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Commentary

Atherosclerosis: A redox-sensitive lipid imbalance suppressible by cyclopentenone prostaglandins

Lucila Ludmila Paula Gutierrez [a,1], *Alexandre Maslinkiewicz* [a,1], *Rui Curi* [b], *Paulo Ivo Homem de Bittencourt Jr.* [a,c,*]

[a] *Laboratory of Cellular Physiology, Department of Physiology, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Rua Sarmento Leite 500 – 2° andar, 90050-170 Porto Alegre, RS, Brazil*
[b] *Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, S. Paulo, SP, Brazil*
[c] *Federal University of Rio Grande do Sul School of Physical Education, Porto Alegre, RS, Brazil*

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ABSTRACT

Disorders concerning the metabolism of plasma and intracellular lipids are hallmarks of atherosclerosis. However, failures in proper control of intracellular cholesterol balance, rather than simple cholesterol overloading due to augmented uptake, could fuel atherogenesis. Therefore, the understanding of atherosclerosis-associated lipid alterations, which feed an inflammatory microenvironment in the arterial wall, requires the meticulous investigation of several aspects of lipid synthesis, uptake and export from cells. In this regard, the presence of reactive cysteines in transcription factors and key enzymes of lipid metabolism may dictate cholesterol accumulation, and therefore the progression of vascular disease. The strong inhibitory effect of cysteine-reactant anti-inflammatory cyclopentenone prostaglandins (CP-PGs) over atherosclerosis progression *in vivo* (LipoCardium technology) symbolizes a new concept of atherosclerosis and its treatment. Results from this laboratory and those from other research groups have unraveled a novel facet in

* *Corresponding author at*: Laboratory of Cellular Physiology, Department of Physiology, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Rua Sarmento Leite 500 – 2° andar, 90050-170 Porto Alegre, RS, Brazil. Tel.: +55 51 33083151; fax: +55 51 33083151.
E-mail address: pauloivo@ufrgs.br (P.I.H. de Bittencourt Jr.).
URL: http://www.ufrgs.br/fisiologia/fisiologiacelular

[1] These authors equally contributed to this work.

Abbreviations: ABC, ATP-binding cassette; ACAT, acyl-coenzyme A:cholesterol acyltransferase; AMPK, 5′-adenosine monophosphate-dependent protein kinase; AMPKK, AMPK kinase; AP-1, activator protein-1; Apo, apolipoprotein; ARE, antioxidant-responsive element; CEH, cholesteryl ester hydrolase; COX, cyclooxygenase; CP-PG, cyclopentenone prostaglandin; FAS, fatty acid synthase; G6PDH, glucose-6-phosphate dehydrogenase; γ-GCS, γ-glutamylcysteine synthetase; GSPx, glutathione peroxidase; GSRd, glutathione disulfide reductase; GST, glutathione S-transferase; HDL, high-density lipoprotein; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; hsp, heat-shock protein; ICAM-1, intercellular adhesion molecule-1; Keap1, Kelch-like ECH-associated protein-1; LDL, low-density lipoprotein; MAPK, mitogen-activated protein kinase; MRP/GS-X pump ATPase, multidrug resistance-associated protein/glutathione S-conjugate export ATPase; NF-κB, nuclear factor κB; NOS, nitric oxide synthase; Nrf2, nuclear factor-erythroid 2 p45-related factor 2; NSAID, non-steroidal anti-inflammatory drug; oxLDL, oxidized low-density lipoprotein; PG, prostaglandin; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2; PPARγ, nuclear factor peroxisome proliferators-activated receptor; RLIP76/GS-X pump, the 76-kDa Ral-binding protein-1 (RalBP1), stress-responsive glutathione S-conjugate export ATPase; ROS/RNS, reactive oxygen/nitrogen species; SR, scavenger receptor; SRE, sterol-regulatory element; SREBP, sterol-regulatory element binding protein; TGFβ, transforming growth factor-β; TNFα, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; VLDL, very-low density lipoprotein.

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prostaglandin research in that CP-PGs may act as redox signals that guide lipid metabolism in atherosclerosis. By modifying enzymes (*e.g.*, HMG-CoA reductase, ACAT and cholesteryl ester hydrolases) and transcription factors (*e.g.*, NF-κB and Keap1) involved in inflammation and lipid metabolism, CP-PGs (especially those of A-series) induce pivotal changes in glutathione and lipid metabolism that completely arrest atherosclerosis progression. Hence, pharmacological manipulation of lipid metabolism by CP-PGs may be a novel and invaluable strategy for treating atherosclerosis. Also, a better understanding of why CP-PGs do not resolve inflammation physiologically may explain many unsolved questions and yield insights into atherogenesis and its termination.

1. Atherogenesis: rephrasing an old disease from new clues

Atherosclerosis-related cardiovascular diseases cause at least one in every three deaths in industrialized countries; in the US this figure may reach the impressive mark of 40% of all deaths considering all age-groups [1]. Remarkable disturbances in plasma and vascular cell lipid metabolism are evident in atherosclerosis. However, many aspects of lipid imbalance that accompany the disease remain unresolved. For instance, inappropriate control of intracellular cholesterol balance, rather than simple cholesterol overloading due to augmented uptake, could fuel atherogenesis. However, this point is often underemphasized. Current human lifestyles (relative physical inactivity, high carbohydrate consumption, inadequate dietary fatty acid balance) likely differ enormously from primitive conditions, such that present-day levels of physical activity and dietary patterns have changed much faster than evolutionary metabolic changes. As a result, prodromal stages of atherosclerotic lesions are observed in humans as early as *in utero* [2]. Hence, the understanding of atherosclerosis-associated lipid alterations requires meticulous investigation of many aspects of lipid metabolism to allow full comprehension of atherogenesis.

Notably, regulation of vascular cell lipid balance is critical for determining atherogenesis and the progression of atherosclerosis. In this regard, the presence of reactive cysteines in transcription factors and key enzymes of lipid metabolism may dictate cholesterol accumulation, and therefore dictate the progression of vascular disease. Nevertheless, few studies have addressed the applicability of such findings. The strong inhibitory effect of cysteine-reactant (electrophilic) cyclopentenone prostaglandins (CP-PGs) over atherosclerosis progression *in vivo* [3] suggests a new view of atherosclerosis and its treatment. Considering the fact that atherosclerosis is a chronic inflammatory disease, recent insights into the resolution of inflammation [4], which notably involves the participation of CP-PGs [5], reinforce the need to explore these new avenues in atherosclerosis research.

In this paper, we discuss how the role of CP-PGs in intracellular lipid balance is an attractive and useful tool for treating atherosclerosis.

2. From cholesterol homeostasis to atherosclerosis

Lipid accumulation within arterial wall cells is a consequence of the progression of atherosclerotic lesions but, at the same time, promotes disturbances in cellular homeostasis, including changes in lipid metabolism, which perpetuates the disease. However, although hyperlipidemia and enhanced uptake of oxidized low-density lipoprotein (oxLDL) particles by blood vessels contribute to atherogenesis, intracellular cholesterol homeostasis in mammalian cells is sustained by a precise balance between cholesterol input and output [6]. Hence, disruption of intracellular cholesterol balance rather than elevated uptake or enhanced *de novo* cholesterol synthesis has emerged as a major topic for investigation. In this regard, the ''output pan'' of this balance is accomplished by cholesterol/cholesteryl ester export towards the extracellular space, *e.g.*, via the ATP-binding cassette (ABC) type A1 (ABCA1) export system and delivery to reverse cholesterol transport towards the liver, especially through apolipoprotein A (ApoA)-containing high-density lipoproteins (HDL). On the other hand, the ''input side'' is defined by a combination of the *de novo* cholesterol synthesis (and subsequent esterification into cholesteryl esters) and cholesterol/cholesteryl ester entry into cells through receptor-mediated endocytosis of LDL (and/or oxLDL) particles.

2.1. *The input portion of intracellular cholesterol balance*

Although strictly necessary for the assembly of cellular membranes, excess cholesterol must be avoided because it stiffens cellular membranes, impairs membrane receptor responses and cell signaling, blocks mitogen-stimulated proliferation of lymphocytes, allows the formation of toxic oxysterols, induces apoptosis and forms insoluble crystals that are toxic to cells [7,8]. Cholesterol overload is a determining factor for the establishment of membrane lipid rafts, *i.e.*, membrane microdomains rich in cholesterol and sphingolipids (*e.g.*, caveolae), which provide a platform for cell signaling proteins and membrane channels. These proteins are preferentially found in rafts because of strong Van der Waals interactions between ordered saturated chains and the liquid-ordered hydrocarbon chains of rafts [7]. Cholesterol-rich lipid raft signaling has

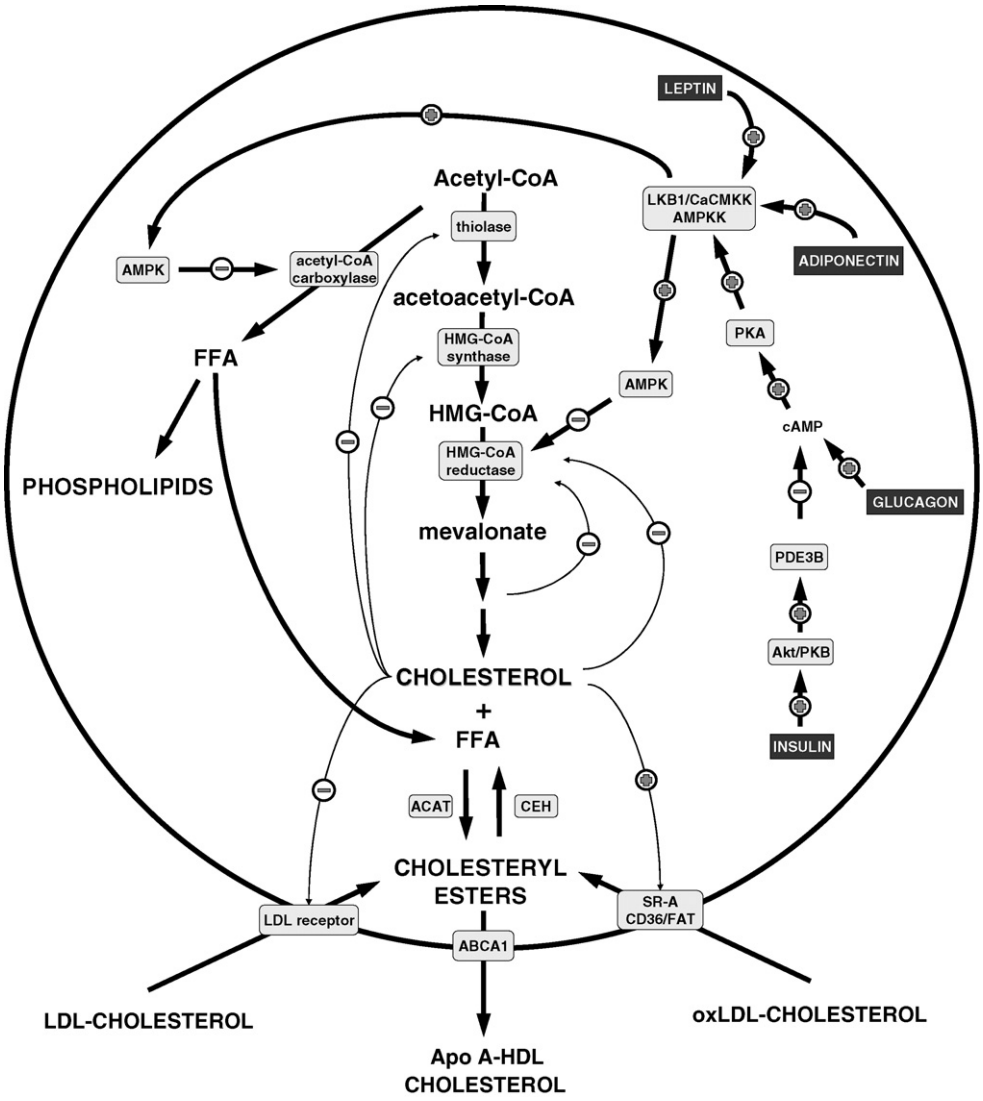

Fig. 1 – Metabolic pathways involved in intracellular cholesterol balance. Metabolic and hormonal regulating points of cholesterogenesis are shown. Minus and plus signals in the thin arrows indicate inhibition and stimulation of gene expression, respectively. Signals in the thicker arrows represent direct influence on the activity of either enzymes or transcription factors accordingly.

emerged as a potential target for the understanding of the pathogenesis of a variety of conditions, including cardiovascular diseases [9].

If cholesterol abundance is to be avoided, a sufficient cholesterol supply is required by cells to ensure proper membrane receptor signaling, whose defects are associated with pathological states. Moreover, cholesterol synthesis is so important that, in cells committed to cell proliferation, cholesterogenesis (the *de novo* synthesis of cholesterol from acetyl-coenzyme A, Fig. 1) precedes DNA synthesis, which characterizes entry into the S-phase of the cell cycle. Indeed, cholesterogenesis is pivotal for cell survival not only for its ultimate bulk product (cholesterol) but also because non-sterol isoprenoid intermediates, such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate, are required for cell signaling via covalent modification of determinant protein substrates (*e.g.*, receptor-coupled G-proteins, lamin B, dolichol, ras) that mediate cell proliferation and differentiation [8].

Because of the tenuous line that separates cholesterol needs from cholesterol overload, cells have evolved a complex machinery to undergo sufficient cholesterogenesis without any undesirable accumulation of cholesterogenic intermediates or cholesterol itself. Therefore, intracellular cholesterol homeostasis is primarily attained by a delicate poise between input and output of cholesterol in which both sides of this metabolic balance are accurately and coordinately regulated to provide sufficient amounts of other lipids to cells while avoiding lipid accumulation (Fig. 1). Accordingly, the metabolic flux through cholesterogenesis is regulated at different levels. Cytosolic acetoacetyl-CoA thiolase, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase and HMG-CoA reductase are susceptible to end-product repression exerted by cholesterol

and inhibits the expression of genes encoding these key enzymes and LDL receptors [8,10]. Notably, HMG-CoA reductase, the rate-limiting step of cholesterogenesis, is subject to regulation at multiple levels, including gene transcription, translation, protein degradation, protein phosphorylation and intracellular redox status [8,10,11], which makes the reductase one of the most precisely and intricately regulated enzymes in nature [6].

Transcription of HMG-CoA reductase and other genes implicated in cholesterol synthesis is dependent upon sterol-regulatory element binding proteins (SREBP), a family of sterol-regulated, endoplasmic-reticulum membrane-bound transcription factors whose translocation to sterol-regulatory element (SRE) binding sites in DNA is triggered by low intracellular cholesterol content. Conversely, SREBP-dependent transcription is prevented by the binding of excess cholesterol to a complex consisting of SREBP cleavage-activating protein (Scap) and insulin-induced gene (Insig) proteins, which arrest SREBP at the endoplasmic-reticulum membrane [10]. In addition, HMG-CoA reductase is regulated by cholesterol availability from LDL and by both sterol and non-sterol (*e.g.*, farnesyl pyrophosphate) end products that accelerate HMG-CoA reductase degradation and block mevalonate synthesis (Fig. 1), thus hindering cholesterol synthesis [6,10].

Indeed, SREBPs regulate not only transcription of cholesterogenic genes, namely HMG-CoA synthase, HMG-CoA reductase, farnesyl diphosphate synthase and squalene synthase, but also genes encoding enzymes of fatty acid synthesis and uptake, such as acetyl-CoA carboxylase, fatty acid synthase (FAS), stearoyl-CoA desaturase-1 and -2, and lipoprotein lipase [8,12]. Thus, SREBPs coordinate the synthesis of the two major building blocks of cell membranes: fatty acids and cholesterol (Fig. 1). Both acetyl-CoA carboxylase and HMG-CoA reductase are negatively regulated by phosphorylation via 5′-adenosine-monophosphate-dependent protein kinase (AMPK), which is considered the ''energy-sensing'' kinase since it responds to AMP/ATP ratio changes during metabolic fuel deprivation, hypoxic conditions and low insulin/glucagon ratios that signal physiological energy stressful situations (*e.g.*, fasting) [13]. HMG-CoA reductase is both phosphorylated and inactivated by AMPK [14] during ATP depletion, and this lowers the rate of cholesterol synthesis and preserves energy stores in the cell.

The importance of cholesterol feedback systems to human health was established by the finding that diets rich in cholesterol (*per se*) and/or saturated fatty acids (which enhance cholesterol efflux from the liver) raises blood cholesterol levels and causes heart attacks. In addition to suppressing endogenous cholesterol synthesis, high cholesterol diets act through a feedback system (Fig. 1) to reduce the liver's uptake of cholesterol by blocking the production of receptors for LDL, thus causing these atherogenic particles to accumulate in the blood [8]. Conversely, the most potent cholesterol lowering drugs currently available, the statins (*e.g.*, lovastatin, simvastatin, atorvastatin), which are HMG-CoA reductase inhibitors, exploit the feedback system by preventing cholesterol synthesis, and enhance the expression of LDL receptor to favor cholesterol uptake and removal from the blood [8].

With regard to the input side of cholesterol metabolic balance, acyl-CoA:cholesterol acyltransferase (ACAT, also known as sterol *O*-acyltransferase [SOAT]) plays a role in unburdening excess unesterified (''free'') cholesterol towards cholesteryl ester stores (Fig. 1). This cellular strategy to alleviate free cholesterol overaccumulation may nevertheless be dangerous since it impedes cholesterol-mediated feedback inhibition over HMG-CoA reductase and LDL receptor, thus causing continual cellular production and uptake of cholesterol. In fact, cholesterol feeding enhances aortic ACAT activity [15]. Conversely, ACAT inhibitors reduce plasma cholesterol levels by suppressing the absorption of dietary cholesterol and by blocking the assembly and secretion of apolipoprotein B (ApoB)-containing lipoprotein particles, such as very-low density lipoproteins (VLDL) in the liver and chylomicrons in the intestine [16]. Consequently, these drugs tend to halt atherosclerosis progression in both animal models and humans.

In a hypercholesterolemic diet, excess of cholesterol-containing plasma LDL particles undergo oxidation and are taken up by a different set of LDL receptors, the scavenger receptors (SR, mainly expressed in macrophages, vascular smooth muscle cells and endothelial cells), which, after being endocytosed, deliver oxLDL to lysosomes where its cholesteryl ester content is hydrolyzed to free cholesterol and fatty acids (Fig. 1). In such a situation, the feedback mechanism of lowering cholesterol synthesis and uptake by LDL receptors is not sufficient to avoid cholesterol accumulation in the face of continuous cholesterol uptake by SR-mediated mechanisms. However, in the absence of an extracellular acceptor, excess free cholesterol undergoes re-esterification by ACAT [17]. Indeed, excess cholesterol taken up by SRs worsens the scenario because cholesterol induces the expression of the scavenger receptor of SR-B type CD36 (also known as FAT, for fatty acid translocase), which enhances cholesterol uptake [18]. As a result, the increase in intracellular cholesteryl ester fluxes through the cholesteryl ester hydrolases (CEH) and this vicious cycle is perpetuated until free cholesterol is used for membrane assembly or expelled into the bile after reverse cholesterol transport to the liver.

2.2. *The output portion of the intracellular cholesterol balance*

Cholesterol efflux pathways from extra-hepatic tissues constitute the ''output'' side of this metabolic balance and are also of value in repressing lipid accumulation and atherosclerosis progression. Since mammalian cells do not metabolize the sterol ring, excess cholesterol can only be eliminated from the body by biliary excretion, so that macrophages and other cells must export any excess cholesterol to extracellular acceptors, such as HDL particles, which can undergo hepatic catabolism [17]. In this regard, cholesterol export through ABCA1 and scavenger receptor SR-B1 favors cells to dispose surplus cholesterol [19]. Excess cholesterol triggers the activation of nuclear receptors of the ligand-dependent LXR transcription factor (from liver X receptors) family, which are mostly expressed in the liver, intestine and macrophages. LXRs coordinately protect cells from cholesterol overload by inducing ABC-transporters, ApoE (which may serve as a cholesterol acceptor to be transported by ABCA1 carrier) and the synthesis of specific fatty acids that may be substrates

for ACAT-mediated esterification of cholesterol [17]. Additionally, a novel surprising mechanism for macrophages to dispose of excess cholesterol is the transfer of cholesterol to different acceptor cells, a process that is both regulated by macrophages and one that is dramatically inhibited in inflammatory situations (please, see, for instance, ref. [20,21]). Hence, any lack of coordination that may lead to even discrete alterations in this delicate balance may account for cholesterol accumulation and atherogenesis.

3. Atherosclerosis: a multifaceted inflammatory process

In 1973, Russell Ross and his colleagues described atherosclerotic lesions in response to localized injury to the lining of the arteries (reviewed in ref. [22]). Since then, it has become apparent that atherosclerosis is a multifactorial inflammatory disease. In fact, atherosclerotic lesions have all the features of an inflammatory response and the major stimuli for the atheroma plaque formation (namely hypertension, high plasma levels of LDL, dyslipidemias, diabetes and obesity) are implicated in vascular wall inflammation. Accordingly, after a chronic risk factor-induced injury to the vessel-lining endothelial cells (which may take years but may also initiate *in utero*), atherosclerotic lesions take place when inflammatory cells (signalized by endothelial markers of injury) are recruited from the circulation and, after contacting the endothelial surface and undergoing transendothelial migration (diapedesis), leukocytes begin a typical inflammatory response [23].

3.1. *Macrophages: a major player in atherogenesis*

Although atherogenesis may involve several blood cell types, such as neutrophils, lymphocytes and platelets, monocytes are the primary actors in the initiation of lesions within the vascular wall. Once in the subendothelial space, they differentiate into macrophages that in turn secrete many different cytokines, reactive oxygen and nitrogen species (ROS/RNS), and take up increasing amount of lipids. They are thus referred to as foam cells (or foamy macrophages) due to their accumulation of cytoplasmic lipid droplets [23]. Foamy macrophages are inflammatory cells that, besides their usual inflammatory attributes, have an extremely active lipid metabolism (taking up, synthesizing and exporting lipids) [3,20,24]. They accumulate large amounts of cholesterol and cholesteryl esters, which enter a cycle of hydrolysis and esterification until the excess of cholesterol is secreted or directed for membrane synthesis [25].

Accumulation of lipids by foamy macrophages is more than enlarging cells with unwanted metabolites; the more plentiful foam cells become, the more intense the inflammation becomes [26]. As inflammatory macrophages, these foam cells feed forward and exacerbate the lesion, which is worsened because atheromata are not commonly inflamed tissues. Rather, their state resembles that of chronic inflammation, in which resolution phases are dysfunctional [4] and physiological feedback mechanisms that regulate intracellular cholesterol are overcome by constant noxious stimuli stated above which exacerbate the arterial wall lesion.

3.2. *NF-κB activation as the convergent point in atherogenesis*

A striking feature of atherosclerosis initiation as an inflammatory disease is the activation, within endothelial cells, of nuclear factor κB (NF-κB) by different endothelial stressors, such as oxLDL and nonlaminar flow-induced shear stress [27]. The existence of κB DNA-binding domains in the promoter regions of many key mediators of inflammation and immune responses has been demonstrated, such as the inducible cyclooxygenase (COX-2), inducible nitric oxide synthase (iNOS encoded by the *NOS-2* gene), interferon-γ, major histocompatibility complexes, interleukin-2, E-selectin, and intercellular adhesion molecules ICAM-1 and VCAM-1 genes [27,28]. In resting (unstimulated) cells, NF-κB dimeric complexes are predominantly found in the cytosol and associate with members of the inhibitory IκB family [28]. Thus, NF-κB gene products are entirely inducible proteins whose activation is dictated by specific stimuli that turn on IκB kinase (IKK) complexes. IKKs, in turn, phosphorylate IκB proteins directing them to proteasome-mediated degradation, which, consequently, sets NF-κB dimers free to bind DNA in the nucleus.

Hemodynamic shear stress occupies an important place, since hypertension- and nonlaminar flow-induced shear stress in the blood stream (especially in arterial bifurcations) specifically stimulates NF-κB-dependent transcription pathways that lead to the production of inflammatory proteins in endothelial cells [27]. Just after an increase in shear stress, endothelial cells express, in an NF-κB-dependent manner, selectins (that mediate rolling and the initial steps of leukocyte attachment to the endothelium [23]) and vascular cell adhesion molecule-1 (VCAM-1 also known as CD106), responsible for the firm attachment of monocytes to the arterial wall. This renders leukocytes prone to transendothelial migration [23]. In fact, it has been recently demonstrated that dynamic flow alterations dictate leukocyte adhesion and response to endovascular interventions in a porcine model [29], while laminar flow induces the transcription of antioxidant-response element (ARE)-mediated genes [30], leading to cell defense against oxidative stress. Then, NF-κB-dependent expression of VCAM-1 (accompanied by that of other leukocyte–endothelium adhesion molecules) in response to altered blood flow in certain territories triggers atherogenesis.

Although the exact mechanisms involved in NF-κB-dependent initiation of atherogenesis still remains under debate, NF-κB is very active in lesion-prone regions where upregulation of NF-κB-commanded genes is considered a driving force of atherosclerotic lesion development, while the expression of the A20 gene, which decreases NF-κB activity, correspondingly diminishes atherosclerosis [31]. Paradoxically, however, Kanters et al. have shown, in a very elegant experimental protocol, that inhibition of NF-κB activation, by using a macrophage-restricted deletion of IκB kinase 2 (IKK2), does increase atherosclerosis in LDL receptor-deficient mice [32]. Interestingly, they also found a major reduction in the anti-inflammatory cytokine interleukin-10 (IL-10), which is NF-κB-dependent, implying that inhibition of the NF-κB pathway in macrophages leads to more severe atherosclerosis in mice, possibly by affecting the pro- and anti-inflammatory balance that controls the development of atherosclerosis [32].

However, the same group also observed that transplantation of bone marrow from NF-κB1-deficient mice into LDL receptor knockout mice results in a nearly 50% reduction of atherosclerotic lesions [33]. Moreover, in macrophages, the inflammatory cytokine TNFα induces the expression of ABCA1 through NF-κB which, in atherosclerotic plaques, may help phagocytic macrophages to efflux excess lipids derived from the ingestion of cholesterol-rich apoptotic corpses [34]. However, since NF-κB activation also guides the expression of genes that are clearly anti-inflammatory, such as the cytokine IL-10, and because pro-atherogenic (IL-1β, IL-12p40), ambiguous (IL-6), and anti-atherogenic (IL-10) cytokines are all increased in the plasma of A20-haploinsufficient mice, which presents increased susceptibility to NF-κB-dependent atherogenesis, it is conceivable that a disruption in the balance between pro- and anti-inflammatory cytokines might trigger atherogenesis in an NF-κB fashion [31]. This proposition is corroborated by the observation that IL-10/ApoE double knockout mice present increased atherosclerosis, thrombosis, and LDL levels [35].

3.3. *Redox status and NF-κB activation*

Oxidative stress may also mediate the activation of NF-κB thus further augmenting leukocyte adhesion to the endothelium [36]. NF-κB was the first redox-sensitive eukaryotic transcrip-

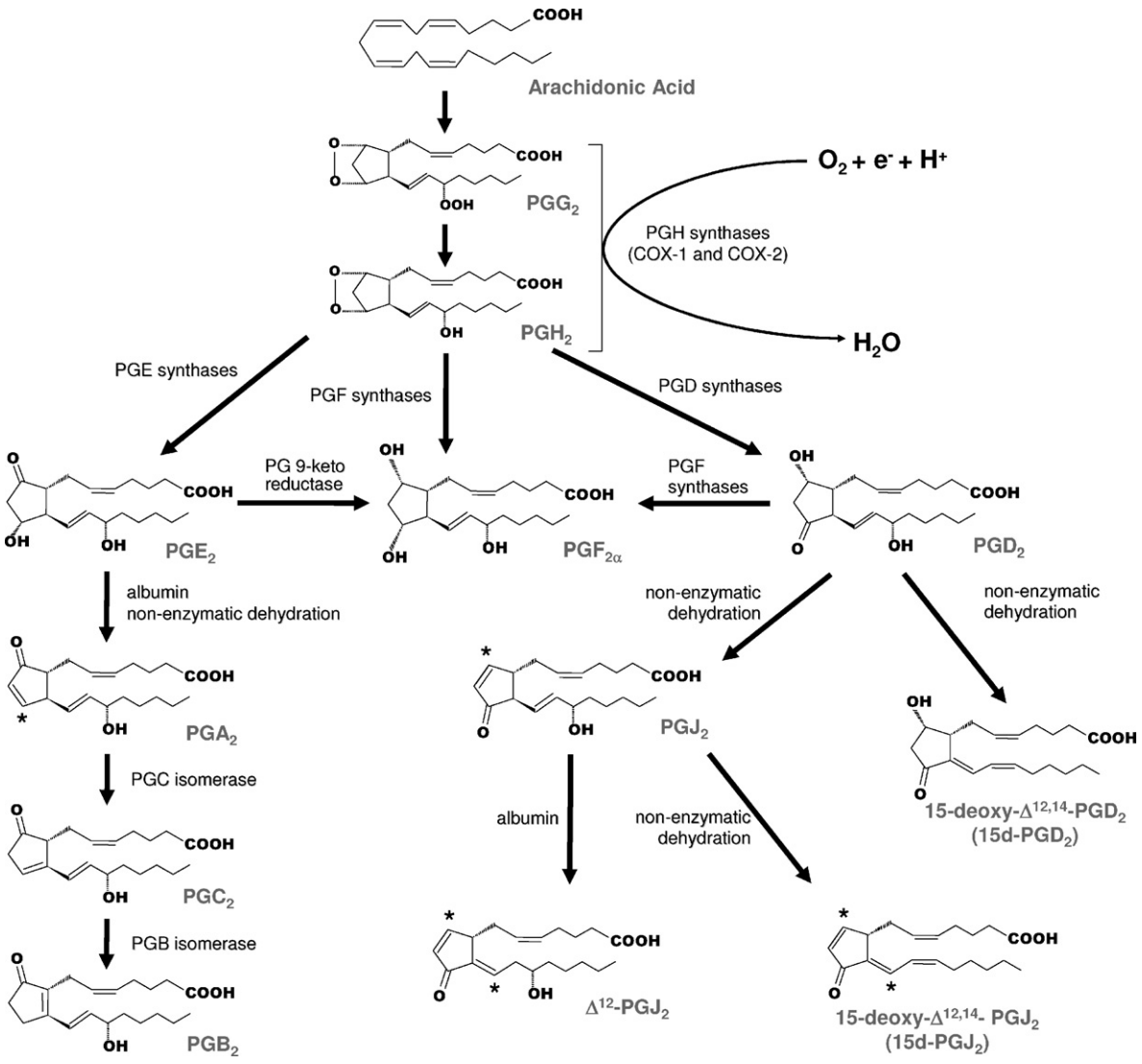

Fig. 2 – Arachidonic acid metabolism highlighting the structures of CP-PGs of A_2 and J_2 families. Physiologically known pathways for the generation of CP-PGs through the consecutive coupling of cyclooxygenases (either COX-1 or COX-2) with PGE synthases and PGD synthases are shown. Asterisks in the reactive CP-PG molecules (only those presenting at least one α,β-conjugated-unsaturation in the cyclopentane ring) denote the electrophilic carbons that are susceptible to Michael addition reaction with nucleophiles, such as reactive sulfhydryls present in GSH molecules and cysteine residues of cellular proteins: C-11 in PGA_2 ring, C-9 in PGJ_2 ring, and C-9 and C-13 in both Δ^{12}-PGJ_2 and 15-d-PGJ_2 molecules. Note that Δ^{12}-PGJ_2 and 15d-PGJ_2 possess additional unsaturations that are sequentially conjugated in α,β,γ and α,β,γ,δ, respectively, which renders these J-series CP-PGs more reactive, *i.e.*, during the formation of Michael's adducts, these specific CP-PGs form stabler resonance intermediates that allow enhanced probability for reaction.

tion factor shown to respond directly to oxidative stress in many cell types [37]. NF-kB activation is inhibited by antioxidants such as cysteine, which explains why some antioxidant therapies may be valuable in arresting the development of atheroma plaques. SR-mediated uptake of oxLDL particles by circulating monocytes, foamy macrophages, smooth muscle cells and endothelial cells of the vascular wall is known to induce an intracellular redox imbalance that triggers NF-κB activation [37] in addition to arterial hypertension. For instance, high-renin hypertension induces monocyte infiltration into the endothelium through a VCAM-1-dependent mechanism [38], while oxLDL autoantibodies, as well as endothelial dysfunction, have been shown to correlate with cardiac transplant-associated atherosclerosis [39]. Oxidized thiols (particularly glutathione disulfide, GSSG) amplify vascular responses to endothelial balloon injury [40] and shear stress has been shown to enhance enzyme of glutathione (GSH) antioxidant metabolism [41].

Either oxidative stress (directly) or shear stress (accompanied or not by an initial redox imbalance) may initiate a vicious cycle that perpetuates NF-κB-centered disease in the vessel wall. Blood flow appears to be sensed by membrane lipid rafts (see Section 2, please) that transmit mechanical signals downstream via extracellular signal-regulated kinase (ERK [42]), through a Rho-like GTPase (Rac1)-assisted p38 mitogen-activated protein kinase (MAPK) activation of different intracellular targets, including NAD(P)H oxidase, which sequentially generate ROS/RNS thus triggering NF-κB-dependent pathways, including VCAM-1 expression (see, for instance, ref. [43]). Hence, any definitive anti-atherosclerosis therapeutics must intervene in the pro-inflammatory branch of NF-κB activation cycle in order to interrupt the inflammation.

4. Cyclopentenone prostaglandins and atherosclerosis

After the discovery in 1997, by Maria Gabriella Santoro's group in Italy, that PGA_1, an antiproliferative CP-PG inhibits NF-κB activation [44], it has become clear that CP-PGs should be studied as potential pharmacological tools against atherosclerosis. CP-PGs (Fig. 2) are naturally occurring 20-carbon antiproliferative fatty acids derived from essential fatty acids, such as the ω-6 arachidonic (type 2 PGs, the most abundant in humans) and eicosatrienoic (type 1 PGs) acids, and the ω-3 eicosapentenoic acid (type 3 PGs). CP-PGs are characterized by an α,β-unsaturated cyclopentane ring that is obligatory for their biological activity (reviewed in ref. [45]).

4.1. *Arachidonic acid metabolism and the generation of CP-PGs*

Physiologically, and depending upon cell type and stimulation, arachidonic acid can provide PGH_2 (the parental PG), via a cyclooxygenase (COX, also known as PGH synthase)-catalyzed reaction that can be induced by both the constitutive COX-1 and the inducible COX-2 isoforms [46]. COX-1 as well as COX-2 may be competitively inhibited by non-steroidal anti-inflammatory drugs (NSAIDs), whereas their activity in generating PGH_2 is completely abolished by GSH, which may act as a reducing agent in the presence of glutathione peroxidase (GSPx), deviating fatty intermediates from the catalytic site [46]. Aside from other eicosanoid metabolites, PGH_2 can be further metabolized by the action of several different PGE synthases or by PGD synthases to generate PGE_2 and PGD_2, respectively [47]. Enzymatic and non-enzymatic conversion of PGE_2 and PGD_2 then furnishes the CP-PGs of A_2 and J_2 families, respectively [48,49]. Interestingly, albumins are able to convert PGE_2 into PGA_2, and the CP-PG PGJ_2 into another CP-PG, namely Δ^{12}-PGJ_2, whereas the formation of remaining CP-PGs (Fig. 2) has been proven to be non-enzymatic [50], although it occurs *in vivo*.

Despite physiological significance and *in vivo* production of CP-PGs, they have remained at the center of considerable controversy since the discovery of PGA_2 in human seminal fluid in 1966 (excellently reviewed in ref. [51]). Convincing proof for the *in vivo* formation of CP-PGs has been provided by different laboratories [50,52], including ours [53,54]. While pharmacological doses of CP-PGs were used in the micromolar range [3], physiological concentrations of CP-PGs range *ca.* 100 ng/mL (316 nM), in inflammatory cells [55,56], whereas urinary Δ^{12}-PGJ_2 amounts *ca.* 850 ng per day in humans, which equals a concentration of about 44 pM [52]. Interestingly, a single dose of 1 mg/kg indomethacin (a COX inhibitor) blocks urinary Δ^{12}-PGJ_2 production by roughly 50% in monkeys [52], while in mesenteric lymph nodes from Walker 256 tumor-bearing rats, concentrations of PGA_2 may reach as much as 7 μg/g tissue [54]. Nevertheless, in the surroundings of inflammatory cells and upon appropriate stimuli, these concentrations may reach a micromolar range since flux through the COXs can be enhanced several times, and because cells are able to actively take up and concentrate CP-PGs [57]. Furthermore, although oxidized lipids may account for the perpetuation of atherosclerosis as an inflammatory disease, anti-inflammatory CP-PG-derivatives may also be produced by free radical-mediated attack directed to membrane phospholipids [58], so that a natural anti-inflammatory response may be physiologically triggered within vascular cell membranes.

CP-PGs of the J-series (only) are the physiological ligands of the nuclear transcription factor PPAR-γ (peroxisome proliferator-activated receptor-γ) [59,60]. PPAR-γ is an important transcription factor related to cell proliferation and differentiation while the serendipitous discovery of its metabolic activation by CP-PGs has shed light on its pivotal role in glucose and lipid homeostasis as well as its potential relevance in metabolic disorders such as diabetes, obesity and atherosclerosis. CP-PGs inhibit cell proliferation in a variety of experimental tumor models, *in vitro* as well as *in vivo* [61]. They are actively and selectively transported into cells by a cell membrane carrier and then transferred to the nucleus where they bind to nuclear proteins and specifically act on the G_1/S interface of the cell cycle [61,62]. Although it has been assumed that physiological CP-PG concentrations may be non-stressful to cells, depending on the doses employed, CP-PGs may be extremely cytotoxic by blocking protein synthesis and causing damage to actin filaments, while they inhibit the expression of β and γ DNA polymerases for the cytoskeleton (please, see ref. [45]). The antiproliferative effect of CP-PGs depends on their uptake by cells, a phenomenon that may be so effective that it makes it difficult to detect such substances *in vivo* or after a few minutes following addition to culture media [57]. Due to CP-PG's intrinsic

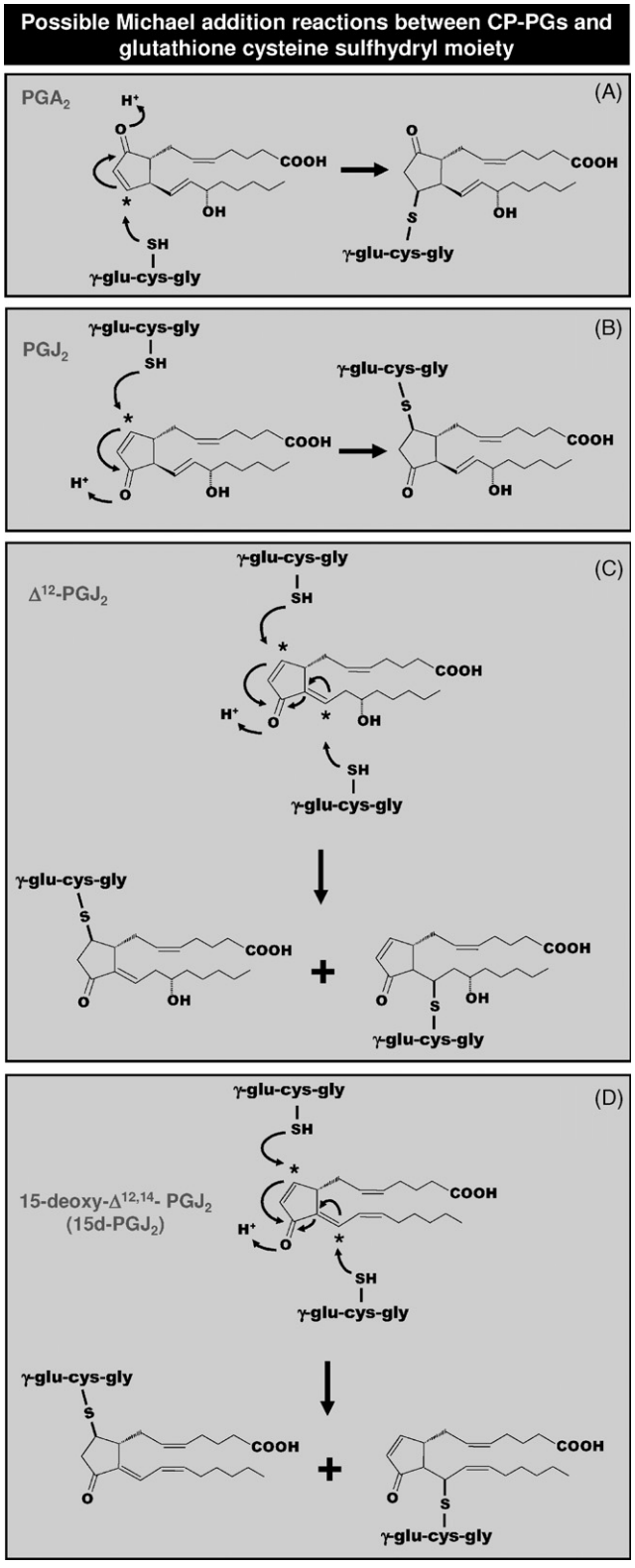

Fig. 3 – Possible Michael addition reactions between CP-PGs and GSH. The formation of Michael's adducts of CP-PGs with the sulfhydryl of the reactive cysteine present in GSH is shown. Note that Δ^{12}-PGJ_2 and 15d-PGJ_2 have two electrophilic centers (asterisks), which allows for the formation of two different adducts.

electrophilicity, a possibility does exist, however, in that CP-PG biological activity may be modulated by the amount of extracellular GSH, protein sulfhydryls and other nucleophiles, such as antioxidant vitamins (*e.g.*, ascorbate). The detection of considerable amounts of CP-PGs in physiological fluids and tissues suggests that at least some CP-PGs may escape neutralization from these agents (se below, please). Additionally, all the CP-PGs so far tested as well as their synthetic analogs, at non-cytotoxic doses, exhibit extremely potent antiviral activity over a number of DNA and RNA viruses, including HIV [63,64].

4.2. *The chemistry of CP-PGs: formation of Michael addition reactions with reactive sulfhydryls*

The presence of an α,β-unsaturated (enone) cyclopentane ring, which is common to PGA_2, PGJ_2, Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (also known as 15d-PGJ_2), makes these CP-PGs highly electrophilic and susceptible to Michael addition reactions with strong electron donors (nucleophiles), such as the free sulfhydryl group of cysteine residues located in GSH (Fig. 3) and cellular proteins (Fig. 4). The existence of multiple conjugated double-bonds observed in J-type derivatives (Fig. 2) accounts for their stronger electrophilicity as compared to A-type CP-PGs. Consequently, the conjugations of unsaturations are proportional to the strength of CP-PG biological effects [45,65].

As powerful electrophiles, CP-PGs exert profound effects on GSH metabolism. Inside the cells, CP-PGs are rapidly conjugated with GSH, via GSH *S*-transferase (GST)-catalyzed reactions, or even non-enzymatically, leading to the formation of CP-PG–GSH S-conjugates [66–68]. Shortly after (minutes to hours) addition to cultured cells, non-toxic concentrations of CP-PGs decrease intracellular GSH content [69,70] lead cells into a redox imbalance state (*i.e.*, GSSG to GSH ratio augments), which, *per se*, is an important regulatory signal that triggers the activation of redox-sensitive transcription factors [37]. Interestingly, the conjugation of CP-PGs with GSH (glutathiolation) completely abrogates CP-PG biological effects (see, for instance, ref. [69], please).

4.3. *CP-PG effects on glutathione metabolism, intracellular redox status and gene function*

CP-PG-mediated GSH depletion elicits a rise in the activity of antioxidant GSH-related enzymes, such as γ-glutamylcysteine synthetase (γ-GCS, also known as glutamylcysteine ligase, GCL, the rate-limiting enzyme for GSH synthesis), GSH *S*-transferases (GSTs, protect cells by conjugating CP-PGs with GSH), and MRP/GS-X pump ATPases (extrude electrophiles from cells as GSH S-conjugates, also known as GS-conjugates). The promoter region of γ-GCS subunits contains activator protein-1 (AP-1), NF-κB and antioxidant-response (ARE, also known as EpRE for electrophile-response element) *cis*-acting elements, which are regulated by oxidative stress and the pro-inflammatory cytokine tumor necrosis factor-α (TNFα) [71]. This is also the case for GST gene promoters, which are regulated by AP-1 and Nrf2 (nuclear factor-erythroid 2 p45-related factor 2) transcription factors, via ARE [72]. Since AP-1 consists of Fos–Jun and Jun–Jun dimers, whose individual

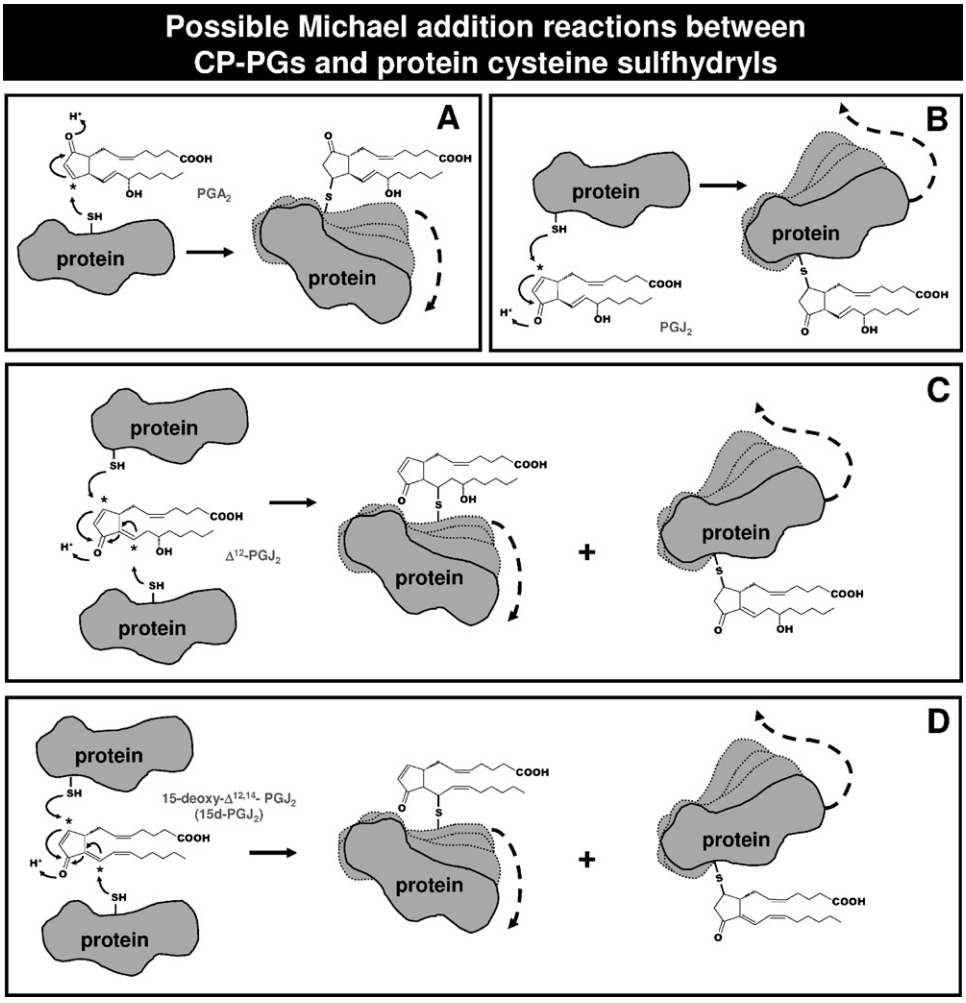

Fig. 4 – Possible Michael addition reactions between CP-PGs and protein cysteine sulfhydryls. The formation of Michael's adducts of CP-PGs with a reactive cysteine sulfhydryl present in a hypothetical protein is shown. Physiologically, reactive cysteine moieties are those in which their location in tertiary (or quaternary) spatial arrangement of a given protein is such that they are oriented to the outside of the protein, that is, not directed to the hydrophobic core of the protein. On the other hand, a critical reactive cysteine is one located near or at the catalytic (or regulatory) site of a protein, where the formation of Michael's adducts profoundly modifies protein function.

expression is NF-κB-dependent, both γ-GCS and GSTs may be modulated, at least in part, by NF-κB when intracellular GSSG/GSH ratio rises. On the other hand, MRPs (from multidrug resistance-associated proteins), particularly MRP/GS-X pump ATPases, are members of the ATP-binding cassette C-family (ABCC) of membrane transporters closely related to CP-PG-mediated alterations in GSH metabolism. MRP1 (ABCC1) and MRP2 (ABCC2, also known as cMOAT or cMRP, from hepatic canalicular biliary multiorganic anion transporter) are frequently co-induced with γ-GCS under oxidative stress conditions, which makes this cellular strategy a major threat to cancer chemotherapy, because electrophiles used in cancer therapeutics (*e.g.*, cisplatin, methotrexate and doxorubicin) may be conjugated to GSH exported from cells [45]. Moreover, the powerful antiproliferative effect of CP-PGs is strongly abrogated by MRP1 expression in cancer cells, while transfection with MRP1 gene clearly induces resistance against CP-PG effects [54].

Although by far more studied, MRP members of the ABC-transporter gene family are not the only GS-conjugate exporting ATPases capable of extruding CP-PGs and GSSG towards the extracellular space. RLIP76 (Ral-binding protein-1, RalBP1), a 76-kDa stress-responsive ATPase, has been recently described as a non-ABC multi-specific transporter of amphiphilic chemotherapeutic drugs (such as doxorubicin) and GS-conjugates [73]. In human cells, RLIP76/GS-X pump accounts for more than two thirds of the transport activity for GS-conjugates and drugs, as opposed to the ABC-transporters including MRP1, which account for less than one-third of this activity [67,68,73]. RLIP76/GS-X pump transports GS-conjugates of lipoperoxide-derivatives, such as 4-hydroxynoneal (4-HNE). Cells exposed to mild oxidative stress show a rapid and transient induction of RLIP76 resulting in an increased efflux of GS-HNE and acquire resistance to oxidative stress-mediated toxicity and apoptosis. Conversely, cells transfected with RLIP76 acquire resistance to doxorubicin through increased

efflux of the drug, suggesting its possible role in the mechanisms of drug-resistance [74], similar to that observed for MRP1 in rat cells [54]. The RLIP76/GS-X pump is responsible for most of the non-MRP transport of GS-conjugates but whether its redox-sensitive expression is related to the activation of a redox-sensitive transcription factor (*e.g.*, NF-κB or Nrf2) remains to be elucidated.

Similar to γ-GCS and GSTs, the expression of both MRP1 [75] and MRP2 [76] genes requires Nrf2-ARE activation pathway, which is triggered by CP-PG-dependent GSH depletion [77]. The expression of GSSG reductase (GSRd, which regenerates GSH from GSSG, in an NADPH-dependent fashion under oxidative stress) [78] and selenium-dependent GSPx [79], is also regulated in the same manner. These alterations lead to delayed (24 h)-augmentation in GSH contents thus reestablishing intracellular redox equilibrium after CP-PG challenge [70], while a similar mechanism of ''acquired resistance'' is observed in cells treated with TNFα, which triggers AP-1 and NF-κB-dependent expression of enzymes of GSH metabolism [71]. Altogether, these strategies promote a CP-PG-based cellular counterattack that may alleviate oxidative stress in inflammation. Meanwhile the powerful anti-inflammatory activity of CP-PGs *per se* has been further demonstrated by Santoro's group who found that these PGs directly inhibit the IKKβ catalytic subunit of IKK complex by reacting with a cysteine in the position 179, by virtue of their α,β-unsaturated ring-dependent electrophilicity [5].

The formation of Michael's adducts between CP-PGs and the nucleophilic centers of reactive cysteine-containing substrates gives rise to significant stereochemical alterations, because CP-PG are highly hydrophobic molecules. As such, they locally disrupt protein microenvironment in addition to causing steric hindrance that impedes further chemical reactions at those centers (Fig. 4). CP-PGs covalently modify transcription factors (as well as their controllers) of at least four important pathways closely related to inflammation and atherosclerosis (reviewed in ref. [77]): NF-κB, AP-1, Nrf2 and PPARγ. Besides the reaction with Cys179 of IKKβ (above), CP-PG-blockage of NF-κB pathway is also based on Michael addition reactions directed to both p50 (Cys38) and p65 (Cys62) κB subunits, which impairs their DNA-binding activity. The same is observed for c-Jun (Cys269), which blocks Jun–Jun and Jun–Fos dimerization and, consequently, halts AP-1 pathway. Conversely, the reaction of CP-PGs with critical cysteines present in Keap1 (Kelch-like ECH-associated protein 1), leads to Nrf2 activation and the eventual transcription of hemeoxygenase, NAD(P)H oxidase (also known as phox, for phagocyte oxidase) [77], and, as discussed above, γ-GCS, GSRd, GST, and MRP/GS-X pumps. The cytosolic regulatory protein Keap1 is an inhibitory protein that, under basal conditions binds tightly to Nrf2, retaining it in the cytoplasm, so that it resembles IκB family components that arrest NF-κB dimers out of the nucleus [80]. Reactive cysteine residues located at positions 257, 273, 288 and 297 in Keap1 function as electrophilic sensors that trigger Nrf2-dependent gene expression [81]. Finally, CP-Gs of J-family (only) also activate PPARγ by an addition reaction at Cys285 thus modifying lipid metabolism [17,77].

Although still under debate, there is evidence for a pro-atherogenic role for PPARγ activation, since it transcribes the scavenger receptor CD36/FAT [17,19], which may overcome any possible beneficial effects of PPARγ activation. On the other hand, studies suggest that PPARα and γ activation decreases atherosclerosis progression not only by correcting metabolic disorders, but also through direct effects on the vascular wall [19]. Even though PPARγ activation enhances the expression of CD36/FAT, the ApoE gene (that encodes cholesterol efflux transporters) is upregulated by PPARγ agonists in macrophages. PPARs may also activate the transcription of ABCG1 (that allows for the efflux of cholesterol to HDL particles) and ABCA1 (that facilitates the efflux of cholesterol to ApoA1 acceptors) in macrophages in response to atherosclerotic lesions [17]. This can lead, on the one hand, to the inhibition of macrophage transformation into foam cells and can contribute to the anti-atherogenic effect of PPARγ ligands. PPARα and γ specific ligands might also influence intracellular cholesterol distribution as a PPRE has been identified in the promoter of cholesteryl ester hydrolase (CEH), the enzyme responsible for hydrolysis of cholesteryl esters in foam cells generating free cholesterol available for efflux. On the other hand, PPARα-deficiency in the ApoE-null model results in lowered atherosclerosis [19]. In the clinic, PPARγ agonists are effective in treating type 2 diabetic patients, and PPARα agonists provide significant anti-dyslipidemic and anti-atherosclerotic outcomes [82]. While further studies are required to assign a possible role for CP = PGs via PPAR activation in the regulation of intracellular cholesterol distribution, evidence also suggests that PPAR agonists may possess additional beneficial effects that are independent from their ability to activate PPARs.

4.4. *Anti-inflammatory and anti-cholesterogenic effects of CP-PGs: resolution of inflammation and anti-atherogenic properties*

The concept of CP-PGs as physiological molecules for the ''resolution of inflammation'' is now well accepted [4,50,77]. However, what is fascinating about the anti-inflammatory effect of CP-PGs is that, besides inhibiting NF-κB activation and stimulating antioxidant responses, they also activate the heat-shock protein (hsp) biochemical pathway, which is *sine qua non* for their full biological actions, and promotes tissue repair because the expression of the chaperone hsp70 confers thermotolerance and cytoprotection [63]. Moreover, and remarkably, hsp70 binds to IKKγ [83] and to NF-κB/IκB complexes [84] preventing IκBα phosphorylation and further degradation. Hence, the complete CP-PG-mediated anti-inflammatory effect has an additional and obligatory component, namely hsp70-mediated interruption of NF-κB pathways. This has led us to investigate the effect of CP-PGs as well as other non-cyclopentenone ring-possessing PGs on foamy macrophages *in vitro* and *in vivo*, using LDL receptor knockout ($ldlr^{-/-}$) mice in order to evaluate the feasibility of a CP-PG-based therapeutic approach against atherosclerosis. Among the eicosanoids tested, PGA_2 has shown the most promising results. To overcome systemic CP-PG toxicity, particularly the strong antiproliferative effect on the immune system and rapidly proliferating cells [45], negatively charged liposomes were prepared containing PGA_2 and antibodies against VCAM-1 in order to specifically direct liposome particles onto injured arterial wall linings, since VCAM-1 is expressed almost

exclusively by endothelial cells and foamy macrophages of atherosclerotic lesions under stress. In these studies, the potential beneficial effects of J-series CP-PGs were surpassed by their solely PPARγ-activating properties. These preparations gave rise to LipoCardium, a formulation that completely reverses atherosclerotic lesions *in vivo* [3]. LipoCardium is proposed to reach its targets in the arterial wall through its binding to VCAM-1, leading to the downregulation of NF-κB-dependent pro-inflammatory and pro-proliferative genes. LipoCardium-elicits blockage of cholesterol metabolism and hsp70-mediated cytoprotection appears to perform the remaining tasks. Similar results have been obtained with PGJ_2 and 2-cyclopenten-1-one which, locally administered on carotid arteries, cause a dose-dependent inhibition of neointimal formation [85].

Previous results from this laboratory have shown that, in addition to its anti-inflammatory effects, PGA_2 has a potent inhibitory effect on cholesterol metabolism of inflammatory macrophages, whose lipid metabolism resembles that of foamy macrophages [24]. From the studies with LipoCardium, it has been peremptorily confirmed that, in foamy macrophages, PGA_2 (and, in a lesser extent, 15d-PGJ_2) shunts lipogenesis deviating acetyl-CoA units from cholesterogenesis to the synthesis of phospholipids, which are promptly exported from cells, while it decreases the uptake of both cholesterol and cholesteryl esters from the extracellular space and increases the export of cholesterol and cholesteryl esters out of cells (Fig. 5). Interestingly, PGA_2 blocks both cholesterol esterification and hydrolysis of cholesteryl esters. These effects are clearly supplementary and PGA_2-based liposome therapy dramatically reduces cholesterol accumulation in foam cells and atherosclerotic lesions *in vivo* [3]. Hence, at least part of the tetravalent action proposed for this PGA_2-based therapy (namely anti-inflammatory, antiproliferative, anticholesterogenic and cytoprotective) might be due to coordinately PGA_2-mediated inhibition of ACAT, CEH and HMG-CoA reductase activities. It is worth noting that PGA_2 even increases HMG-CoA reductase mRNA expression [3], which suggests that PGA_2-elicited anticholesterogenic effects should involve modulation of HMG-CoA reductase activity or state of activation. Recent results from this laboratory confirms this assumption, since PGA_2 was shown to promptly block the reductase activity while doubling the expression and its latent activity in 24-h treated rat macrophages, in a dithiothreitol-reversible fashion (LLP Gutierrez et al., unpublished observations). In these studies, the phosphorylation state of the reductase appears to be unaffected, which was indeed expected, since CP-PGs inhibit LKB1/AMPKK [86] (see below, please), the enzyme responsible for the AMPK-dependent inactivation of HMG-CoA reductase by phosphorylation (Fig. 1). Thus, CP-PGs (and, particularly, PGAs) emerge as a novel class of non-statin HMG-CoA reductase inhibitors with promise for the treatment of cardiovascular diseases.

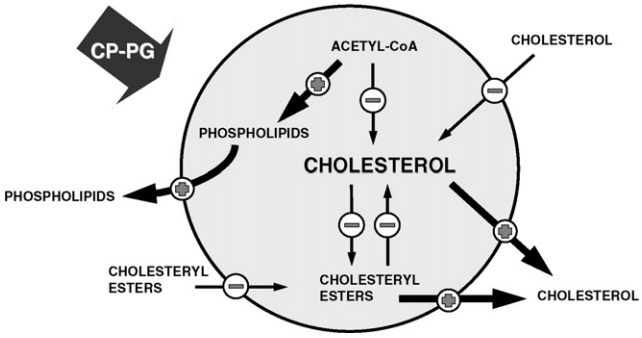

Fig. 5 – CP-PG effects on cholesterol balance. Reported effects of CP-PGs on cholesterogenesis, cholesteryl ester formation and hydrolysis, as well as cholesterol uptake and export in a typical foam cell are schematically represented.

The presence of critical reactive cysteine residues located near or at the catalytic sites of HMG-CoA reductase [87], ACATs [88] and CEHs (neutral and acid forms) [89,90] has therefore shed new light on the mechanism of CP-PG-mediated anti-atherogenic activity, in addition to its powerful anti-NF-κB effects: the modulation of lipid metabolism through the binding to reactive cysteines via Michael's adducts with the molecules of potentially pro-atherogenic key enzymes.

5. Reactive cysteines in atherosclerosis-prone key enzymes of lipid metabolism

5.1. *Redox-sensitive lipogenic pathways*

Curiously, it seems that nature has evolved a singular way to regulate lipid metabolism through reactive cysteine residues in key enzymes and transcription factors that promptly respond to tiny alterations in intracellular redox status. This renders lipid metabolism prone to redox signals. Not fortuitously, the NADPH-generating pathways, catalyzed by glucose-6-phosphate dehydrogenase (G6PDH, in the cytosolic pentose-phosphate shunt) and malic enzyme, are coordinately regulated by both oxidative stress and lipogenic stimuli that drive Nrf2-mediated gene expression (Fig. 6). G6PDH, the principal NADPH flux-generating system, actually works as a ''housekeeping'' enzyme present in all cells but its expression is also transcriptionally and post-transcriptionally regulated by oxidative stress [81]. On the other hand, insulin-dependent lipogenic signals also activate the Keap1/Nrf2/ARE pathway, which increases the expression and activity of NADPH-generating and lipid synthesizing enzymes (outstandingly reviewed in ref. [13]). Therefore, lipogenesis and oxidative stress are two sides of the same coin, in that they always convey information to both reductant-generating and redox-protecting pathways that are brought together by Keap1/Nrf2/ARE routes, as above discussed. Because of this, either the activation of lipogenic pathways or the challenge of redox metabolism trigger, in GSH metabolism, its generation (γ-GCS), regeneration from GSSG (GSRd and G6PDH) and extrusion (GST and GS-X pumps), which, taken as a whole, protect the highly reductive intracellular milieu from the accumulation of electrophiles, ROS/RNS and GSSG. Altogether, these strategies sustain the reducing power needed to accomplish lipid biosynthesis.

Actually, all known mammalian lipogenic pathways are redox-sensitive. Accordingly, coenzyme A (CoA-SH), which is necessary for the activation of virtually all lipid-reacting intermediates (*e.g.*, fatty acids), is a very reactive cysteine-containing thiol. NADPH, which is redox-sensitive and acts

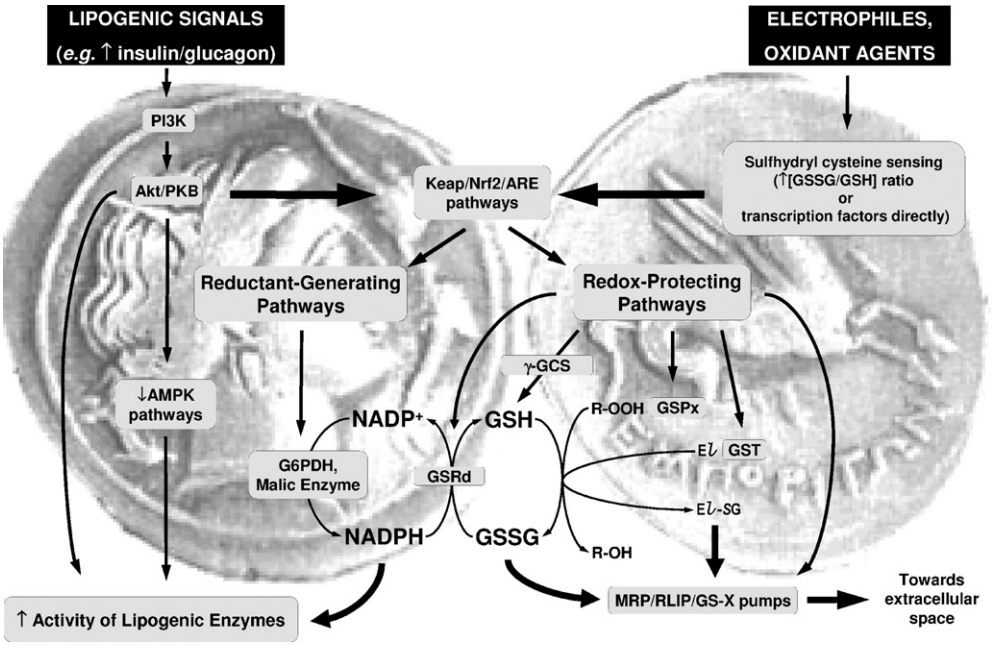

Fig. 6 – Confluence of lipogenic and redox pathways in arterial wall cells: two sides of the same coin. Lipogenic routes utilize intracellular cascades that activate both reductant-generating and redox-protecting pathways that are brought together in the Keap/Nrf2/ARE transcriptional complex. Electrophiles and oxidant agents also trigger the same cascades. Therefore, lipogenesis and redox status, which may be regulated by CP-PGs, are two sides of the same coin, so that CP-PGs may reestablish intracellular lipid balance in chronic redox-depending lipid disequilibria, such as atherosclerosis. El represents electrophilic substances, whereas R-OOH and R-OH mean, respectively, hydroperoxides and their reduced alcohol forms, whose metabolism proceeds via glutathione-peroxidase-catalyzed reactions.

with dehydrogenases (oxidoreductases) as a soluble electron carrier, is required for the synthesis, elongation and desaturation of fatty acids, plasmalogens, sphingolipids, cholesterol (HMG-CoA reductase, squalene synthase, squalene monooxygenase), and cytochrome P450-reductase, desmolase-dependent production of pregnenolone. Fatty acid synthase (FAS, or acyl-carrier protein synthase) has two reactive cysteines that are responsible for the inhibition of the condensation components by strong thiol reagents [91]. PG synthesis, via the concerted action of COX and PG synthases, is redox-sensitive, since COX-1 and COX-2 may be inhibited by high GSH/GSPx contents, but autocatalytically activated by hydroperoxides [46], while GSH-dependent PGE and PGD synthases have also been described. Therefore, it is expected that the electrophilic CP-PGs may somehow interfere in lipid metabolism.

GSSG to GSH ratio ([GSSG]/[GSH]) is considered an index of redox status because all intracellular redox reactions are found in equilibrium with GSSG–GSH redox coupling and the formation of mixed disulfides of GSSG with intracellular sulfhydryls depends on ln [GSSG]/[GSH], which influences the ΔG of these processes. On the other hand, CP-PGs promptly react with the cysteine moiety of GSH, which, *per se*, promotes oxidative stress (*i.e.*, there is a rise in [GSSG]/[GSH]), and immediately triggers the consumption of NADPH to regenerate GSH from GSSG via a reaction catalyzed by GSRd, whose expression is also elevated under these situations [78]. As a consequence, in addition to the other cellular effects that disruptions in GSH metabolism may cause, CP-PG-mediated decrease in GSH contents results in NADPH deficit. This deficit, in turn, endangers the redox-dependent lipid metabolism (Figs. 6 and 7), but may be pharmacologically useful for treating atherosclerosis.

5.2. *Fatty acid synthesis*

Notably, although FAS may theoretically be susceptible of undergoing Michael addition reactions with CP-PG, treatment of non-foam cell macrophages with CP-PGs has no effect on fatty acid synthesis, whereas CP-PGs enhance fatty acid synthesis from acetyl-CoA in oxLDL-elicited foamy macrophages [3,24]. We speculate that this behavior may be due to the direct inhibition of the tumor suppressor LKB1 kinase [86], which has been identified to be identical to AMPK kinase (AMPKK) and calcium-calmodulin kinase–kinase (CaCMKK) [92]. The binding of A- and J-series CP-PGs to Cys210 in LKB1 leads to the formation of Michael's adducts that explain some well-known effects of CP-PGs that are independent of either NF-κB inhibition or PPARγ activation. Adiponectin, which is cardioprotective, and leptin, whose metabolic defects are implicated in obesity and diabetes, increase fat "burning" through the activation of the AMPK pathways [92] (Fig. 1). Hence, physiological inhibition of AMPKK by CP-PGs may have important consequences to metabolic regulation and account for the activation of acetyl-CoA carboxylase, thus enhancing metabolic flux through the FAS.

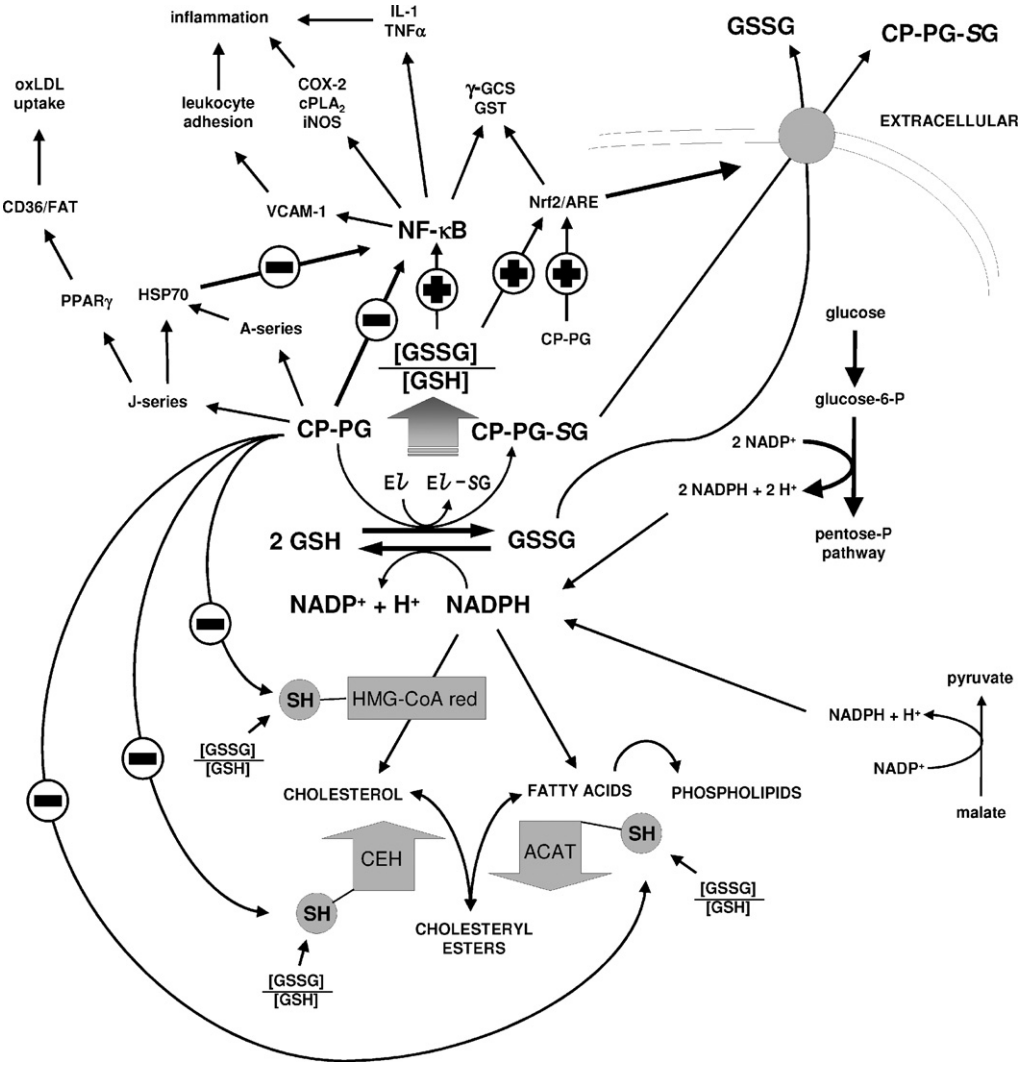

Fig. 7 – Overview of redox-sensitive pathways involved in cholesterol metabolism in atherosclerosis and putative points of regulation by CP-PG. GSH metabolism plays a central role in dictating changes in redox-sensitive pathways that may lead to chronic inflammation and atherogenesis. On the other hand, CP-PGs may simultaneously interfere in GSH metabolism, redox status and the activity of enzymes of lipid metabolism. Additionally, CP-PGs block inflammation and inflammation-derived changes in lipid metabolism, which are metabolically brought together at a reactive-cysteine GSH metabolic-pathway convergence point. The [GSSG]/[GSH] ratio represents intracellular redox status that may either activate NF-κB-dependent pro-inflammatory cascades or repress them by triggering antioxidant and redox-protecting pathways. The importance of CP-PG-mediated induction of hsp70, which is both anti-inflammatory and cytoprotective is also shown. In this regard, the crucial difference between J- and A-series CP-PGs in, respectively, activating or not PPARγ pro-atherogenic pathways is also indicated. El represents electrophilic substances.

5.3. *Cholesterogenesis*

As discussed above, the accompanying inhibitory effect of CP-PGs over HMG-CoA reductase reduces intracellular cholesterol availability so that SREBPs can target their DNA-binding domains at HMG-CoA reductase and FAS promoters [6,12]. These actions may result from the CP-PG-elicited enhancement of fatty acid synthesis previously reported [3,24] concomitantly with the presence of an apparently paradoxical increase of HMG-CoA reductase mRNA expression [3]. HMG-CoA reductase is extremely sensitive to oxidative inhibition by micromolar concentrations of GSSG and presents a thiol/disulfide exchange with GSH and GSSG, depending on the [GSSG]/[GSH] ratio [87]. Also, results from our group as well as several other lines of evidence suggest that the strong CP-PG-inhibiting effect on HMG-CoA reductase may be due to Michael addition reactions at critical cysteine residues of the reductase, similar to that observed for other sulfhydryl reagents [11,87]. Indeed, it is assumed that HMG-CoA reductase activity is GSH-dependent and allosterically modulated by the redox status under physiological conditions [11]. Furthermore, reactive cysteines in the catalytic (Cys266) and allosteric (Cys323) sites of HMG-

CoA synthase are responsible for its redox-sensitivity and inactivation under high [GSSG]/[GSH] environments.

5.4. *Cholesteryl ester synthesis and hydrolysis*

ACAT-catalyzed esterification of cholesterol with fatty acids is also subject to redox regulation. Macrophage-type ACAT1, which is allosterically activated by cholesterol, contains nine critical cysteine residues that bring about extremely high redox sensitivity [88], while Cys467 modification leads to complete ACAT inactivation [93]. Recent work from our laboratory suggests that this is the case in foamy macrophages treated with 1 μM PGA_2 for 24 h (LLP Gutierrez et al., unpublished observations). In support of this proposition is the fact that depletion of GSH leads to the inhibition of cholesteryl ester formation in human macrophages [94]. Therefore, ACAT is also a potential pharmacological target for CP-PG-based anti-atherogenic approaches.

According to the above discussion, CEH activity is involved in lipid accumulation and atherosclerosis [95]. In this regard, there are different enzymes that hydrolyze cholesterol esters. On the basis of their optimal working pH, they are characterized as acid (lysosomal and microsomal) CEH and neutral (cytosolic and hormone-sensitive lipase) CEH. During the development of atherosclerosis, high cholesteryl ester uptake through SRs, which are expressed by stimulated foamy macrophages (mainly) and endothelial cells, contributes to cholesterol accumulation in cytoplasmic lipid droplets. Hence, a deficient expression/activity of lysosomal CEH alone may be atherogenic because the hydrolysis of triacylglycerols and cholesteryl esters stimulates fatty acid utilization (energy production) and cholesterols to be used in cell membrane turnover or export to the extracellular space [96]. This is notable in foamy macrophages, where oxLDL does not readily enter the ACAT substrate pool due to impaired lysosomal degradation of oxidized components of this modified lipoprotein particle [97]. Nevertheless, the simultaneous inhibition of ACAT and CEH may have an opposite (protector) effect, since CP-PGs block both pathways in foamy macrophages [3] avoiding both cholesterol and cholesteryl ester overload. This is because neither cholesterol nor cholesteryl esters may enter this cycle that perpetuates foam cell lipid accumulation.

Human lysosomal acid CEH, which catalyzes the deacylation of triacylglycerols and cholesteryl esters, has six reactive cysteines. Interestingly, specific mutations on Cys227 decrease cholesteryl esterase, but not triacylglycerol lipase activity, while mutations in Cys236, Cys240 and Cys244 variably impair both activities. Nonetheless, the catalytic activity over both substrate types is never completely abrogated, which suggest that although it is redox-sensitive, lysosomal CEH has no cysteine residues at the active site and thus is not considered a thiol enzyme [89]. Mutation in Cys240 of human lysosomal CEH brings about an almost complete reduction in cholesteryl esterase and LDL degrading activities [98]. Microsomal acid CEH is also a specific cholesteryl ester hydrolase that is redox-sensitive [90]. Hormone-sensitive lipase (HSL), which is the major neutral CEH of macrophages [95] and an important factor dictating pathological conditions, such as atherosclerosis, is not a thiol-enzyme, but is regulated by protein kinases (*e.g.*, AMPK and ERK), which are redox-regulated [99]. On the other hand, neutral cytosolic CEH gene has an SRE *cis* element that is regulated by cholesterol, whose metabolism is redox-sensitive.

5.5. *Michael's adducts of CP-PGs with reactive cysteines: impairment of lipid accumulation in atherosclerosis*

Although Michael addition reactions of CP-PGs with reactive protein sulfhydryls have not been reported to occur physiologically at the level of enzymes involved in lipid metabolism, this is a likely possibility as CP-PGs may be produced at physiologically relevant intracellular concentrations. In fact, Shibata et al. [55] observed the formation of considerable amounts of the strong electrophilic CP-PG 15d-PGJ_2 (reaching up to 79 nM in 12-h incubations) by macrophages during inflammatory processes, while 15d-PGJ_2 production has been found to be localized in the cytoplasm of foamy macrophages obtained from human atherosclerotic lesions. Yano et al. [56] recently demonstrated intracellular production of 15d-PGJ_2 in statin-stimulated macrophages that reach *ca.* 100 ng/mL (316 nM), at the same time that statins increase intracellular levels of PGE_2, the precursor of PGA_2, another CP-PG. Combined expression of the arachidonic-acid-releasing enzyme cytosolic phospholipase A_2 ($cPLA_2$) with COX-2 and PGD synthase, which favors intracellular formation of J-series CP-PG, results in the complete inhibition of TNFα-induced IKK activation and IκB phosphorylation and degradation, whereas overexpression of each pair of enzymes (that is, $cPLA_2$/PGD synthase or COX-2/PGD synthase) is insufficient for inhibition. This suggests that levels of CP-PG that inhibit NF-κB activation can be obtained in vivo [5]. Indeed, it is assumed that CP-PGs are endogenous electrophiles that induce many cellular effects through the formation of Michael's adducts with critical protein cysteines [55]. However, taking into account low physiological intracellular CP-PG concentrations, which range from picomolar to nanomolar, it is conceivable that physiologically, CP-PGs may serve as switches that turn on or shut off critical biochemical pathways through the modification of transcription factors and regulatory proteins, processes that require only minute amounts of such eicosanoids. Nevertheless, one cannot exclude the possibility that under conditions of high-throughput CP-PG production (*e.g.*, in inflammatory sites), CP-PGs may be taken up by cells that accumulate high intracellular concentrations, since the active transport system that selectively imports CP-PGs from the extracellular space is able to concentrate CP-PGs more than 20-fold as compared to the extracellular concentration [57]. CP-PG-based pharmacological studies may have an enormous impact on human atherosclerosis progression, as proved in the mouse model [3]. This may be at least partially due to the fact that HMG-CoA reductase, ACATs, CEHs, and many other redox-sensitive lipogenic pathways are potential intracellular targets for Michael addition reactions directed to reactive cysteines located at critical sites.

6. Concluding remarks

CP-PG-mediated cell signaling by cysteine modification is well documented [65,71,77,86]. What is novel, however, is the

possible role of eicosanoids for the regulation of lipid metabolism by acting as cysteine reactants, particularly their potential usage as HMG-CoA reductase inhibitors and anti-atherosclerotic agents. Results from this laboratory and those from other research groups have unraveled a novel facet in prostaglandin research in which CP-PGs physiologically acts as a redox signal that guides lipid metabolism in atherosclerosis. At pharmacological levels (micromolar range), CP-PG-based LipoCardium *in vivo* effects corroborate this assumption. Of note, PGA_2 shows an impressive anti-atherosclerosis potential, which seems to be a summation of different interconnected effects (Fig. 7). Firstly, its electrophilicity over reactive cysteines present in lipid metabolic enzymes, such as HMG-CoA reductase, ACAT and CEH, brings about a marked reduction of intracellular cholesterol and cholesteryl ester contents. These actions are accompanied by a rise in the activity of acetyl-CoA carboxylase (probably due to CP-PG-mediated impairment of LKB1/AMPKK activity; Fig. 1) as well as the enhancement of metabolic fluctuations through FAS, which is likely to occur because the CP-PG-elicited decrease in cholesterol content triggers SREBP-dependent fatty acid synthesis in the presence of low AMPK activity. This elevation in phospholipid synthesis [3,24] may also be cytoprotective, since stimulated macrophages have intense cholesterol metabolism, and are capable of entirely recycling their membranes once every half an hour. Secondly, PGA_2 has profound effects on redox status that by turning on Keap1/Nrf2 cascades, culminates in the activation of both redox-generating and redox-protecting pathways (Fig. 6); these events are also cytoprotective and anti-atherogenic. It is remarkable that the CP-PG-mediated rise in MRP/GS-X pump ATPase activity functions as a GSSG extruding system, which, in turn, relieves intracellular oxidative stress of foamy macrophages by dislocating GSH ↔ GSSG equilibrium to the left (Figs. 6 and 7). The transient redox imbalance imposed by CP-PGs may also increase GSSG extrusion through the RLIP76/GS-X pump. Actually, PGA_2 thoroughly upregulates GSH metabolism, which is cytoprotective, while PGA_2-mediated increases in GSRd avoid overloading pentose-phosphate and malic enzyme pathways and preserve the energetic reserves of macrophages. Finally, PGA_2's ability to block NF-κB activation *per se* and PGA_2-dependent triggering of the anti-inflammatory hsp70 pathway physiologically resolves inflammation, in a PPARγ-independent way (Fig. 7).

Although CP-PGs of J-series may provide useful pharmacological tools against atherosclerosis [85], A-family CP-PGs seem to have an overall higher beneficial effect as gauged by the disequilibria found in lipid metabolism and cellular homeostasis that accompany cardiovascular diseases. While such a different behavior might be ascribed to the fact that PGAs lack any PPAR-γ-activating capacity, evidence suggests that both CP-PG types may act intracellularly as physiological protectors against chronic inflammation, not only defending cells via hsp70 induction, but also by sparing GSH and deviating lipid metabolism in a favorable fashion. Hence, considering that chronic inflammatory diseases, such as atherosclerosis, may originate from a failure in the resolution of inflammation [4], a better understanding of why CP-PGs apparently do not cope with the resolution of inflammation physiologically may explain many unsolved questions and yield novel insights into atherogenesis and its treatment.

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