residue using competition assays (Oeste et al., 2011; Zorrilla, Garzón & Pérez-Sala, 2010). In addition bifunctional and/or fluorescent cysteine reagents have been used in combination with cyPG or other electrophiles to get insight into the nature of protein modification (Oeste et al., 2011; Sánchez-Gómez et al., 2010).

Given the high sensitivity of the avidin-biotin-based detection techniques, the proportion of a given protein that is modified in cells, and thus the functional relevance of the modification, remains an issue. Several attempts have been made to estimate the proportion of adducts formed upon exposure of cells to biotinylated cyPG analogs. Using a semi-quantitative method based on the use of biotinylated standards the proportion of 15d-PGJ_2-B bound to protein in endothelial cells was found to be approximately 1.5 pmol of thiol per microgram of total protein (Landar et al., 2006a). In fibroblasts treated with PGA_1-B, protein elongation factors (EF-1α and EF-2) were amongst the most extensively modified targets as estimated from the retention on avidin of a 3-6% of the amount detected in total lysates (Garzón et al., 2010). Notably, GSH depletion may increase the modification of certain proteins by cyPG, and a 3-fold increase in avidin retention of AKR enzymes (30% of the total immunoreactive signal) has been observed in cells incubated with PGA_1-B after treatment with buthionine sulfoximine (Díez-Dacal et al., 2011). Thus, several strategies can been used to modulate the extent of cyPG-protein adducts formation and potentially improve their detection. Remarkably, quantitative proteomic approaches have so far not been used to assess the extent of protein modification by cyPG in cells. Regarding the functional outcome, it should be taken into account that the repercussion of the modification of a given protein may depend on its specific function and on the biological context. Thus, inactivation of a small proportion of a constitutively active protein may have little impact in the cell. In contrast, activation of even a small proportion of an otherwise inactive protein may provoke relevant biological consequences.

In the context of the extensive use of biotinylated cyPG analogs for target detection and purification several drawbacks need to be mentioned. The need for the use of stringent conditions to minimize non-specific retention in avidin-based matrices has been stressed (Landar et al., 2006b). Avidin-biotin interaction is very stable. Therefore, in theory nearly denaturing conditions can be used. The use of stringent conditions allowed reducing the retention of interacting partners of the direct targets, and therefore, the purification of individual components of multiprotein complexes, like the mitochondrial multi-subunit electron transfer complex III (Landar et al., 2006b). In lack of direct detection of the modified peptides, target validation through complementary strategies is needed, like immunoprecipitation followed by detection of the biotin signal associated with the target proteins (Garzón et al., 2010; Shibata et al., 2003).

As deduced from the above, strategies based on the use of tagged cyPG analogs present limitations. Therefore, it will be necessary to develop tag-free methods for detection of electrophilic lipid-protein adducts.

VII. CONCLUDING REMARKS

Cyclopentenone prostaglandins constitute valuable tools to increase our knowledge on the signaling and mechanisms of action of electrophilic mediators. On one hand, the stability of the Michael adducts of cyPG with proteins has allowed to perform hypothesis-driven or high throughput proteomic studies to identify their targets. In addition, their structural variety has allowed observing distinct structural requirements for their binding to proteins thus unveiling phenomena like the intra and inter-molecular selectivity of the modification and the influence of context and steric factors. cyPG establish reciprocal interactions with redox regulation at multiple levels and ultimately, at the level of protein modification. The cysteine residues targeted by cyPG are often subjected to other redox-induced modifications or to the addition of other electrophilic species. Thus, in any given situation, multiple species of every protein will coexist bearing different proportions of structurally different modifications, which in some cases will lead to different functional outcomes. Moreover, there is a reciprocal regulation between cyPG and the synthesis and/or availability of redox-active small molecules. Therefore, an additional output of the study of cyPG targets is the identification of previously unknown mechanisms for redox regulation. Nevertheless, to understand the complex picture of cyPG signaling many challenges remain ahead, including the identification and characterization of the proteins modified by endogenously generated cyPG and the interplay of these modifications with other electrophilic and oxidative modifications of varied structure and stability in the cellular context. Finally, learning how these modifications can be used in the fight against disease remains the ultimate goal.

VIII. ABBREVIATIONS

AKR	aldo-keto reductase
COX	cyclooxygenases
cyPG	cyclopentenone prostaglandins
GSH	glutathione
γGCS	γ-glutamilcysteine synthetase
GST	glutathione S-transferase
HO-1	hemooxygenase-1
HSF	heat shock factor
Hsp	heat shock protein
iNOS	inducible nitric oxide synthase (NOS2)

MEF	mouse embryonic fibroblasts
PPAR	peroxisome proliferator activated receptor
15d-PGJ_2	15-deoxy-$\Delta^{12,14}$-prostaglandin J_2
ROS	reactive oxygen species
Triphenylphosphonium	TPP
Trx	thioredoxin

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Table 1 Selected targets for modification by cyclopentenone prostaglandins which illustrate the interplay with redox regulated mechanisms

TARGET PROTEIN	RESIDUE MODIFIED	cyPG	REFERENCE
Keap1	Cys273 and Cys288	15d-PGJ_2	Kobayashi et al., 2009
AP-1	Cys269 in c-Jun, not identified in c-Fos	15d-PGJ_2	Pérez-Sala et al., 2003
NF-κB p50	Cys62	15d-PGJ_2	Cernuda-Morollón et al., 2001
NF-κB p65	Cys38	15d-PGJ_2	Strauss et al., 2000
Thioredoxin	Cys35 and Cys69	15d-PGJ_2	Shibata et al., 2003
Thioredoxin reductase	Not identified	PGA_1	Moos et al., 2003
H-Ras	Cys118, Cys181 and Cys184	15d-PGJ_2 and PGA_1	Oliva et al., 2003 Renedo et al., 2007
GSTP1-1	Cys47 and Cys101	15d-PGJ_2	Sánchez-Gómez 2007
Hsp90	Not identified	15d-PGJ_2 and PGA_1	Stamatakis et al., 2006 Gharbi et al., 2007
Hsp70	Not identified	PGA_1	Gharbi et al., 2007
Catalase	Not identified	15d-PGJ_2	Landar et al., 2006
Aldehyde dehydrogenase	Not identified	15d-PGJ_2	Landar et al., 2006
Biliverdin reductase A	Not identified	15d-PGJ_2	Aldini et al., 2007

Figure legends

FIGURE 1. Formation of cyPG and covalent binding to proteins through Michael addition. cyPG (in shaded ovals) are generated by spontaneous dehydration of their parent PG, which in turn are formed by the action of cyclooxygenases on unsaturated fatty acids but also by non-enzymatic processes like the isoprostane pathway (via epimerization of isoprostanes, represented by dashed lines). cyPG possess one or more electrophilic carbons (marked by asterisks), which can suffer the attack of nucleophilic groups such as thiol groups in proteins and form Michael adducts, as illustrated at the bottom for 15d-PGJ_2.

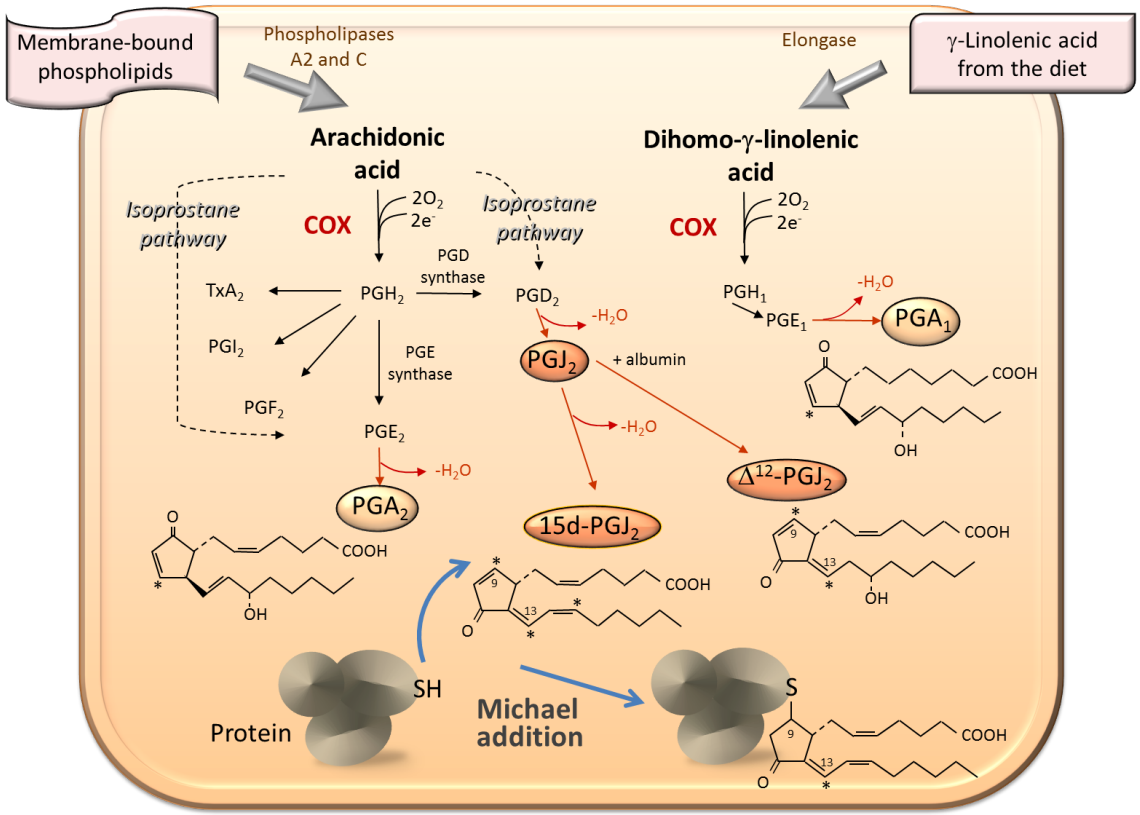
FIGURE 2. Modifications affecting the C-terminal end of H-Ras protein. H-Ras possesses three cysteine residues at its C-terminal end (depicted in green). Cys186 is the site for farnesylation, which is considered an irreversible modification (depicted in blue). Amino acids shown in grey are lost upon farnesylation. Cysteines 181 and 184 are normally the sites for palmitoylation, a reversible modification. These cysteine residues are also targets for modification by various electrophiles of different structure, which are schematically shown, leading to diverse functional outcomes.

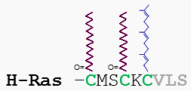
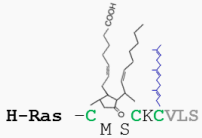
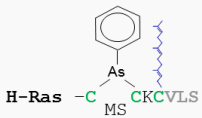
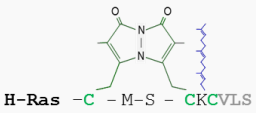
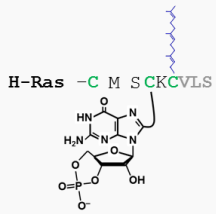
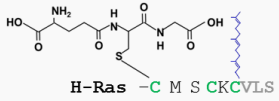
FIGURE 3. Modifications induced in GSTP1-1 by treatment with cyPG. GSTP1-1 exists in the cytosol as an active homodimer. GSTP1-1 possesses four cysteine residues, Cys14, 47, 101 and 169, of which, Cys 101 is located towards the dimer interface. Treatment of cells with 15d-PGJ_2 induces oxidative stress resulting in GSTP1-1 thiolation and formation of intra-or inter-molecular disulphide bonds, either with other GST monomers or small molecule thiols. In addition, 15d-PGJ_2 induces irreversible protein cross-links both in vitro and in cells, leading to the formation of highly stable tetramers. GSH or GSH analogs, like nitrobenzyl glutathione (NBG) protect GSTP1-1 from the covalent binding of cyPG which results in inactivation. In addition, 15d-PGJ_2 may inhibit GSTP1-1 through non-covalent interactions.

FIGURE 4. Typical workflows used for identification and characterization of cyPG targets.

FIGURE 5. Example of the MS analysis of H-Ras proteins incubated in vitro with PGA_1 in the presence of DTT. **A**: Peptide mass fingerprint. In bold, peptides for which the MALDI-TOF-TOF MS/MS analysis is shown. **B**: MALDI-TOF-TOF MS/MS analysis of the peptide of *m/z* 984.48, showing a fragmentation pattern indicative of the incorporation of a PGA_1 molecule into the peptide CDLAAR (*m/z*=648.31). Adapted with permission from Renedo et al., (2007)

Biochemistry 46:6607-6616. Copyright 2007 American Chemical Society. **C**: MALDI-TOF-TOF MS/MS analysis of the peptide of *m/z* 1136.49, showing a fragmentation pattern compatible with the incorporation of one DTT (mass 152.2) plus one PGA_1 (mass 336.5) molecule into the CDLAAR peptide.


Membrane-bound phospholipids
Phospholipases A2 and C
Elongase
γ-Linolenic acid from the diet
Arachidonic acid
Dihomo-γ-linolenic acid
Isoprostane pathway
COX
2O2
2e-
PGD synthase
PGE synthase
TxA2
PGH2
PGI2
PGF2
PGE2
PGD2
PGH1
PGE1
-H2O
PGA2
PGA1
PGJ2
+ albumin
Δ12-PGJ2
15d-PGJ2
COOH
OH
Protein
SH
S
Michael addition

Modification	Schematic structure	Functional outcome	Reference
Palmitoylation		Cycle of palmitoylation/ depalmitoylation and traffic between Golgi and plasma membrane	Hancock, 1989 Rocks, 2005
Lipoxidation (15d-PGJ$_2$)		Activation	Oliva, 2003 Renedo, 2007 Oeste, 2011
PAO		Activation on endomembranes	Oeste, 2011
DBB		Activation on endomembranes	Oeste, 2011
Guanylation		Activation at plasma membrane	Nishida, 2012
Glutathionylation		Inhibition of palmitoylation and plasma membrane association	Mallis, 2001 Burgoyne, 2012

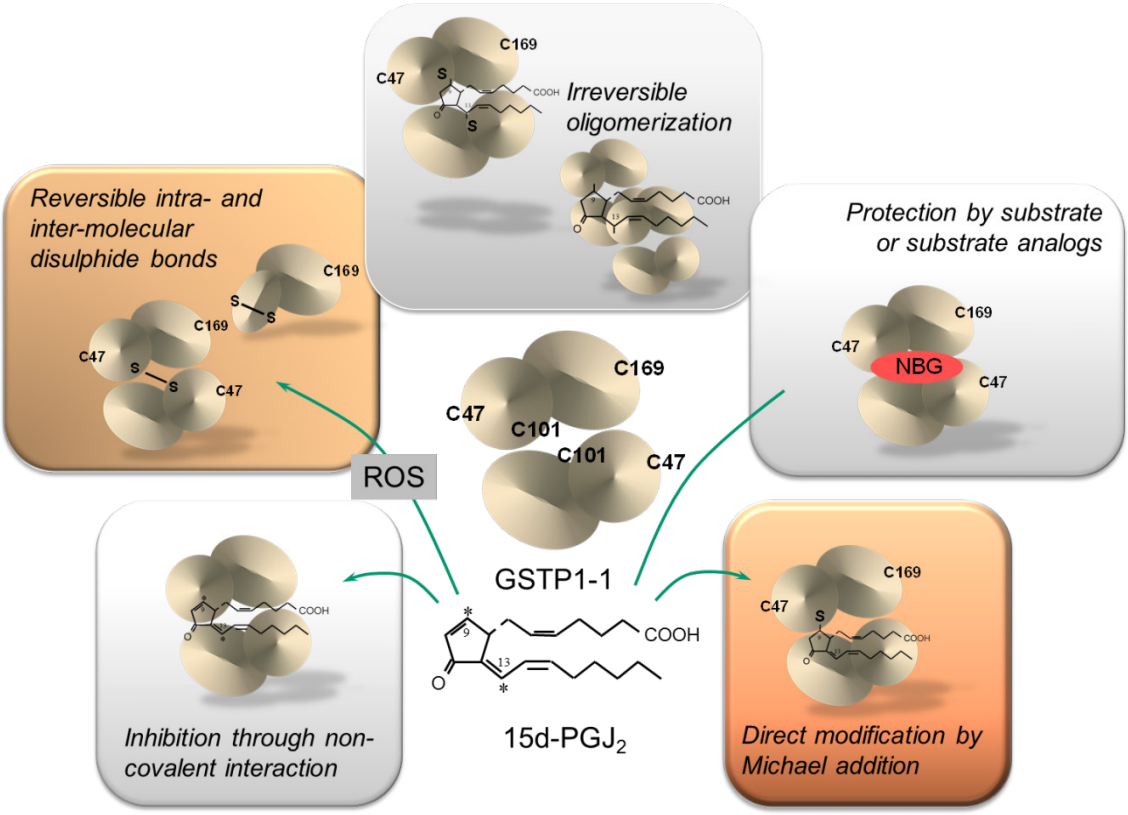

C169
C47
S
S
COOH
Irreversible
oligomerization
COOH
Reversible intra- and
inter-molecular
disulphide bonds
C169
S
S
C169
C47
S
S
C47
Protection by substrate
or substrate analogs
C169
C47
NBG
C47
C169
C47
C101
C101
C47
ROS
GSTP1-1
COOH
15d-PGJ2
COOH
Inhibition through non-
covalent interaction
C169
C47
S
COOH
Direct modification by
Michael addition

Fig. 3

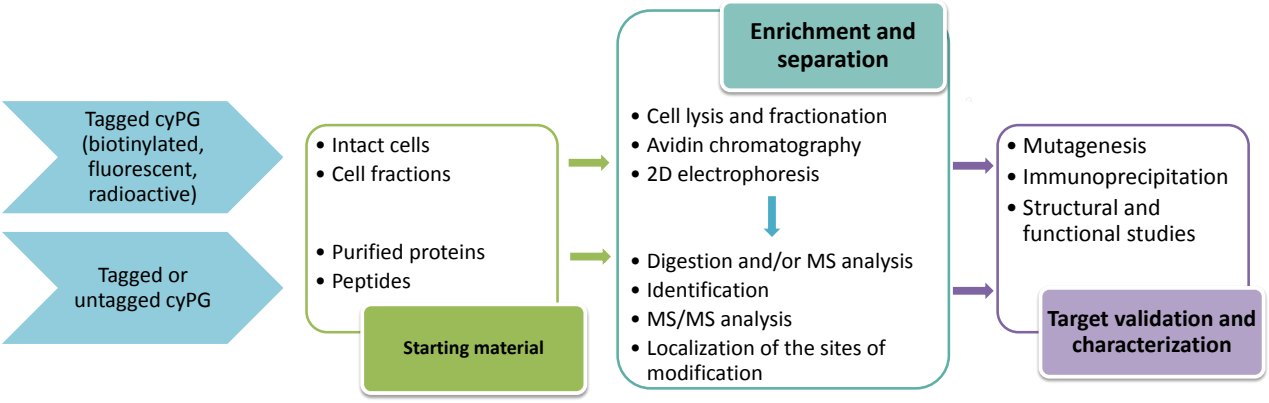

Tagged cyPG (biotinylated, fluorescent, radioactive)
Tagged or untagged cyPG
• Intact cells
• Cell fractions
• Purified proteins
• Peptides
Starting material
Enrichment and separation
• Cell lysis and fractionation
• Avidin chromatography
• 2D electrophoresis
• Digestion and/or MS analysis
• Identification
• MS/MS analysis
• Localization of the sites of modification
• Mutagenesis
• Immunoprecipitation
• Structural and functional studies
Target validation and characterization

Fig. 4

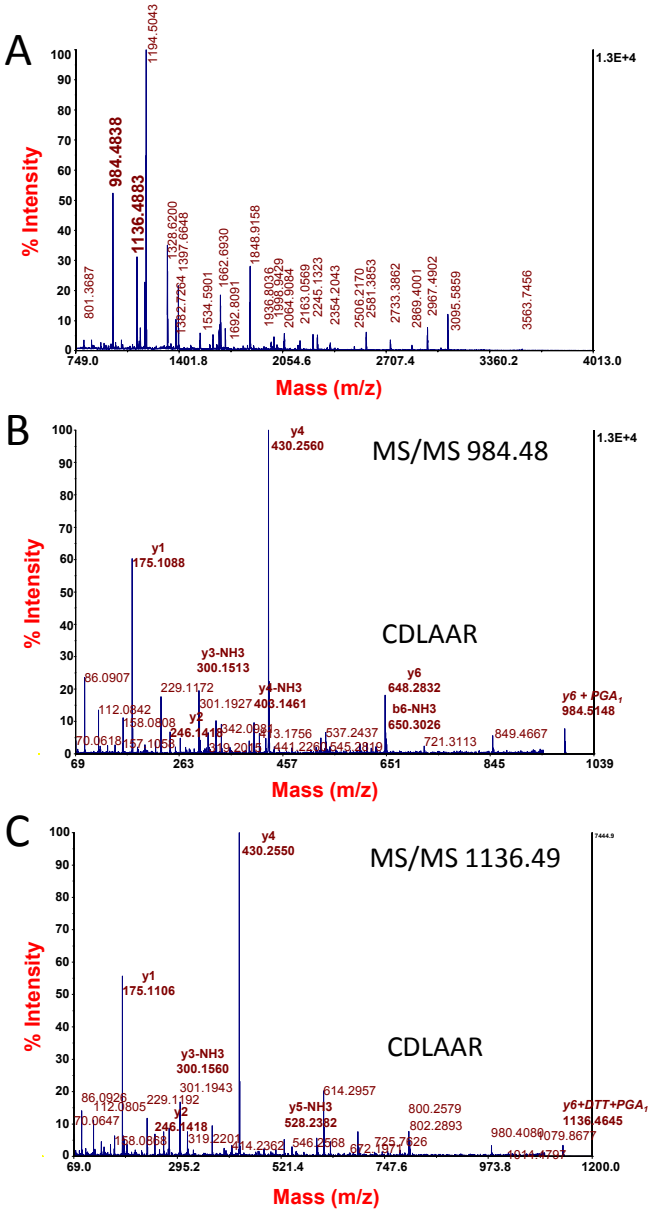

A
% Intensity
Mass (m/z)
1.3E+4
984.4838
1136.4883
1194.5043
B
MS/MS 984.48
CDLAAR
y4
430.2560
y1
175.1088
y3-NH3
300.1513
y6
648.2832
b6-NH3
650.3026
y6 + PGA,
984.5148
C
MS/MS 1136.49
CDLAAR
y4
430.2550
y1
175.1106
y3-NH3
300.1560
y5-NH3
528.2382
y6+DTT+PGA,
1136.4645

Fig. 5