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Forum Review

The Cyclopentenone (A_2/J_2) Isoprostanes—Unique, Highly Reactive Products of Arachidonate Peroxidation

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ABSTRACT

Cyclopentenone (A_2/J_2) isoprostanes (IsoPs) are a group of prostaglandin (PG)-like compounds generated *in vivo* from the free radical-induced peroxidation of arachidonic acid. Unlike other classes of IsoPs, cyclopentenone IsoPs contain highly reactive unsaturated carbonyl moieties on the prostane ring analogous to cyclooxygenase-derived PGA_2 and PGJ_2 that readily adduct relevant biomolecules such as thiols via Michael addition. The purpose of this review is to summarize our knowledge of the A_2/J_2-IsoPs. As a starting point, we will briefly discuss the formation and biological properties of PGA_2 and PGJ_2. Next, we will review studies definitively showing that cyclopentenone IsoPs are formed in large amounts *in vivo*. This is in marked contrast to cyclopentenone PGs, for which little evidence exists that they are endogenously produced. Subsequently, we will discuss studies related to the chemical syntheses of the 15-A_2-IsoP series of cyclopentenone IsoPs. The successful synthesis of these compounds provides the recent impetus to explore the metabolism and biological properties of A-ring IsoPs, particularly as modulators of inflammation, and this work will be discussed. Finally, the formation of cyclopentenone IsoP-like compounds from other fatty acids such as linolenic acid and docosahexaenoic acid will be detailed. *Antioxid. Redox Signal.* 7, 210–220.

BACKGROUND

OXIDATIVE STRESS has been implicated in a number of human diseases, including atherosclerosis, cancer, neurodegenerative disorders, and even the normal aging process (2, 26, 28, 63). Damage to tissue biomolecules, including lipids, proteins, and DNA, is believed to play a key role in the pathophysiology of these diseases. Lipid peroxidation is a central feature of oxidant stress. Unsaturated lipids, such as arachidonic acid (AA), are particularly susceptible to oxidative modification because they contain readily extractable bisallylic hydrogens.

AA is oxidized via several important pathways *in vivo* resulting in the formation of a number of biologically active products. AA can be peroxidized enzymatically either by lipoxygenases (LOXs), to yield hydroperoxyeicosatetraenoic acids, or by the cyclooxygenases (COXs), COX-1 and COX-2, to generate the unstable intermediate prostaglandin (PG) H_2. PGH_2 can then be metabolized by various enzymes to generate the parent PGs which include $PGF_{2\alpha}$, PGD_2, PGE_2, PGI_2 (prostacyclin), and thromboxane (Tx) A_2 (Fig. 1). Additionally, PGD_2 and PGE_2 can dehydrate to yield the cyclopentenone PGs PGJ_2 and PGA_2, respectively. This dehydration has been definitively shown to occur *in vitro* and may occur *in vivo* (7).

AA can also be oxidized *in vivo* independent of LOX or COX enzymes via free radical-induced mechanisms. This route of oxidation leads to the formation of a family of PG-like compounds termed isoprostanes (IsoPs). The mechanism by which IsoPs form has been reviewed and is shown in Fig. 2 (18). A key structural difference between IsoPs and COX-derived PGs is that the side chains of the IsoPs are predominantly oriented *cis* in relation to the prostane ring, whereas the side chains of PGs are oriented *trans*. IsoPs containing F-type prostane rings (F_2-IsoPs), E/D-prostane rings (E_2/D_2-IsoPs), and A/J-prostane rings (A_2/J_2-IsoPs), as well as Tx-like compounds (IsoTxs), are formed from the free radical-catalyzed oxidation of AA. The structures of these molecules have been extensively characterized, and highly robust mass

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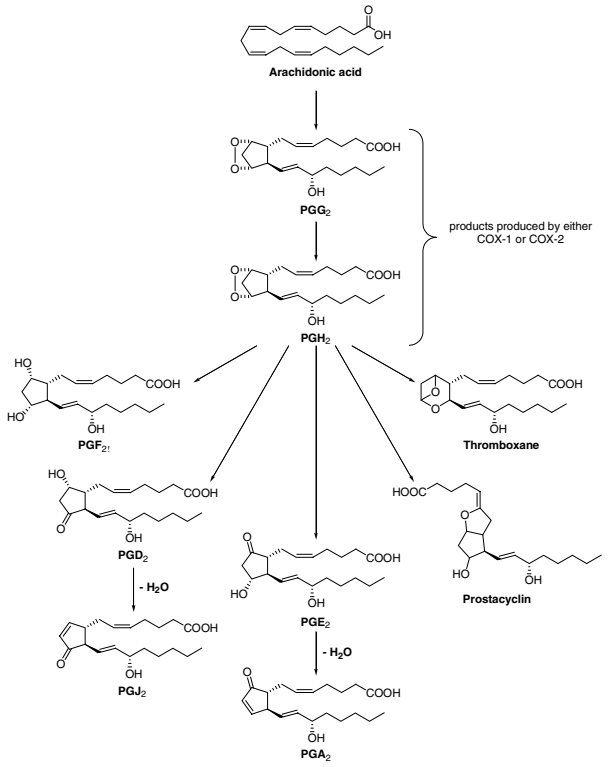

FIG. 1. Products of the oxidation of AA by the COX enzymes. The COXs convert AA to PGH_2, which is then metabolized to the PGs and Tx. PGE_2 and PGD_2 can subsequently dehydrate to PGA_2 and PGJ_2, respectively.

spectrometric (MS) techniques have been developed to measure them in biological samples. IsoPs are now widely accepted as accurate and sensitive markers of oxidant injury in human diseases and in animal models of oxidant stress.

Unlike other classes of IsoPs, the A_2/J_2-IsoPs contain unsaturated carbonyl moieties that render them highly reactive and capable of adducting relevant biomolecules such as thiols via Michael addition. This property likely contributes importantly to their biological activities and provides a basis for understanding their metabolism *in vitro* and *in vivo*. It is the purpose herein to summarize our current knowledge of the A_2/J_2-IsoPs. This review will (a) briefly discuss the cyclopentenone PGs and the controversy surrounding their generation *in vivo*, (b) summarize the discovery and characterization of the cyclopentenone IsoPs *in vitro* and *in vivo*, (c) highlight the formation of cyclopentenone eicosanoids from lipid substrates other than AA, and (d) discuss the metabolism and bioactivity of A_2-IsoPs.

CYCLOPENTENONE PROSTAGLANDINS

The cyclopentenone PGs have recently been extensively reviewed by Straus and Glass (64). Herein, these molecules will only be briefly discussed in order to put into perspective our current understanding of cyclopentenone IsoPs.

PGA_2 was the first cyclopentenone PG discovered in the mid-1960s by Hamberg and Samuelsson (27). They reported that PGA_2 was formed *in vitro* from PGE_2 in human seminal plasma after treatment with sodium hydroxide. It was not, however, until 15 years later that Hayaishi and colleagues discovered PGJ_2 in the course of studying the antitumor activity of PGD_2 (23). They showed that PGJ_2 is formed in aqueous solution from the spontaneous dehydration of PGD_2, and they and others went on to determine that PGJ_2 can isomerize *in vitro* in the presence of albumin to Δ^{12}-PGJ_2 and then undergo dehydration to yield 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15-dPGJ_2) (Fig. 3) (20, 22).

As a class, cyclopentenone PGs are characterized by the presence of an unsaturated carbonyl group on the prostane ring. This moiety is highly electrophilic, which renders cyclopentenone PGs susceptible to Michael addition with nucleophilic biomolecules. 15-dPGJ_2 is particularly reactive because it contains two unsaturated carbonyl groups. Cyclopentenone PGs have been shown to conjugate readily with the

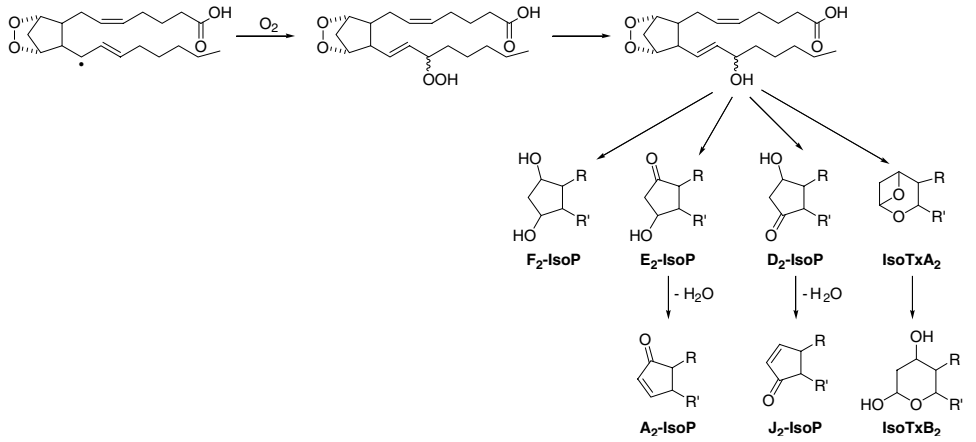

FIG. 2. Mechanism of IsoP formation from the free radical-initiated peroxidation of AA. Similar to the generation of PGs, endoperoxides are formed that are then metabolized to the parent IsoPs. E_2/D_2-IsoPs can subsequently dehydrate to A_2/J_2-IsoPs. For simplicity, stereochemistry is not indicated.

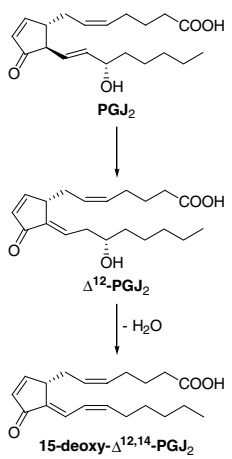

FIG. 3. Conversion of PGJ_2 to Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15-$dPGJ_2$).

cysteine residue of glutathione (GSH) and with cysteines in cellular proteins (3, 4, 9, 32, 53). Reaction with various cellular biomolecules is the primary path by which cyclopentenone PGs are thought to exert their biological activity (11, 44, 50, 61). Further, conjugation of cyclopentenone PGs with GSH has been shown to render them biologically inactive (32).

Biological activity of cyclopentenone PGs

The cyclopentenone PGs, and particularly 15-$dPGJ_2$, elicit a number of biological responses *in vitro*. Much of the work on the biological activity of these compounds has focused on their role in modulating inflammation and tumorigenesis, although the data are frequently conflicting. 15-$dPGJ_2$ has been shown to exert antiinflammatory properties both by acting as a ligand for the peroxisome proliferator-activated receptor-γ (PPARγ) and by attenuating the formation of cytokines, adhesion molecules, and other proinflammatory proteins through PPARγ-independent pathways, such as modulation of nuclear factor-κB (NF-κB)-signaling mechanisms (16, 24, 64, 65). However, in other reports, 15-$dPGJ_2$ enhances the formation of the proinflammatory chemokine interleukin (IL)-8 (21, 29, 39, 70). It has also been shown that when various cell lines are exposed to low micromolar concentrations of cyclopentenone PGs, proinflammatory cytokine expression is potentiated (8). Equally conflicting is literature on the role of 15-$dPGJ_2$ in tumorgenesis. 15-$dPGJ_2$ has been shown to be antiangiogenic and to prevent the proliferation of tumors (67). Conversely, it has also been reported that cyclopentenone PGs promote tumor proliferation by inducing, among other molecules, vascular endothelial growth factor (VEGF) (6, 14, 30, 44), which has been identified as a central mediator of tumor angiogenesis. Further, these compounds can activate the protein H-Ras (50), which is a critical component of signal transduction mechanisms that modulate cell proliferation and differentiation. Thus, it is apparent that cyclopentenone PGs possess a variety of conflicting biological activities that vary among cell lines. As a consequence, the physiological roles of cyclopentenone PGs *in vivo* remain unknown.

Formation of cyclopentenone PGs in vivo

Equally unclear is the extent to which cyclopentenone PGs are formed in humans. This subject has been extensively debated for the past three decades (5, 31, 38, 46) and many investigators have concluded that these compounds are not formed *in vivo*. Recently, however, a study by FitzGerald and co-workers, using a highly sensitive and selective liquid chromatographic (LC)/MS assay, reported that 15-$dPGJ_2$ is produced *in vivo,* but only in very small amounts (~5 p*M*) (7). Importantly, the compound is not present *in vivo* in quantities sufficient to effect the biological activities it has been shown to produce *in vitro*. Interestingly, in this report, unconjugated 15-$dPGJ_2$ was quantified. However, we have previously shown that in cell cultures PGJ_2, as well as the cyclopentenone IsoP 15-A_{2t}-IsoP, readily conjugates with GSH (15, 47). These results suggest that conjugation of cyclopentenone eicosanoids with GSH might be a major route of metabolism of these compounds *in vivo* and be one reason why the cyclopentenone PGs have been difficult to detect unconjugated in biological fluids (see below).

CYCLOPENTENONE ISOPROSTANES

Identification and characterization

Analogous to the dehydration of PGE_2 and PGD_2 to form PGA_2 and PGJ_2, respectively, we have recently found that E_2/D_2-IsoPs readily dehydrate to form cyclopentenone IsoPs with structures analogous to PGA_2 and PGJ_2, respectively (Fig. 2). Initially, we determined that these compounds can be generated in large amounts from the peroxidation of AA *in vitro*. Gas chromatographic (GC)/MS analysis of the oxidation mixture revealed a series of compounds with the predicted molecular weight for A_2/J_2-IsoPs and which had similar retention times to a PGA_2 standard (12). A representative GC/MS chromatogram of putative A_2/J_2-IsoPs and a [$^2H_4$]-PGA_2 standard is depicted in Fig. 4. On the upper *m/z* 434 chromatogram are a group of peaks representing different A_2/J_2-IsoP stereoisomers, whereas on the lower *m/z* 438 chromatogram is the [$^2H_4$]-PGA_2 standard denoted by two methyloxime isomer peaks. Further characterization using various chemical derivatizations confirmed that the compounds in the *m/z* 434 chromatogram contained three double bonds, one carbonyl group, and one hydroxyl group as would be predicted for A_2/J_2-IsoPs. Additionally, LC/MS analysis utilizing collision-induced dissociation (CID) revealed that these compounds fragmented in a manner consistent with them being A_2/J_2-IsoPs. The quantity of cyclopentenone IsoPs formed during the *in vitro* oxidation of arachidonate was 529 ± 135 ng/mg of AA (mean ± SE, $n = 4$) (12).

Subsequently, cyclopentenone IsoPs were also shown to be formed *in vivo* esterified in liver phospholipids from normal and carbon tetrachloride (CCl_4)-treated rats (12, 13). MS analysis of compounds revealed a pattern of chromatographic peaks virtually identical to that shown in Fig. 4. Administration of CCl_4 to rodents induces an intense oxidant stress *in vivo.* Compared with D_2/E_2-IsoPs, A_2/J_2-IsoPs were present at approximately one-half to one-third the level in liver tissue, indicating that the extent to which D_2/E_2-IsoPs undergo dehy-

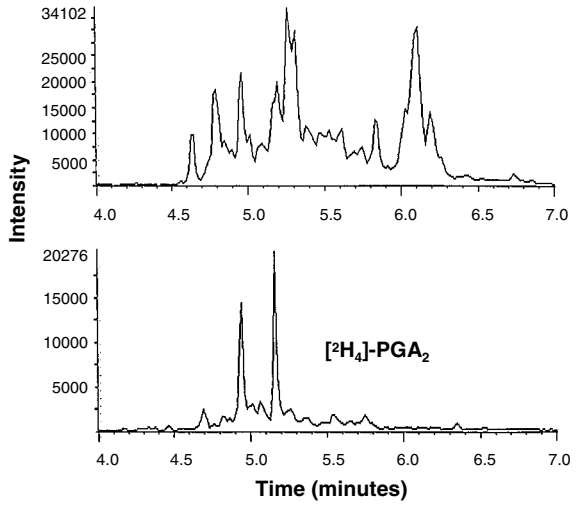

FIG. 4. GC/MS analysis of putative A_2/J_2-IsoPs generated *in vitro* from the peroxidation of arachidonate as pentafluorobenzyl ester, *O*-methyloxime, trimethylsilyl ether derivatives. On the upper *m/z* 434 chromatogram are a series of peaks representing various A_2/J_2-IsoPs. On the lower *m/z* 438 chromatogram is a $[^2H_4]$-PGA_2 internal standard represented by two methyloxime isomer peaks.

dration *in vivo* is significant. Quantities of A_2/J_2-IsoPs in livers from untreated rats are ~5 ng/g of liver. Following administration of CCl_4, levels of both A_2/J_2-IsoPs and D_2/E_2-IsoPs increased dramatically and to a similar extent by a mean of 23.9- and 21.2-fold, respectively.

Subsequently, we undertook additional studies that showed that one cyclopentenone IsoP, 15-A_{2t}-IsoP, is one of the major A_2-IsoPs formed *in vivo* (13). These latter experiments are particularly important because the formation of 15-A_{2t}-IsoP *in vivo* provides a rationale to explore its metabolism and biological properties. In summary, studies carried out over the past several years have definitively shown that cyclopentenone IsoPs are generated in abundance *in vivo*. These findings stand in marked contrast to those that question the extent to which cyclopentenone PGs are generated *in vivo* and suggest that work focusing on the biological properties of 15-A_{2t}-IsoP and other cyclopentenone IsoPs is likely of more relevance than is examining the bioactivity of cyclopentenone PGs.

Synthesis of cyclopentenone IsoPs

Because 15-A_{2t}-IsoP is produced in abundance *in vivo*, exploring its metabolism and biological properties is of obvious importance in order to determine its role as a mediator of oxidant stress. We previously reported that we have been able to synthesize 15-A_{2t}-IsoP from 15-E_{2t}-IsoP either by dehydration in the presence of hydrochloric acid (12, 13) or via two sequential lipase reactions (47), but yields of 15-A_{2t}-IsoP are very small, in the 1–5% range. The primary reason for the relatively low yields is the instability of the product. Under neutral, and particularly acidic, conditions required for the synthesis of 15-A_{2t}-IsoP using these approaches, the *cis*-configuration of the side chains of 15-A_{2t}-IsoP isomerizes to the thermodynamically favored *trans*-configuration, thus yielding PGA_2 as one of the major dehydration products of 15-E_{2t}-IsoP.

Within the past 2 years, Zanoni and co-workers have reported the complete chemical synthesis of a natural mixture of either A_2-IsoPs or J_2-IsoPs (68, 69). Isomerization to A- or J-ring PGs does not occur because the syntheses do not involve prolonged exposure of cyclopentenone IsoPs to protic or acidic conditions. The first compounds synthesized were a natural mixture of 15-A_2-IsoPs that includes 15-A_{2t}-IsoP, 15-*epi*-A_{2t}-IsoP, 15-A_{2c}-IsoP, and 15-*epi*-A_{2c}-IsoP (Fig. 5) (68). The authors were able to synthesize these compounds in milligram quantities as an equal mixture of epimers using a stereoselective Julia–Lythgoe olefination that preserved the *cis* stereochemistry of the side chains. For subsequent studies related to the metabolism and biology of specific A_2-IsoPs such as 15-A_{2t}-IsoP, we have separated the different isomers using chiral HPLC (60).

Zanoni and co-workers have also reported the synthesis of the natural mixture of 15-J_2-IsoPs using the same route he employed for A-ring IsoPs. A 1,3-allylic transposition toward the end of the synthesis allowed the A-type prostane ring to be converted to a J-ring (69). Thus, milligram quantities of the natural mixture of 15-J_2-IsoPs can be readily synthesized. Having methods by which significant quantities of cyclopentenone IsoPs can be generated will thus allow for a number of studies to explore their biological effects.

Reactivity and metabolism of cyclopentenone IsoPs

Despite the fact that we can readily identify cyclopentenone IsoPs esterified in phospholipids from livers of rats, nonesterified A_2/J_2-IsoPs cannot be detected in body fluids such as plasma or urine even following administration of CCl_4 to animals. This finding is in marked contrast to our being able to detect significant concentrations of free F_2-IsoPs and D_2/E_2-IsoPs in rat plasma after CCl_4 treatment. We hypothesized that our inability to detect free cyclopentenone

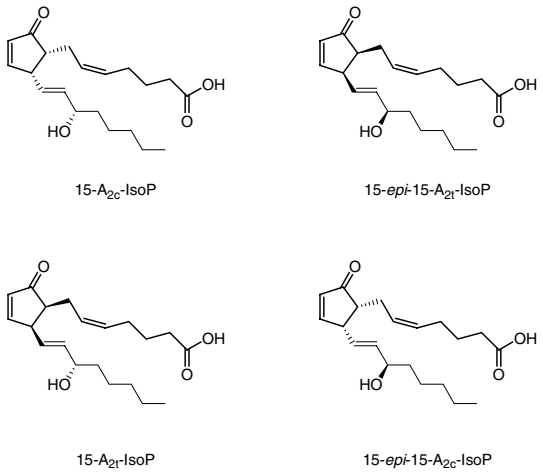

FIG. 5. Compounds comprising the mixture of four different 15-A_2-IsoPs synthesized by Zanoni *et al.* (68).

IsoPs might be due to the marked reactivity of these compounds and their adduction to various biomolecules via Michael addition. Like cyclopentenone PGs, cyclopentenone IsoP reactivity is dependent on the unsaturated carbonyl moiety in the prostane ring of these compounds. As noted, this functional group renders cyclopentenone IsoPs electrophilic and thus susceptible to nucleophilic addition reactions with biomolecules containing thiols such as GSH or cysteine residues in cellular proteins.

To date, most studies have focused on the interaction of cyclopentenone IsoPs, in particular 15-A_{2t}-IsoP, with GSH, but there are also data showing that 15-A_{2t}-IsoP readily conjugates cellular proteins. For example, we found that 15-A_{2t}-IsoP adducts albumin *in vitro* and that after 1 h almost 50% of the IsoP was conjugated with albumin. More recently, Levonen and co-workers have shown that 15-A_{2t}-IsoP conjugates the cysteine-rich protein Keap1 and activates important cytoprotective antioxidant response elements in the cell. The authors propose that cells use electrophilic lipids such as cyclopentenone IsoPs to sense oxidative stress (44).

As noted, the vast majority of research exploring the reactivity of cyclopentenone IsoPs has focused on the ability of these compounds to form adducts with GSH. In our initial report of cyclopentenone IsoPs, we showed that 15-A_{2t}-IsoP rapidly conjugated GSH *in vitro* in the presence of a mixture of bovine liver glutathione transferases (GSTs) (12). Approximately 70% of the 15-A_{2t}-IsoP adducted GSH within 2 min, and the conjugation was complete after 8 min. In further experiments that examined the ability of various human and animal GSTs to conjugate 15-A_{2t}-IsoP with GSH, we found that both the human and the rat GST A4–4 exhibited high catalytic efficiencies for the reaction (34). This finding is of considerable interest because GST A4–4 is ubiquitously expressed in animals and humans and has been shown to possess prominent activity toward other lipids containing unsaturated carbonyl groups, such as 4-hydroxynonenal (33). Together these findings support the contention that 15-A_{2t}-IsoP rapidly adducts thiol-containing molecules, and lend credence to our hypothesis that free cyclopentenone IsoPs cannot be detected *in vivo* because of their marked proclivity to undergo Michael addition.

To explore further the notion that cyclopentenone IsoPs readily conjugate molecules such as GSH *in vivo*, a tracer quantity of radiolabeled 15-A_{2t}-IsoP was infused into a normal human volunteer and the urinary excretion of radiolabel was determined (12). Approximately 95% of the radioactivity recovered in the urine was excreted during the first 4 h following the infusion, representing ~30% of the total amount of radioactivity infused. In order to characterize the product, the urine was first extracted with methylene chloride. Free unconjugated 15-A_{2t}-IsoP would be predicted to extract into methylene chloride, whereas polar (*i.e.*, GSH) conjugates would remain in aqueous solution. Therefore, to determine the percentage of 15-A_{2t}-IsoP excreted as a polar conjugate(s) in urine, the quantity of radioactivity that did not extract into methylene chloride was measured. Only 5% of the radioactivity present in urine was recovered in the organic extract, suggesting that all, or almost all, of the compounds were present in the form of a polar conjugate(s). The characteristics of the extraction did not change with treatment of the urine with either glucuronidase or sulfatase, suggesting that the metabolite was not a glucuronide or sulfate conjugate. On the other hand, GSH or metabolized GSH conjugates are base-labile. Thus, an aliquot of urine was treated with potassium hydroxide prior to methylene chloride extraction. Interestingly, after extraction, ~90% of the material was present in the organic layer, suggesting that 15-A_{2t}-IsoP was excreted as a GSH or metabolized GSH conjugate. It has been shown that various carbonyl-containing compounds are frequently excreted in urine from animals and humans as mercapturic acid conjugates (1, 17). Ongoing studies are aimed at further identifying this urinary metabolite(s) of 15-A_{2t}-IsoP *in vivo*.

Cellular metabolism of cyclopentenone IsoPs

To complement the *in vitro* and human metabolism studies of 15-A_{2t}-IsoP, we have also examined the metabolic fate of this cyclopentenone IsoP in the human hepatoma cell line HepG2 (47). These cells contain a number of GSTs and have been found to efficiently conjugate cyclopentenone PGs with GSH (4). We have determined that ~60% of 15-A_{2t}-IsoP added to HepG2 cells in culture is in the form of a water-soluble conjugate(s) within 6 h of incubation. Structural characterization of this conjugate(s) by LC/MS/MS revealed four major products depending on the duration of the incubation (Fig. 6). After 4 h, the major metabolites of 15-A_{2t}-IsoP are the GSH conjugate and the GSH conjugate in which the carbonyl at C-9 of 15-A_{2t}-IsoP is reduced. After 10 h, the GSH conjugates are no longer the major metabolites; a 15-A_{2t}-IsoP-cysteine conjugate and a cysteine conjugate in which the carbonyl at C-9 of 15-A_{2t}-IsoP is reduced are most abundant.

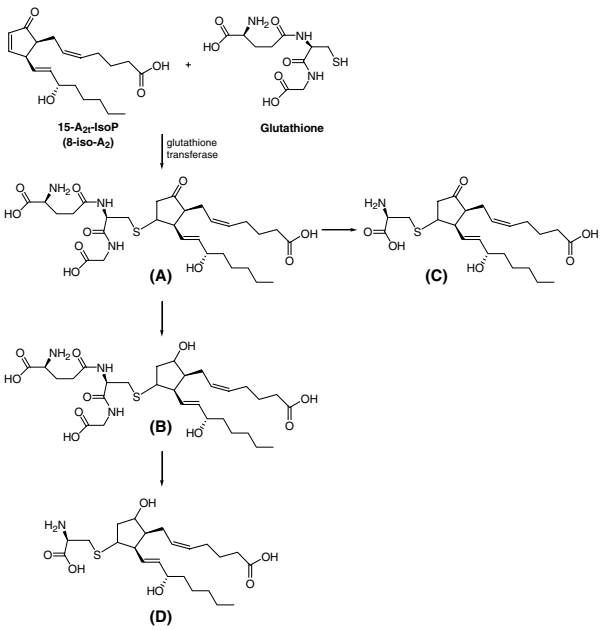

FIG. 6. Conjugation of 15-A_{2t}-IsoP with GSH in HepG2 cells. Two major products are detected at early time points: (**A**) the 15-A_{2t}-IsoP-GSH conjugate and (**B**) the 15-A_{2t}-IsoP-GSH conjugate in which the carbonyl at C-9 is reduced. At later time points, the major metabolites are (**C**) the 15-A_{2t}-IsoP-cysteine conjugate and (**D**) the 15-A_{2t}-IsoP-cysteine conjugate in which the carbonyl at C-9 is reduced.

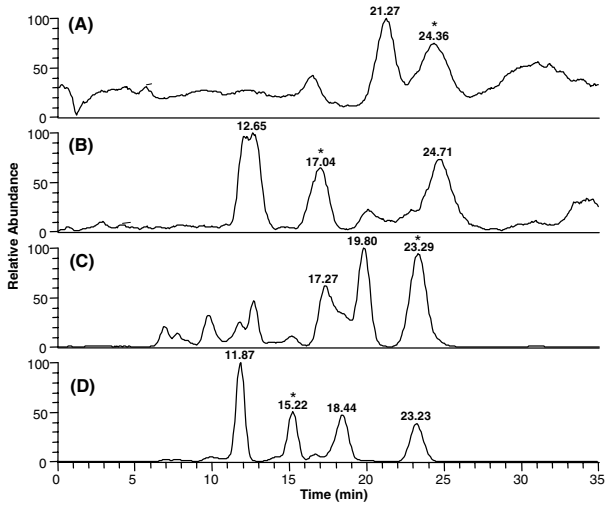

FIG. 7. Selective reaction monitoring analysis of the extract from an incubation of 15-A_2-IsoPs with HepG2 cells. The peaks denoted by asterisks (*) represent the 15-A_{2t}-IsoP conjugates and the peaks denoted by various other retention times are similar conjugates of other 15-A_2-isoP isomers. (**A**) 15-A_2-IsoP-GSH conjugate (parent ion = *m/z* 642) after a 4-h incubation. (**B**) 15-A_2-IsoP-GSH conjugate where the C-9 carbonyl is reduced (parent ion = *m/z* 644) after a 4-h incubation. (**C**) 15-A_2-IsoP-cysteine conjugate (parent ion = *m/z* 456) after a 10-h incubation. (**D**) 15-A_2-IsoP-cysteine conjugate where the C-9 carbonyl is reduced (parent ion = *m/z* 456) after a 10-h incubation.

Thus, these studies further support the contention that conjugation with GSH represents a major route of metabolic disposition of 15-A_{2t}-IsoP.

In the experiments summarized above, the metabolism of a single A_2-IsoP isomer, 15-A_{2t}-IsoP, was examined. However, a mixture of four 15-A_2-IsoP isomers is generated from arachidonate peroxidation *in vitro* and *in vivo* because the formation of these compounds is nonenzymatic. As discussed above, this natural isomeric mixture was synthesized by Zanoni and co-workers (68), and we sought to determine whether the metabolism of the isomers other than 15-A_{2t}-IsoP is similar to that of 15-A_{2t}-IsoP in HepG2 cells. Herein, we report, for the first time, that other 15-A_2-IsoP isomers, in addition to 15-A_{2t}-IsoP, are conjugated with GSH in HepG2 cells. As with 15-A_{2t}-IsoP, the major metabolites of the four 15-A_2-IsoP isomers at 4 h were GSH conjugates and GSH conjugates in which the C-9 carbonyl on the prostane ring was reduced (Fig. 7A and B), whereas the major metabolites at 10 h were the corresponding cysteine conjugates (Fig. 7C and D). These conjugates were characterized by MS as described for 15-A_{2t}-IsoP, and their identification was confirmed using various chemical derivatizations and CID (47). In Fig. 7, the starred peak in each chromatogram represents the metabolite of 15-A_{2t}-IsoP, whereas the other peaks denoted at various elution times represent corresponding metabolites of other 15-A_2-IsoPs.

Bioactivity of cyclopentenone IsoPs

As noted, the cyclopentenone PGs possess potent biological actions, largely ascribed to their chemical reactivity. Importantly, cyclopentenone PGs have been shown to possess significant antiinflammatory properties associated with their respective abilities to suppress NF-κB transcriptional activity, at least in part (57, 65). Cyclopentenone PGs have been reported to decrease the production of numerous proinflammatory mediators, including nitric oxide, PGs, and cytokines (41), in many cell types, including activated macrophages (42), microglia and astrocytes (54), and epithelial cells (59). The antiinflammatory properties of cyclopentenone PGs have been attributed to several mechanisms, including direct ad-

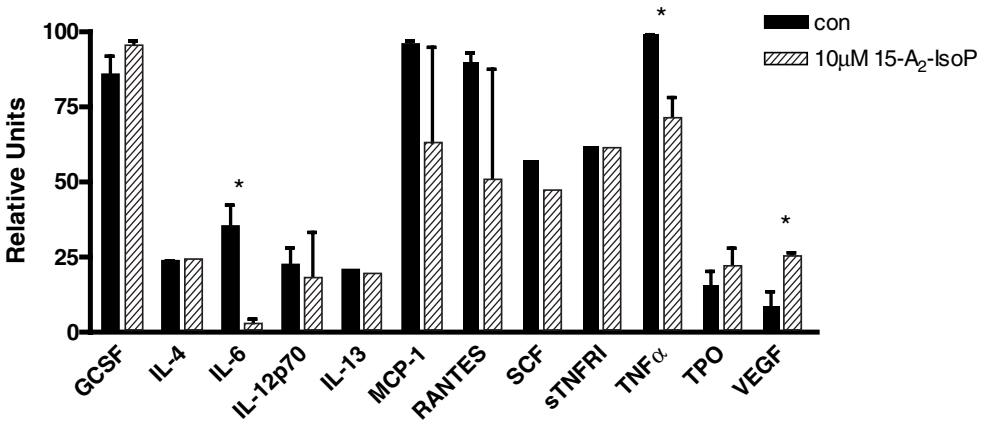

FIG. 8. 15-A_2-IsoPs modulate cytokine production in stimulated macrophages. Conditioned medium from RAW 264.7 macrophages treated with 100 ng/ml LPS plus vehicle or 10 μ*M* 15-A_2-IsoPs was assayed for cytokine protein levels by cytokine array. Of the cytokines analyzed, the three denoted by asterisks (*) were most significantly affected by 15-A_2-IsoP treatment. Modulation indicates change in protein level in 15-A_2-IsoP-treated samples versus control. GCSF, granulocyte-colony stimulating factor; RANTES, regulated upon activation, normal T-cell expressed, and presumably secreted; SCF, stem cell factor; sTNFR1, soluble tumor necrosis factor receptor 1; TPO, thrombopoietin.

duction of IκB kinase (58), perturbation of NF-κB DNA binding ability (65), and induction of the antiinflammatory proteins heat shock protein 70 (35) and heme oxygenase-1 (42). In addition, 15-dPGJ$_2$ is a ligand for the PPARγ receptor. Due to the structural similarity of 15-A$_2$-IsoP isomers to this molecule, we hypothesized that 15-A$_2$-IsoPs might also demonstrate antiinflammatory effects. We tested this hypothesis here for the first time in an activated macrophage cell line. RAW 264.7 murine macrophages were preincubated in serum-free medium with either ethanol vehicle or 10 μ*M* of the synthetic mixture of four 15-A$_2$-IsoP isomers for 30 min, then stimulated with 100 ng/ml lipopolysaccharide (LPS) in the presence of the 15-A$_2$-IsoPs or vehicle for 8 h. Conditioned medium was then collected, and the levels of 12 different cytokines were measured in this medium using a mouse cytokine array (RayBiotech, Norcross, GA, U.S.A.). Of the 12 cytokines monitored, three showed substantial modulation by 15-A$_2$-IsoPs (Fig. 8). Treatment with 15-A$_2$-IsoPs suppressed IL-6 production by 93%, and reduced tumor necrosis factor-α (TNFα) levels by 27%. These findings are in keeping with an antiinflammatory effect for 15-A$_2$-IsoPs, as both IL-6 and TNFα play crucial proinflammatory roles. Interestingly, 15-A$_2$-IsoP treatment also caused a 3.3-fold increase in macrophage VEGF production, as previously described for 15-dPGJ$_2$ (30). VEGF, a key proangiogenic growth factor, not only plays a role in vasculogenesis associated with inflammation and wound repair, but is also crucial for tumor angiogenesis and is a highly tumorigenic agent. Thus, it is clear that, like their PG counterparts, 15-A$_2$-IsoPs have potent biological actions, including antiinflammatory and proangiogenic effects.

OTHER CYCLOPENTENONE EICOSANOIDS

Epoxycyclopentenone IsoPs

Cyclopentenone IsoPs, like other IsoPs, are initially formed esterified to phospholipids and are subsequently hydrolyzed from this storage site by various phospholipases. The biological activity of cyclopentenone IsoPs has been studied primarily with nonesterified compounds. Recently, however, Subbanagounder and colleagues identified and characterized a biologically active phospholipid-bound epoxycyclopentenone IsoP, 1-palmitoyl-2-(5,6-epoxycyclopentenone)-*sn*-glycero-3-phosphatidylcholine (PECPC) (Fig. 9A), which is an oxidation product of 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylcholine that is present in minimally oxidized low-density lipoproteins. The authors reported that this compound accumulated in cells exposed to cytokines and in atherosclerotic lesions. Furthermore, PECPC was shown to induce the monocyte-binding molecules monocyte chemotactic protein-1 (MCP-1) and IL-8 on endothelial cells. MCP-1 and IL-8 are known to mediate the recruitment of monocytes to blood vessel walls during atherogenesis. This biological activity of PECPC and its accumulation in atherosclerotic lesions suggest that PECPC might play a role in the pathogenesis of atherosclerosis. It is important to note that the epoxide portion of the molecule, rather than the unsaturated carbonyl on the prostane ring, may be responsible for the biological activity of PECPC.

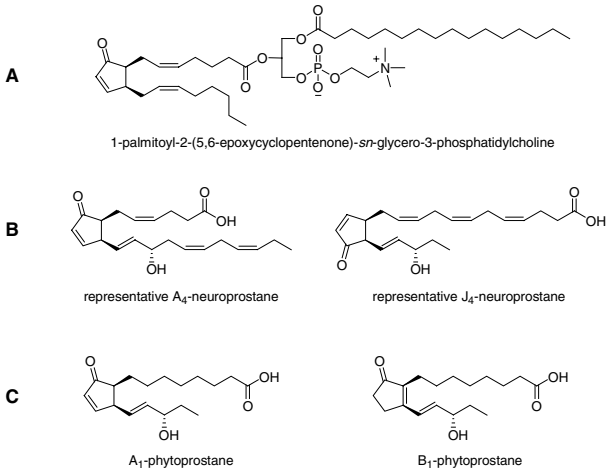

FIG. 9. Structures of cyclopentenone eicosanoids formed from lipid subtrates other than AA.

Docosahexaenoic acid (DHA)-derived cyclopentenone eicosanoids

AA is one of a number of polyunsaturated fatty acids that can be oxidized to generate IsoP-like compounds. DHA (C22:6ω3) is an ω-3 polyunsaturated fatty acid that is estimated to account for ~30% of the total fatty acids in brain tissue aminophospholipids in animals and humans (10, 62). Oxidation of DHA has been an area of intense research because lipid peroxidation has been implicated in the pathogenesis of various central nervous system disorders, such as Alzheimer's disease (26, 40, 45, 48, 55). Previously, we reported that 22-carbon IsoP-like compounds, termed neuroprostanes (NPs), of both the F-series (F$_4$-NPs) and the D/E-series (D$_4$/E$_4$-NPs), can be generated during the oxidation of DHA (49, 55, 56). More recently, we reported that, analogous to the IsoPs, cyclopentenone NPs (A$_4$/J$_4$-NPs) are generated from the dehydration of D$_4$/E$_4$-NPs (Fig. 9B) (19). These compounds were shown to be formed when DHA is oxidized *in vitro* using an iron/ADP/ascorbate mixture and during the *in vitro* oxidation of synaptosomes isolated from adult rat brain. Further, A$_4$/J$_4$-NPs were present esterified in brain lipids from normal rats and in postmortem human temporal lobe brain tissues. Interestingly, both *in vitro* and *in vivo*, quantities of A$_4$-NPs and J$_4$-NPs significantly exceed those of D$_4$/E$_4$-NPs and F$_4$-NPs by up to five-fold, supporting the concept that the formation of these compounds represents an important pathway of the oxidative metabolism of DHA in the central nervous system.

IsoP-like compounds from α-linolenic acid

Oxidation of polyunsaturated lipids is important not only in animals, but also in plants. The major polyunsaturated fatty acid in plants is α-linolenic acid (C18:3); higher plants do not have the enzymatic capacity to make AA (C20:4). α-Linolenic acid, analogous to AA *in vivo*, can be oxidized both enzymatically and nonenzymatically *in planta*. Enzymatic oxidation leads to the generation of PG-like compounds, termed jasmonates (25, 52), whereas non-enzymatic oxidation leads to the formation of several classes of IsoP-like molecules,

termed dinor isoprostanes and phytoprostanes (36, 37, 51). In 2003, Thoma and co-workers reported for the first time that cyclopentenone phytoprostanes, specifically the A_1- and B_1-phytoprostanes (Fig. 9C), are present in healthy, untreated plants in quantities comparable to levels of not only other phytoprostanes (E_1- and F_1-phytoprostanes), but also the enzymatically produced jasmonates (66). These compounds, in analogous fashion to the cyclopentenone products formed during the oxidation of AA and DHA, are generated from the dehydration of E_1-phytoprostane. In their report, Thoma *et al.* also showed that formation of the cyclopentenone phytoprostanes was enhanced in tobacco cell cultures after peroxide stress and in tomato plants after infection with a necrotrophic fungus. Although the exact role and function of these compounds in plant physiology have not yet been elucidated, the cyclopentenone phytoprostanes possess a number of biological activites *in planta*. When these compounds are exogenously administered to plants, they rapidly activate the mitogen-activated protein kinase and induce GSTs, defense genes, and phytoalexins (66). Based on these results, it has been proposed that cyclopentenone phytoprostanes are components of an oxidant injury-sensing signaling system that induces a number of plant defense mechanisms.

CONCLUSIONS

The discovery that cyclopentenone (A_2/J_2) IsoPs are formed from the free radical-initiated oxidation of AA both *in vitro* and *in vivo* is an important advance in our understanding of potential mediators of oxidative stress. These compounds, like enzymatically generated cyclopentenone PGs, are electrophilic, due to the presence of an α,β-unsaturated carbonyl group on the prostane ring, and thus react readily with nucleophilic biomolecules such as GSH. The recent synthesis of one A-ring IsoP, 15-A_{2t}-IsoP, has enabled a number of studies to be undertaken examining the biological activities of this and related molecules. In preliminary work presented herein, these compounds were shown to exert antiinflammatory and proangiogenic properties. Additionally, A-ring IsoPs have been proposed to act as sensors of oxidative stress by adducting to proteins and activating antioxidant defense mechanisms in both animals (43) and plants (66). Besides exerting these biological effects, cyclopentenone IsoPs readily conjugate with GSH both *in vitro* in the presence of GSTs and in HepG2 cells. This rapid metabolism by conjugation of GSH is likely a relevant pathway of metabolism *in vivo* and accounts for one reason that cyclopentenone IsoPs are only detectable esterified in lipids and not as free acids. In the future, the availability of other synthetically pure A_2- and J_2-IsoPs will allow more extensive exploration of the reactivity and metabolism of cyclopentenone IsoPs *in vivo*.

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ABBREVIATIONS

15-A_2-IsoPs, a synthetic mixture of A-ring isoprostanes containing 15-A_{2t}-IsoP, 15-A_{2c}-IsoP, 15-*epi*-A_{2t}-IsoP, and 15-*epi*-A_{2c}-IsoP; 15-dPGJ$_2$, 15-deoxy-$\Delta^{12,14}$-PGJ$_2$; AA, arachidonic acid; CCl_4, carbon tetrachloride; CID, collision-induced dissociation; COX, cyclooxygenase; DHA, docosahexaenoic acid; GC, gas chromatography; GSH, glutathione; GST, glutathione transferase; IL, interleukin; IsoP, isoprostane; LC, liquid chromatography; LOX, lipoxygenase; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; MS, mass spectrometry; *m/z*, mass-to-charge ratio; NF-κB, nuclear factor-κB; NP, neuroprostane; PECPC, 1-palmitoyl-2-(5,6-epoxycyclopentenone)-*sn*-glycero-3-phosphatidylcholine; PG, prostaglandin; PPARγ, peroxisome proliferator-activated receptor-γ; TNFα, tumor necrosis factor-α; Tx, thromboxane; VEGF, vascular endothelial growth factor.

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