Formation of Reactive Cyclopentenone Compounds in Vivo as Products of the Isoprostane Pathway*

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Cyclopentenone prostaglandins A2 and J2 are reactive compounds that possess unique biological activities. However, the extent to which they are formed in vivo remains unclear. In this study, we explored whether D2/E2-isoprostanes undergo dehydration in vivo to form A2/J2-isoprostanes. Oxidation of arachidonic acid in vitro generated a series of compounds that were confirmed to be A2/J2-isoprostanes by mass spectrometric analyses. A2/J2-isoprostanes were detected in vivo esterified to lipids in livers from normal rats at a level of 5.1 ± 2.3 ng/g, and levels increased dramatically by a mean of 24-fold following administration of CCI4. An A2-isoprostane, 15-A2-isoprostane, was obtained and found to readily undergo Michael addition with glutathione and to adduct covalently to protein. A2/J2-isoprostanes could not be detected in the circulation, even following CCI4 administration, which we hypothesized might be explained by rapid formation of adducts. This was supported by finding that essentially all the radioactivity excreted into the urine following infusion of radiolabeled 15-A2-isoprostane into a human volunteer was in the form of a polar conjugate(s). These data identify a new class of reactive compounds that are produced in vivo as products of the isoprostane pathway that can exert biological effects relevant to the pathobiology of oxidant injury.

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The abbreviations used are: CP, cyclopentenone; PG, prostaglandin; IsoP, isoprostane; HPLC, high pressure liquid chromatography; GC, gas chromatography; NICI, negative ion chemical ionization; MS, mass spectrometry; PFB, pentafluorobenzyl; TMS, trimethylsilyl; BSTFA, N,O-bis(trimethylsilyl)triﬂuoroacetamide.

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EXPERIMENTAL PROCEDURES

Conversion of 15-E2t-IsoP to 15-A2t-IsoP—8-Iso-[3H]PGA2, termed 15-A2t-IsoP according to the approved nomenclature for IsoPs (28), was obtained by treatment of 15-E2t-[3H]IsoP (8-iso-PGE2) (Cayman Chemical Co., Inc., Ann Arbor, MI) with 0.1 n HCl (29, 30), purified by normal phase HPLC, and analyzed by NMR for structural confirmation.

Analysis of A2t/J2-IsoP—A2t-IsoPs were analyzed by GC/NICI/MS using a modification of methods described for the analysis of other IsoPs (31). Briefly, 10 ng of [3H]PGA2 was added to samples as an internal standard, after which compounds were partially purified using C18 and silica Sep-Pak cartridges (Waters), methoximated, and converted to pentafluorobenzyl (PFB) esters. Compounds were then purified by chromatography on silica 60ALK6D TLC plates (Whatman) using a solvent system of hexane/acetone (70:30, v/v). Compounds migrating in the region 15.5 cm below the PFB ester of PGA2 to 1 cm above the PFB ester of PGA2 were scraped, extracted with ethyl acetate, converted to a trimethylsilyl (TMS) ether derivative, and then analyzed by GC/NICI/MS. The major ions generated in the NICI mass spectra of the PFB esters, O-methoxime, TMS ether derivative of A2t-IsoPs and [3H]PGA2, are the carboxylate anions at m/z 434 and m/z 438, respectively. Quantitation was performed by integration of the peak areas.

Analysis of A2t/J2-IsoPs as a Piperidyl-enol-trimethylsilyl Ether Derivative—Treatment of PGA with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and piperidine has been shown to convert this derivative to a piperidyl-enol-TMS ether derivative, which is specific for A-ring prostanooids (32). Thus, we analyzed for the formation of this derivative with CP-IsoPs. PFB esters of putative CP-IsoPs were treated with a 1:1 mixture of BSTFA/piperidine for 1 h at 60 °C and analyzed by GC/NICI/MS. The formation of the carboxylate anions at m/z 592 for the IsoPs and m/z 566 for [3H]PGA2.

Analysis of A2t/J2-IsoP by GC/Electron Ionization/MS—Putative A2t/J2-IsoPs generated during oxidation of arachidonic acid in vitro were subjected to purification using two HPLC systems: (a) normal-phase HPLC on a 5-μm Econosil 31 column using an isoocratic solvent system of hexane/isopropyl alcohol/acetic acid (97:3:0.1, v/v/v) and (b) reversed-phase HPLC on a 5-μm Econosil C18 column using an iso-acetic acid solvent system of acetonitrile/water/acetic acid (38:62:0.1, v/v/v). 15-A2t-[3H]IsoP was added prior to HPLC purification, and compounds that coeluted with 15-A2t-[3H]IsoP were then converted to a PFB ester, O-methoxime, TMS ether derivative and analyzed by GC/electron ionization/MS.

Analysis of D2/E2-IsoPs by GC/NICI/MS—Analysis of D2/E2-IsoPs by GC/NICI/MS was performed as described (27). The amounts of D2/E2-IsoPs reported in this study differ from those in our previous reports (27). In our previous reports, quantitation was based on the intensity of a single prominent peak, whereas in this study, the integrated area under all peaks was used for quantification.

Oxidation of Arachidonic Acid in Vitro—Non-esterified arachidonic acid was dissolved in ethanol and then oxidized in 50 mM Tris buffer (pH 8.0) for 45 min using an iron/ADP/ascorbate oxidizing system as described (33). The lipid extracts were then hydrolyzed enzymatically using Apis mellifera bee venom phospholipase A2 (27).

Conjugation of 15-A2t-IsoP and PGA2 with GSH in Vitro—Radiolabeled 15-A2t-IsoP and PGA2 were incubated at 37 °C with GSH in phosphate buffer (pH 6.5) in the presence of 19 units/ml glutathione S-transferase (Sigma). The molar ratio of the prostanooids to GSH was 1:10. At the designated time points, aliquots were removed, acidified immediately with 50% TCA, extracted twice with 2 volumes of methylene chloride. The formation of a GSH conjugate was assessed by determining the percent of radioactivity that did not extract into organic solvent (14). We predetermined that 93 ± 4% (mean ± S.E.) of PGA extracts into methylene chloride from buffer solutions at pH 3. Control incubations were carried out in the absence of GSH and enzyme.

Formation of 15-A2t-IsoP and PGA2 Covalent Adducts with Albumin—Radiolabeled PGA2 and 15-A2t-IsoP were incubated at 37 °C with human serum albumin at a molar ratio of prostanoids to albumin was 1:20. At the designated time points, aliquots were withdrawn, and 10 volumes of cold ethanol were immediately added to precipitate the albumin. The formation of covalent albumin adducts was assessed by determining the percent of radioactivity present in the albumin precipitate after centrifugation. Control incubations were carried out in the absence of albumin.

Formation of Polar Conjugates of 15-A2t-IsoP in Humans—After informed consent was obtained, a small tracer quantity (1 μCi) of 15-A2t-[3H]IsoP (150 Ci/mmol) was dissolved in 200 μl of ethanol, diluted to 10 ml with sterile saline, and infused over 10 min into the antecubital vein of a normal male volunteer. Individual voided urine specimens were collected over a period of 10 h and assayed for radioactivity. The formation of polar conjugates was assessed by determining the percent of radioactivity in a 1-ml aliquot of urine that did not extract into 2 volumes of methylene chloride at pH 3.

RESULTS

Initially, we explored whether A2t/J2-IsoPs are formed during oxidation of arachidonic acid in vitro (Fig. 1). In the upper m/z 434 chromatogram are multiple peaks with a similar retention time as the m/z 438 ion current chromatogram representing the syn- and anti-O-methyloxime isomers of the [3H]PGA2 internal standard. In the m/z 434 chromatogram are a series of peaks with a m/z 438 ion current chromatogram representing the syn- and anti-O-methyloxime isomers of the [3H]PGA2 internal standard. In the m/z 434 chromatogram are a series of peaks consistent with the presence of A2t-IsoPs. The summed total amount of the putative A2t-IsoPs formed was 529 ng/mg of arachidonic acid.

20 mg/ml human serum albumin in phosphate buffer (pH 7.4). The molar ratio of prostanoids to albumin was 1:20. At the designated time points, aliquots were withdrawn, and 10 volumes of cold ethanol were immediately added to precipitate the albumin. The formation of covalent albumin adducts was assessed by determining the percent of radioactivity present in the albumin precipitate after centrifugation. Control incubations were carried out in the absence of albumin.

Formation of Polar Conjugates of 15-A2t-IsoP in Humans—After informed consent was obtained, a small tracer quantity (1 μCi) of 15-A2t-[3H]IsoP (150 Ci/mmol) was dissolved in 200 μl of ethanol, diluted to 10 ml with sterile saline, and infused over 10 min into the antecubital vein of a normal male volunteer. Individual voided urine specimens were collected over a period of 10 h and assayed for radioactivity. The formation of polar conjugates was assessed by determining the percent of radioactivity in a 1-ml aliquot of urine that did not extract into 2 volumes of methylene chloride at pH 3.

Additional analyses further supported the identity of these compounds as A2t-IsoPs. First, no m/z 433 peaks were present, indicating that the m/z 434 peaks were not natural isotope peaks of compounds generating an ion less than m/z 434. When analyzed as [3H]TMS ether and O-[3H]methyloxime derivatives, all of the original m/z 434 peaks disappeared, and an identical pattern of new peaks appeared 9 and 3 Da higher, respectively (data not shown), indicating that all of the compounds had one hydroxyl and one carbonyl group. When analyzed following catalytic hydrogenation, there was a disappearance of the m/z 434 peaks and the appearance of new intense peaks 6 Da higher at m/z 440 (Fig. 2). No peaks were detected at m/z 346, 348, 349, or 442. This indicated that the compounds...
FIG. 2. Analysis of the putative A2/J2-IsoPs formed during oxidation of arachidonic acid in vitro prior to and after catalytic hydrogenation. A, analysis of compounds prior to hydrogenation. The peaks in the m/z 434 ion current chromatogram represent putative A2/J2-IsoPs, and the peaks in the m/z 438 chromatogram represent the [2H4]PGA2 internal standard. No compounds were detected 6 Da above m/z 434 at m/z 440 prior to hydrogenation (not shown). B, analysis of compounds following hydrogenation. Both the internal standard and the m/z 434 peaks in A have shifted upwards 6 Da following hydrogenation, indicating the presence of three double bonds.

Further evidence for the identity of these compounds as CP-IsoPs was obtained by analyzing the compounds by electron ionization/MS to obtain complete mass spectra. Compounds were analyzed as a PFB ester, O-methyloxime, TMS ether derivative. One of the mass spectra obtained is shown in Fig. 4. As noted, a molecular ion is present at m/z 615. Other prominent ions are present at m/z 600 (M – 15, loss of CH3), m/z 584 (M – 31, loss of OCH3), m/z 544 (M – 71, loss of CH2(CH2)2CH3, m/z 525 (M – 90, loss of Me5SiOH), m/z 494 (M – (90 + 31)), m/z 308 (M – 307, loss of CH2 CH=CH(CH2)3COOC6F5), m/z 277 (M – (307 + 31)), m/z 218 (M – (307 + 90)), m/z 199 (CH=CH–CH(Me5SiOH) CH2=CH3), m/z 181 (base) (CH=CHC6F5), and m/z 173 (Me5SiO+ = CH(CH2)3CH2). These ions or analogous ions and losses of other derivatives are also intense ions in the mass spectra of CP-PGs (34), indicating that this was a mass spectrum of a 15-series CP-IsoP in which the side chain hydroxyl is located at C-15. Although the structure depicted in Fig. 4 is an A-ring IsoP, ions are not present that would allow a differentiation between an A-ring and a J-ring compound.

We then explored whether CP-IsoPs are also formed in vivo. Previously, we showed that IsoPs are initially formed in situ esterified in tissue phospholipids (26). Therefore, we assayed for the presence of A2/J2-IsoPs esterified in livers from rats that had been treated with CCl4 to induce lipid peroxidation (Fig. 5A). Again, a series of m/z 434 peaks were present similar to what was found following oxidation of arachidonic acid in vitro, although the pattern of the peaks differed somewhat. This may be explained by our observation that there appears to be a steric influence of phospholipids on the formation of different IsoP isomers. In support of this notion, the pattern of peaks present following oxidation of arachidonoylphosphatidylcholine in vitro was found to be very similar to the pattern of peaks detected in esterified lipids in liver (Fig. 5B).

Further evidence for the identity of these compounds generated in vivo as A2/J2-IsoPs was obtained utilizing similar approaches used for the structural identification of the compounds formed in vitro. Analysis as a [2H9]TMS ether derivative, an O-[2H3]methyloxime derivative, and following catalytic hydrogenation indicated that all of the compounds had one hydroxyl group, one carbonyl, and three double bonds (data not shown). Furthermore, the compounds formed a piperidyl-enol-TMS ether derivative (Fig. 6), and the pattern of peaks was almost identical to that formed by the CP-IsoPs generated in vitro (Fig. 3), although the relative abundance of individual peaks differed somewhat.

We then compared the relative amounts of A2/J2-IsoPs and D2/E2-IsoPs present in livers from both normal and CCl4-treated rats to determine the extent to which D2/E2-IsoPs undergo dehydration in vivo (Fig. 7). Of interest was the finding that A2/J2-IsoPs could be detected esterified in the livers of normal rats at a level of 5.1 ng/g of liver. Relative to the amounts of D2/E2-IsoPs measured, A2/J2-IsoPs were present at levels indicating that the extent to which D2/E2-IsoPs undergo dehydration in vivo is not inconsequential. Following administration of CCl4, the levels of both A2/J2-IsoPs and D2/E2-IsoPs increased dramatically and to a similar extent by a mean of 23.9- and 21.2-fold, respectively.

We then carried out experiments to exclude the possibility that dehydration of D2/E2-IsoPs ex vivo during sample processing contributed significantly to the levels measured in liver. That this was not the case was supported by the finding that <1% of either PGE2 or PGD2 that was added to a lipid extract
of liver and then processed and analyzed had undergone dehydration to form PGA₂ and PGJ₂, respectively.

Our major interest in the possibility that CP-IsoPs may be formed in vivo is because these compounds should be reactive molecules that are susceptible to nucleophilic addition reactions. Therefore, we compared the ability of one of the CP-IsoPs, 15-A₂t-IsoP, and PGA₂ to conjugate with GSH and adduct to protein, using albumin as a model. We recently showed that one of the E₂t-IsoPs that is produced in vivo is 15-E₂t-IsoP (25). Its dehydration product, 15-A₂t-IsoP should therefore also be one of the CP-IsoPs that is produced in vivo. We initially determined the time course of conjugation of GSH with radio-labeled 15-A₂t-IsoP and PGA₂ in the presence of glutathione S-transferase. Formation of GSH conjugates was assessed by
determining the percent of radioactivity that did not extract into methylene chloride at pH 3. Approximately 70% of 15-A_2t-IsoP had conjugated with GSH within 2 min, and the conjugation was complete by 8 min (Fig. 8). The time course for the conjugation of PGA_2 with GSH was found to be essentially identical. In the absence of glutathione S-transferase, no appreciable conjugation occurred with either compound over the same time period (data not shown). Similarly, the time course for the formation of covalent adducts with albumin was essentially identical for both PGA_2 and 15-A_2t-IsoP (Fig. 9).

Following administration of CCl_4 to rats, plasma concentrations of non-esterified F_2-IsoPs and D_2/E_2-IsoPs increase to very high levels (25, 27). However, we unable to detect CP-IsoPs in the circulation of rats following administration of CCl_4 in the experiments described above. We hypothesized that this may be due to rapid formation of polar conjugates, e.g. with GSH, following the hydrolysis of the compounds from phospholipids. To test this hypothesis, we infused a tracer quantity of radiolabeled 15-A_2t-IsoP into a normal human volunteer and determined the percent of radioactivity excreted into the urine that did not extract into methylene chloride. 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. Only ~5% of the radioactivity present in the 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).

**DISCUSSION**

We report the finding that PGA_2- and PGJ_2-like compounds are formed in vivo as products of the IsoP pathway. Important and interesting was the finding that these compounds not only were detected in abundant quantities esterified in rat livers following induction of an oxidant injury, but were also present in readily detectable quantities in the livers of normal rats.

The discovery of the formation of CP-IsoPs opens up numerous new avenues for scientific inquiry. The reactive nature of these compounds conferred by the \( \alpha,\beta \)-unsaturated carbonyl moiety that characterizes these molecules provides a basis for hypotheses regarding their potential role in the pathogenesis of oxidant injury. As mentioned previously, CP prostanoids exert unique biological effects. They inhibit cellular proliferation via their ability to modulate a variety of growth-related and stress-induced genes, induce apoptosis, and activate peroxisome proliferator-activated receptor-\( \gamma \) (3–11). The reactive \( \alpha,\beta \)-unsaturated carbonyl seems essential for many of these biological actions (15). In this regard, one might anticipate that all of the CP-IsoPs might exert similar biological effects, lessening the potential importance of elucidating the precise structures of individual compounds in the mixture. As shown in Fig. 8, PGA_2 and 15-A_2t-IsoP covalently adduct to protein. Interestingly, however, in intact cells, PGA_2 seems to preferentially bind to particular proteins, the functions of which remain to be elucidated (35). As shown in Fig. 7, PGA_2 and 15-A_2t-IsoP also rapidly undergo glutathione S-transferase-catalyzed conjugation with GSH. We have previously shown that \( \Delta^{15} \)-PGJ_2 also rapidly conjugates with GSH, but does require catalysis by glutathione S-transferase (14). However, we (36) and others (37) have shown that conjugation with GSH acts to prevent the ability of CP-PGs to inhibit cellular proliferation and to induce apoptosis. Therefore, much remains to be known about the underlying molecular mechanisms that mediate the biological effects of CP prostanoids. Furthermore, the breadth of our understanding of the biological actions of CP prostanoids is probably far from complete. For example, virtually nothing is known about the biological consequences of the addition of these molecules with DNA in regards to their potential mutagenicity.

**Dehydration of PGE_2 and PGD_2 occurs in physiologic buffers, but has also been shown to be catalyzed by plasma and albumin and more recently also by human semen (21, 28, 38, 39). Plasma components could be involved in promoting dehydration of E-ring and D-ring IsoPs following their release from tissue phospholipids into the circulation. However, this study, we identified the presence of CP-IsoPs esterified in tissue membrane lipids. Whether there are also factors in cellular membranes that promote the dehydration of D_2/E_2-IsoPs remains to be explored. Nonetheless, identification of factors that may promote this dehydration may reveal potential avenues for**
intervention to modulate the formation of CP-IsoPs in vivo.

Our observation that 15-\(\alpha\)-IsoP undergoes extensive conjugation in vivo in humans is consistent with our previous findings that \(\Delta^{12}\)-PGJ\(_{2}\) also undergoes extensive conjugation in rats (14). These observations have potentially very important implications. This may explain the difficulty in the past in demonstrating the formation of CP-PGs in vivo because efforts were focused on detecting these compounds in free form. Even if detected in free form in small amounts, e.g. in urine as reported by Hirata et al. (19), this could potentially lead to the erroneous conclusion that the amounts formed in vivo may not be biologically relevant. However, quantification of the conjugated form(s) of CP-IsoPs in plasma and/or urine may provide an approach that more accurately assesses the magnitude of endogenous production of both CP-PGs and CP-IsoPs. In the case of CP-IsoPs, it is possible to assess their formation esterified in tissues of interest, but this is not amenable to human investigation. More important, as well, measurement of a conjugate of CP-PGs and CP-IsoPs would completely eliminate the confounding problem of artifactual formation ex vivo of what is being measured. This clearly provides the impetus for future studies aimed at elucidating the nature of these conjugates.

In summary, we have reported the discovery of a new class of reactive products of lipid peroxidation that are formed in vivo via the IsoP pathway. This provides a rational basis to explore in depth the biological activities of these novel molecules that may provide new insights into the biological consequences of their formation as it relates to the pathobiology of oxidant injury.

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Addendum—Since submission of the manuscript, we carried out a study to determine if 15-\(\alpha\)-IsoP (8-isop-GPA\(_{2}\)) is one of the CP-IsoPs that is produced in vivo. We found that a single compound isolated from the livers of CCl\(_4\)-treated rats coeluted with radiolabeled 15-\(\alpha\)-IsoP through four high resolving HPLC purification procedures, indicating that 15-\(\alpha\)-IsoP is in fact one of the CP-IsoPs formed in vivo. Furthermore, the data indicated that the first two HPLC procedures completely eliminated all other CP-IsoPs. This suggests that the mass spectrum of the compound shown in Fig. 4 that coeluted with radiolabeled 15-\(\alpha\)-IsoP through two HPLC purification procedures is likely a mass spectrum of 15-\(\alpha\)-IsoP.

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