Free Radical-induced Generation of Isoprostanes in Vivo

EVIDENCE FOR THE FORMATION OF D-RING AND E-RING ISOPROSTANES*

(Received for publication, August 17, 1993, and in revised form, October 19, 1993)

Jason D. Morrow†‡, Tanya A. Minton‡, Chetan R. Mukundan‡, Michelle D. Campbelli, William E. Zackerti, Vincent C. Danieli, Kamal F. Badri, Ian A. Blair‡, and L. Jackson Roberts, II§**

From the Departments of 1Pharmacology and §Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-6602 and the §Division of Nephrology, Department of Medicine, Emory University and Veterans Affairs Medical Center, Atlanta, Georgia 30033

We recently reported the discovery that a series of novel prostaglandin (PG)F2-like compounds (F2-isoprostanes) are produced in vivo independent of the cyclooxygenase as products of free radical-catalyzed lipid peroxidation. F2-isoprostanes are initially formed in situ from arachidonic acid esterified to phospholipids and then released preformed. We have now investigated whether PGD2/E2-like isoprostanes are also produced by rearrangement of the PGG2-like intermediates involved in isoprostane formation. Using a variety of approaches utilizing mass spectrometry, compelling evidence was obtained for the presence of D2/E2-isoprostane-containing phospholipidoids in the liver (85 ± 23 ng/g of liver) and free D2/E2-isoprostanes in the circulation (215 ± 90 pg/ml) of rats treated with TCAI to induce lipid peroxidation. In untreated rats, levels of D2/E2-isoprostanes esterified in liver phospholipids were much lower (0.90 ± 0.10 ng/g), and free compounds could not be detected in the circulation (<5 pg/ml). Interestingly, one of the E2-isoprostanes that would be expected to be formed in abundance, 8-epi-PGE2, was found to be a potent renal vasconstrictor, and these effects could be abrogated by SQ29548, a thromboxane receptor antagonist. Further understanding of the biological consequences of the formation of these novel compounds and factors that influence their formation may provide valuable insights into the pathophysiology of oxidant injury.

Free radical-catalyzed lipid peroxidation has been implicated in the pathogenesis of a wide variety of human disorders (1–4). Nonetheless, much remains to be understood about mechanisms of oxidant injury in vivo. Recently, we reported the discovery that a series of prostaglandin (PG)F2-like compounds (F2-isoprostanes) capable of exerting potent biological activity are produced in vivo in humans as products of free radical-catalyzed peroxidation of arachidonic acid (5). Formation of these compounds occurs independently of the cyclooxygenase enzyme, which had heretofore been considered obligatory for endogenous prostanoic biosynthesis. Circulating levels of these compounds increase dramatically in animal models of free radical injury, and quantification of F2-isoprostanes has proven to be an important advance in our ability to assess oxidant status in vivo (6). Formation of F2-isoprostanes proceeds through intermediates comprised of four positional peroxyl radical isomers of arachidonic acid which undergo endocyclization to yield PGG2-like bicyclic endoperoxides. The endoperoxides are then reduced to F-ring isoprostanes (5). Recently, we reported the finding that F2-isoprostanes are primarily formed in situ from arachidonic acid esterified in phospholipids and subsequently released preformed, presumably by a phospholipase(s) (7). This mechanism of formation is in contradistinction to the formation of cyclooxygenase-derived prostanoids in which arachidonic acid esterified to phospholipids must first be released prior to oxygenation.

Because the formation of isoprostanes proceeds through endoperoxide intermediates, we investigated the possibility that, in addition to F-ring isoprostanes, compounds with a prostane D-ring and E-ring (D2/E2-isoprostanes) may also be formed in vivo by rearrangement of the endoperoxide intermediates. We present evidence that D2/E2-isoprostanes are, in fact, produced in vivo, that they are present both esterified to phospholipids and in free form, and that they are capable of exerting potent biological activity.

EXPERIMENTAL PROCEDURES

Reagents—Methoxyamine HCl, pentafluorobenzyl bromide, diisopropylethylamine, and Apis mellifica venom phospholipase A2 were obtained from Sigma. Dimethylformamide and undecane were obtained from Aldrich. N,O-Bis(trimethylsilyl)trifluoroacetamide was obtained from Supelco (Bellefonte, PA). [1H12,13]Bis(trimethylsilyl)trifluoroacetamide and [1H12]methoxyamine HCl were obtained from Regis Chemical Co. (Morton Grove, IL). 1-Butanethiolic acid was obtained from Applied Science Laboratories, State College, PA. All organic solvents were obtained from Baxter Healthcare (Burkid and Jackson Brand, McGaw Park, IL). C-18 and silica Sep-Paks were purchased from Waters Associates (Milford, MA). TLC was performed on silica gel 60ALG6 plates (Whatman International Ltd., Maidstone, U.K.). The E2-isoprostane 8-epi-PGE2 was generously donated by Dr. Douglass Taber, University of Delaware (Newark, DE). [1H3,15]Arachidonic acid was biosynthesized as described previously (9). Other prostaglandin standards were obtained from Cayman Chemical (Ann Arbor, MI). Phosphatidylcholine with palmitate at the sn-1 position and [1H3]arachidonic acid at the sn-2 position was purchased from DuPont NEN.

Analysis of D2/E2-isoprostanes—D2/E2-isoprostanes were analyzed by gas chromatography (GC) and liquid chromatography (HPLC) using a modification of methods described previously for the analysis of PGD2 and PGE2 (10). Briefly, 1.5 ng of [1H3]PGE2 internal standard is initially added to a biological fluid and adjusted to pH 3 with 1 M HCl. The sample is then applied to a C-18 Sep-Pak cartridge that has been prewashed with 5 ml of methanol and...
5 ml of H2O (pH 3). The cartridge is then washed with 10 ml of H2O (pH 3) followed by 10 ml of heptane, and compounds are then eluted with 10 ml of ethyl acetate and evaporated to dryness under nitrogen. Compounds were then methoximated by treatment with a 2% solution of aqueous methoxamine-HCl for 30 min at room temperature. Compounds are then extracted with 1 ml of ethyl acetate and the organic layer evaporated under nitrogen. Compounds are converted to a pentafluorobenzyl (PFB) ester by the addition of 40 ml of a 10% solution of pentafluorobenzyl bromide in acetonitrile and 20 ml of a solution of 10% diisopropylethylamine in acetonitrile and allowed to incubate for 30 min at 37 °C. Reagents are dried under nitrogen and the residue reconstituted in 30 μl of chloroform and 20 μl of methanol and chromatographed on a silica TLC plate to 13 cm in a solvent system of ethyl acetate-methanol (98:2, v/v). The methyl ester of PGF2α, and the O-methyl ester, 0-methyloxime, and PFB ester derivatives (approximate m/z 528) are chromatographed on a separate lane and visualized with 10% phosphomolybdic acid in ethanol by heating. The Rf of PGF2α methyl ester in this solvent system is ≈0.25, whereas the Rf of the O-methylxime, PFB ester derivatives of PGF2α is ≈0.60. Compounds migrating in the region 1 cm above the PGF2α standard to 0.5 cm below the PGF2α standard are scraped from the TLC plate, extracted with 1 ml of ethyl acetate, and dried under nitrogen.

Following TLC purification, compounds are converted to trimethylsilyl (TMS) ether derivatives by the addition of 20 μl of N,O-bis(trimethylsilyl) trifluoroacetamide and 10 μl of dimethylformamide. The sample is then dried under nitrogen and the residue is reconstituted in 50 μl of m/z 524. The major ion generated in the GC/NICI mass spectra of the PFB ester, O-methylxime, TMS ether derivative of PGF2α and PGF2β, which would be the same ion generated by D5z-isoprostanes, is the m/z 524 carboxylic acid anion (M-181 (M-CH2=CHCH3)). The corresponding ion generated by the [5H4]-PGE2 internal standard is m/z 528. Levels of endogenous D5z-isoprostanes in a biological sample are calculated from the ratio of intensities of the ions m/z 524 to 528. In some experiments, compounds were reacted with 1-butanol and subjected to catalytic hydrogenation following TLC purification as described (11). D5z-isoprostanes were also analyzed by GC/electron ionization-MS as methyl ester, O-methylxime, TMS ether derivatives. Purification and derivatization of compounds for analysis by GC/electron ionization-MS was as noted above except the methyl ester derivatives were formed by treatment of compounds with excess ethereal diazomethane.

Analysis of D5z-isoprostanes—Purification, derivatization, and analysis of D5z-isoprostanes by GC/NICI-MS were performed as described (11).

Animal Model of Free Radical-induced Lipid Peroxidation—Free radical-catalyzed lipid peroxidation was induced in rats by intragastric administration of CCl4 as described previously (12). In some cases, radical-catalyzed lipid peroxidation was also induced in rats by the administration of 8-epi-PGF2α at a rate of 2.0 μg/kg/min (n = 3) or 4.0 μg/kg/min (n = 3) in the presence of a thromboxane A2 receptor antagonist, SQ29548, administered by a continuous intravenous infusion of 3 mg/kg/h. Treatment with SQ29548 was maintained for 30 min before the determination of lipid peroxidation. Levels of endogenous PGF2α were monitored, and the renal plasma flow (RPF) and arterial blood pressure were measured, suggesting that the compounds detected in stored plasma were formed during storage.

Biological Effects of 8-Epi-PGF2α in the Kidney—Renal clearance studies were performed using 200-250 male Sprague-Dawley rats as described elsewhere (16). In the first group, time control rats (n = 3), base-line measurements were performed and repeated during a 30-min infusion of vehicle (0.1% phosphate-buffered saline). In the second group of rats (n = 9), whole kidney measurements were performed during base-line conditions and during the infusion of 8-epi-PGF2α as described (11) in the following doses; 1.0 μg/kg/min (n = 3), 2.0 μg/kg/min (n = 3), and 4.0 μg/kg/min (n = 3). In a third group of rats (n = 6), 8-epi-PGF2α was administered at a rate of 2.0 μg/kg/min (n = 3) or 4.0 μg/kg/min (n = 3) in the presence of a thromboxane A2 receptor antagonist, SQ29548, administered by a continuous intravenous infusion of 3 mg/kg/h.

RESULTS

Evidence for the Formation of D5z/E5z-isoprostanes in Vitro during Storage of Plasma—Previously, we had shown that plasma arachidonic acid undergoes autodissociation during storage of plasma at -20 °C for several months, resulting in the formation of large quantities of F5z-isoprostanes (11). Concentrations of F5z-isoprostanes in fresh plasma that has not been stored are approximately 20 pg/ml, and storage of plasma for several months increases levels consistently to greater than 1,000 pg/ml. Therefore, we initially explored whether D5z/E5z-isoprostanes are also formed in vitro by analyzing plasma that had been subjected to storage at -20 °C for approximately 6 months. The selected ion current chromatograms obtained from the analysis using the LKB-1100 model-1000 liquid chromatograph are shown in Fig. 1. When analyzed in an analogous fashion, F5z-isoprostanes are detected as a series of peaks eluting from the GC column over an approximate 30-s interval (11). Similarly, in the upper m/z 524 chromatogram in Fig. 1, a series of peaks are present which eluted from the GC column over an approximate 40-s interval. The two peaks in the lower m/z 528 chromatogram represent syn and anti O-methylxime isomers of [5H4]PGE2. Quantification of compounds in the m/z 524 chromatogram is based on a comparison of the ratios of the starred (★) m/z 524 peak with that of the starred (★) peak in the m/z 528 chromatogram. The concentration of the compounds in the m/z 524 chromatogram was calculated to be 2,500 pg/ml. Four additional plasma samples that had been stored for various periods of time were then analyzed, and the concentrations found ranged from between 90 and 21,000 pg/ml. In contrast, these compounds could not be detected (lower limits of detection = 5 ppb) when fresh plasma from normal volunteers was analyzed, suggesting that the compounds detected in stored plasma were formed during storage.

The finding that large quantities of a series of compounds were formed during storage of plasma that had TLC and GC/MS properties similar to those of PGE2 would be consistent
Formation of \( D_2/E_2 \)-isoprostanes 4319

**Fig. 1.** Analysis of plasma which had been stored at \(-20\) °C for several months for \( D_2/E_2 \)-isoprostanes by GC/MS. The two peaks in the \( m/z \) 528 ion current chromatogram represent syn and anti \( O \)-methylxime isomers of the \( [\text{H}_3] \text{PGF}_2 \) internal standard. In the \( m/z \) 524 ion current chromatogram are a series of peaks consistent with the presence of \( D_2/E_2 \)-isoprostanes. Levels of compounds are based on a comparison of the ratio of the starred (*) peak in the \( m/z \) 524 chromatogram to the starred (*) peak in the \( m/z \) 528 chromatogram.

with their being \( D/E \)-ring isoprostanes. Since PGD\(_2\) and PGE\(_2\) have similar chromatographic properties on TLC and GC and have identical molecular weights and thus generate the same major M-181 ion when analyzed by NICI-MS, it is not possible to differentiate whether the putative isoprostane compounds detected in the \( m/z \) 524 ion current chromatogram in Fig. 1 have a D-type or E-type prostane ring. However, since the endoperoxide PGE\(_2\) derived from the cyclooxygenase rearranges in aqueous solutions to form both PGD\(_2\) and PGE\(_2\) (17), one would expect that the isoprostane endoperoxide intermediates would also rearrange to form both D-ring and E-ring isoprostanes.

Additional experimental approaches were then employed in an attempt to obtain further evidence that the compounds detected in stored plasma were \( D_2/E_2 \)-isoprostanes. First, no peaks were present when \( m/z \) 523 was monitored, indicating that the \( m/z \) 524 peaks were not natural isotope peaks of compounds generating an ion less than 524 Da. When the compounds were analyzed as \( [\text{H}_3] \text{TMS} \) ether derivatives, the \( m/z \) 524 peaks all shifted upwards 18 Da, indicating that they contain one carbonyl group. These data thus indicate that these compounds have the same functional groups as PGD\(_2\) and PGE\(_2\) and are consistent with their being \( D_2/E_2 \)-isoprostanes.

**Analysis for the Presence of \( D_2/E_2 \)-isoprostanes Esterified to Phospholipids in Vivo**—Since the above results suggested that \( D_2/E_2 \)-isoprostanes could be formed in vitro, we investigated whether these compounds may also be formed in vivo. Previously, we had shown that PGD\(_2\) isoprostanes are initially formed in situ from arachidonic acid esterified in tissue phospholipids and subsequently released preformed (7). Therefore, we examined whether \( D_2/E_2 \)-isoprostanes might be esterified to phospholipids in livers of rats that had been treated with CCl\(_4\) to induce lipid peroxidation. To investigate this, lipids were extracted from the livers, subjected to hydrolysis using A. mellif-era venom phospholipase \( A_2\), and subsequently analyzed for free compounds. Enzymatic hydrolysis of phospholipids was employed to circumvent the problem of dehydration of the prostane D/E-rings during chemical hydrolysis with strong base. The results of this analysis are shown in Fig. 2. A series of \( m/z \) 524 peaks were present in a pattern very similar to that obtained from analysis of stored plasma, although the relative abundances of the various peaks differ somewhat (cf. Fig. 1). Table I compares the amounts of the putative \( D_2/E_2 \)-isoprostanes with the amounts of \( F_2 \)-isoprostanes measured following hydrolysis of lipids from the same livers of both untreated and CCl\(_4\)-treated rats. The quantities of \( D_2/E_2 \)-isoprostanes measured following hydrolysis of lipids from livers of CCl\(_4\)-treated rats were approximately 100-fold higher than those in untreated rats. Levels of free \( D_2/E_2 \)-isoprostanes measured in lipid extracts that were not subjected to hydrolysis were < 1% of the levels measured following hydrolysis (\( n = 4 \)), suggesting that the compounds detected following treatment with phospholipase \( A_2\) were released from an acyl linkage on phospholipids. Pretreatment of animals with indomethacin, prior to CCl\(_4\) administration with a dosage regimen shown previously to inhibit cyclooxygenase activity by >90% (13) did not affect levels of the compounds measured (\( p > 0.8 \), Student's \( t \) test, \( n = 4 \)), indicating that the cyclooxygenase enzyme is not involved in their formation. Previously we had shown that butylated hydroxytoluene markedly suppresses the formation of \( F_2 \)-isoprostanes by autoxidation in vitro (11). The presence of butylated hydroxytoluene (0.005%) in the extraction solution, however, did not affect levels of \( D_2/E_2 \)-isoprostanes measured (\( p > 0.8 \), \( n = 4 \)), arguing that these compounds are not formed \( ex \) \( vivo \) by autoxidation during sample processing.

Experiments were then carried out to obtain further evidence for the identity of the compounds represented by the \( m/z \) 524 peaks in Fig. 2 as \( D_2/E_2 \)-isoprostanes. First, analysis of compounds obtained from the hydrolysis of liver extracts as a \( [\text{H}_3] \text{TMS} \) ether derivative resulted in a shift of each of the \( m/z \) 524 peaks upward 18 Da, indicating the presence of two hy-
indicated that the compounds represented by the rnlz 524 peaks bonds as would be expected for DE-ring isoprostanes. The appearance of the rnlz 524 peaks was a result of the presence of one carbonyl group. When the compounds were esterified to phospholipids. In this experiment, phospholipids extracted from livers of CCl₄-treated rats were first reduced by methanolic KOH. The hydrolysate was then analyzed for the presence of F₂-isoprostanes with trans-cyclopentane ring hydroxyls. The rationale of this experimental approach is based on the fact that we had shown previously that the cyclopentane ring hydroxyls forms when treated with 1-butaneboronic acid. A cyclic boronate derivative will form only if the cyclopentane ring hydroxyls are oriented cis (19).

The results of the analyses for F₂-isoprostanes hydrolyzed from phospholipids that had been reduced with NaBH₄ are shown in Fig. 4. At the top of Fig. 4A is shown the m/z 569 selected ion current chromatogram obtained from the analysis for F₂-isoprostanes hydrolyzed from phospholipids that had not been reduced with NaBH₄. In this analysis, compounds were not treated with 1-butaneboronic acid. In the lower three chromatograms are the results obtained following treatment of these compounds with n-butylboronic acid. The major ion generated by F₂-isoprostanes as a PFB ester, TMS ether, cyclic butylboronate derivative is the M₁₈I ion, m/z 491 (11). As is evident, the m/z 569 peaks disappear when reacted with 1-butaneboronic acid, and there is a coincident appearance of intense peaks in the m/z 491 chromatogram shown at the bottom of Fig. 4A. These results thus indicate that essentially all of the F₂-isoprostanes formed a cyclic boronate derivative. Fig. 4B shows the results of similar analyses for F₂-isoprostanes hydrolyzed from phospholipids that had first been reduced with NaBH₄. The m/z 569 chromatogram at the top of Fig. 4B was obtained when the compounds were not treated with 1-butaneboronic acid. The pattern of the m/z 569 peaks is similar to that obtained from the analysis of the hydrolysate of lipids that had not been reduced (Fig. 4A, top chromatogram). However, the level of compounds measured is approximately 1.6-fold higher in 4B than in 4A. In the lower set of three chromatograms are the results obtained from the analysis of these compounds following treatment with 1-butaneboronic acid. Similar to the results in Fig. 4A, intense m/z 491 peaks appear (Fig. 4B, bottom chromatogram), indicative of compounds that formed a cyclic boronate derivative. In contrast to the results shown in Fig. 4A, however, peaks also remain in the m/z 569 chromatogram after treatment with 1-butaneboronic acid. This suggested the presence of F-ring compounds with trans cyclopentane hydroxyls. Of note, the quantity of the compounds that did whether a cyclic boronate derivative bridging the ring hydroxyls forms when treated with 1-butaneboronic acid.

**TABLE I**

| Levels of isoprostanes in hydrolyzed lipid extracts from liver tissue of rats with and without CCl₄ treatment (n = 6 animals/group) | D₂/E₂-isoprostanes | F₂-isoprostanes |
|---|---|---|
| Untreated rats | 0.90 ± 0.10 | 2.8 ± 0.4 |
| CCl₄-treated rats | 85 ± 33 | 459 ± 126 |

**Fig. 2.** Analysis for D₂/E₂-isoprostanes by GC/NICI-MS following hydrolysis of a lipid extract from the liver of a rat treated with CCl₄ to induce lipid peroxidation. A series of peaks are present in the m/z 524 ion current chromatogram representing putative D₂/E₂-isoprostanes; the m/z 526 chromatogram represents the [⁴H₅]PGE₂ internal standard. Levels of compounds are based on a comparison of the ratio of the starred (*) peak in the m/z 524 chromatogram to that in the m/z 528 chromatogram.
not form a cyclic boronate derivative was calculated to be approximately 215 ng/g of liver. Since chemical reduction of the carbonyl on D/E-ring compounds produces a mixture of compounds with both cis- and trans-cyclopentane hydroxyls, it can be estimated that the total quantity D/E-ring compounds that were present would be approximately $215 \times 2 = 450$ ng/g of liver. This figure is almost exactly the difference found in the levels of F-ring compounds in hydrolysates of lipids that had been reduced with NaBH$_4$ (1230 ng/g of liver; Fig. 4B, top chromatogram) and the levels measured in the hydrolysate of lipids that had not been reduced by NaBH$_4$ (750 ng/g of liver, Fig. 4A, top chromatogram). This suggests that the quantity of D/E-ring isoprostanes that are formed esterified to phospholipids in vivo is approximately 40% of the quantity of esterified F$_2$-isoprostanes that are formed. However, this may be somewhat of an underestimate. For our analyses of D/E-ring isoprostanes, we have arbitrarily scraped the region of the TLC plate which lies between the location where the methyl ester of PGF$_{2\alpha}$ migrates up to 0.5 cm below the location where the O-methyloxime, PFB ester derivative of PGD$_2$ migrates. However, we have also analyzed the region on the TLC plate 0.5 cm below to 1 cm above the O-methyloxime, PFB ester of PGD$_2$ migrates for D$_2$/E$_2$-isoprostanes and have detected additional m/z 524 peaks that also elute from the GC with a retention time similar to that of PGE$_2$. Although these compounds may likely represent additional D/E-ring isoprostanes, they have not been scrutinized carefully, e.g. as to the number and type of functional groups that they contain, etc. Therefore, we cannot conclude with confidence that these compounds represent additional D$_2$E$_2$-isoprostanes. We have noted, however, that the levels of these compounds increase in parallel in animal models of oxidant injury with the m/z 524 peaks routinely quantified.

Analysis for the Presence of Free Concentrations of D$_2$/E$_2$-isoprostanes in the Circulation in CC1$_4$-treated Rats—We have demonstrated previously that F$_2$-isoprostanes are initially formed esterified to tissue phospholipids in CC1$_4$-treated rats and subsequently released into the circulation preformed (7). Since the above results suggested that D$_2$/E$_2$-isoprostanes are also formed esterified to phospholipids, we examined whether increased concentrations of these isoprostanes could also be detected free in the circulation of rats 4 h following administration of CC1$_4$ to induce lipid peroxidation. As was found in fresh human plasma, compounds presumably representing D/E-ring isoprostanes could not be detected in plasma from normal rats that had not been treated with CC1$_4$ (lower limit of detection = 5 pg/ml, n = 4). However, following treatment of rats with CC1$_4$, putative D$_2$/E$_2$-isoprostanes were detected in plasma at concentrations of $215 \pm 90$ pg/ml (n = 4), representing an increase of greater than 43-fold. The pattern of peaks was essentially identical to that shown in Fig. 2. Concentrations of F$_2$-isoprostanes measured in the same plasma samples were $640 \pm 70$ pg/ml (n = 4). The increased levels of D/E-ring isoprostanes in the circulation of CC1$_4$-treated rats were not affected by pretreatment of animals with indomethacin (286 $\pm$ 72 pg/ml (n = 3), p > 0.5, Student’s t test). As before, analysis of these compounds in the circulation as deuterated TMS ether and deuterated O-methyloxime derivatives also confirmed that they had, as expected, two hydroxyl groups and one carbonyl group.

Although the above results were consistent with the identity of the compounds detected in the circulation as D$_2$/E$_2$-isoprostanes, we also considered the possibility that they might represent metabolites of F$_2$-isoprostanes in which the side chain hydroxyl had been metabolized by a dehydrogenase to a keto group. First, analogous to cyclooxygenase-derived PGF$_{2\alpha}$ in which metabolism to 15-keto compounds represents a major pathway of metabolism (19), a dehydrogenase may also be involved in metabolism of F$_2$-isoprostanes. In fact, we reported recently the identification of urinary metabolites of F$_2$-isopros-
Formation of D_2/E_2-isoprostanes

A.

FIG. 4. GC/NICI-MS analysis of F-ring isoprostanes hydrolyzed by methanolic KOH from a lipid extract obtained from the liver of a rat treated with CCl_4. Compounds were analyzed both with and without treatment with 1-butaneboronic acid to assess whether compounds would form a cyclic boronate derivative. Peaks in the m/z 569 ion current chromatograms represent F-ring isoprostanes that either were not treated with 1-butaneboronic acid or did not form a cyclic boronate derivative. The peak in the m/z 576 ion current chromatograms represents the [^2H_5,^2H_6]-11β-PGF₂ internal standard. Peaks present in the m/z 491 ion current chromatograms represent F-ring isoprostanes that formed a cyclic boronate derivative. Panel A, analysis of a hydrolysate of liver lipids that had not been reduced with NaBH₄ prior to hydrolysis. The top two ion current chromatograms represent F₉-isoprostanes that had not been treated with 1-butaneboronic acid. The bottom three ion current chromatograms are the results obtained when compounds in the hydrolysate were treated with 1-butaneboronic acid. Panel B, analysis of a hydrolysate of liver lipids that had been reduced with NaBH₄ prior to hydrolysis. The top two ion current chromatograms represent F₉-isoprostanes that had not been treated with 1-butaneboronic acid. The bottom three ion current chromatograms are the results obtained when compounds in the hydrolysate were treated with 1-butaneboronic acid.

Retention Time (min)

Retention Time (min)

Retention Time (min)

Retention Time (min)

tanes which contained a keto group (20). Second, metabolites of F₉-isoprostanes with a keto group would have the same functional groups as D_{9}/E_{2}-isoprostanes, i.e. two hydroxyl groups and one keto group. These metabolites, therefore, would generate the same M-181 ion, m/z 524, as D_{9}/E_{2}-isoprostanes when analyzed as a PFBA or O-methyl oxime, TMS ether derivative by GC/NICI-MS. However, it should be possible to differentiate metabolites of F-ring isoprostanes with a keto group on a side chain from D_{9}/E_{2}-isoprostanes by determining whether the compounds form a cyclic boronate derivative. Cyclic boronate derivatives will only form bridging vicinal hydroxyls or hydroxyls separated by no more than a single carbon atom. Thus, F₉-isoprostane metabolites will form a cyclic boronate derivative, whereas D_{9}/E_{2}-isoprostanes will not. When we treated the compounds detected in the circulation with 1-butaneboronic acid, they did not form a cyclic boronate derivative (not shown).

Analysis of Lipid Extracts by HPLC for Phospholipids Containing D_{9}/E_{2}-isoprostanes—The results obtained up to this point provided evidence suggesting strongly that D_{9}/E_{2}-isoprostanes are formed in vivo esterified to phospholipids. We had demonstrated previously that F₈-isoprostane-containing phospholipids exhibited much more polar characteristics on normal phase HPLC than nonoxidized phospholipids (7). One would expect, therefore, that D/E-ring isoprostane containing phospholipids would also exhibit polar characteristics on HPLC. Therefore, to substantiate further the existence of D/E-ring isoprostane-containing phospholipids, lipid extracts from livers of CCl_4-treated rats were subjected to normal phase HPLC analysis using a solvent system that separates phosphatidylcholine from other phospholipids and neutral lipids (21-23). To detect lipids containing esterified D_{9}/E_{2}-isoprostanes, aliquots of fractions collected were hydrolyzed with bee venom phospholipase A_2 and free D_{9}/E_{2}-isoprostanes quantified by GC/MS. Consistent with the presence of D/E-ring isoprostane-containing species of phosphatidylcholine, fractions in which the majority (~70%) of free D_{9}/E_{2}-isoprostanes were detected after hydrolysis eluted at a much more polar retention volume (25-41 ml) than nonoxidized phosphatidylcholine, which eluted between 14 and 22 ml (Fig. 5). In addition, fractions also eluted between 2 and 8 ml in which free D_{9}/E_{2}-isoprostanes were detected after hydrolysis. Although not analyzed further, these were thought likely to represent phospholipid species other
than phosphatidylcholine-containing esterified D_{2}/E_{2} isoprostanes.

Analysis of D/E-ring Isoprostanes by Electron Ionization-MS—To obtain more direct evidence that the compounds analyzed by selected ion monitoring MS were D_{2}/E_{2}-isoprostanes, the compounds hydrolyzed from the polar lipids eluted from the HPLC column shown in Fig. 5 were analyzed as methyl esters, O-methyloximes, and TMS ethers by electron ionization MS. This analysis yielded multiple similar mass spectra of compounds eluting over approximately a 40-s period from the capillary GC column with characteristics of the electron ionization mass spectra of the same derivative of PGD_{2} and PGE_{2} (24). A mass spectrum obtained from the major eluting peak is shown in Fig. 6. The other mass spectra obtained were similar to that shown in Fig. 6, differing primarily in the relative ion abundances of some of the fragment ions. In the mass spectrum shown, there is an intense molecular ion present at the expected m/z 539. Other characteristic high mass ions present are: m/z 524 (M-15, loss of CH_{3}), m/z 508 (M-31, loss of OCH_{3}); m/z 468 (M-71, loss of CH_{3}(CH_{2})_{2}CH_{3}); m/z 449 (M-90, loss of Me_{2}SOH); m/z 438 (M-101, loss of CH_{2}(CH_{2})_{2}COOCH_{3}), m/z 418 (M-90+31), m/z 398 (M-141, loss of CH_{2}CH=CH(CH_{2})_{2}COOCH_{3}), and m/z 378 (M-90+71). The intense ions resulting from the loss of 71 Da and the ion at m/z 398 resulting from the loss of 141 Da suggest that this compound has the same basic structure as cyclooxygenase-derived prostaglandins with a cyclopentane ring at carbons C-8 through C-12. Prominent low mass ions are also present which are also prominent ions in the mass spectra of PGD_{2} and PGE_{2} such as m/z 199 representing the lower side chain ("CH_{3}CH=CH(Me_{2}SOH)(CH_{2})_{2}CH_{3}) and m/z 173 (Me_{2}SOH=CH(CH_{2})_{2}CH_{3}). The origins of the other low mass ions present remain to be established but presumably arise from coeluting different positional isomeric compounds that are present in this mixture. These data thus provided further evidence for the identity of these compounds as D_{2}/E_{2}-isoprostanes.

Analysis of D_{2}/E_{2}-Isoprostane-containing Phospholipids by Negative Liquid Secondary Ion Tandem Mass Spectrometry—We sought to obtain further direct evidence for the presence of phospholipids containing esterified D_{2}/E_{2}-isoprostanes by analyzing the polar lipids eluted from the HPLC column (see Fig. 5) by negative liquid secondary ion mass spectrometry. The negative liquid secondary ion mass spectrum of the these phospholipid species showed expected high mass intense ions at m/z 843 and 798, corresponding to M-15 [M-CH_{3}] and M-60 [M-RN(CH_{3})_{2}], respectively, of phosphatidylcholine containing stearate and a D_{2}/E_{2}-isoprostane (7, 25, 26). An expected ion at m/z 772 [M-CH_{2}CH=CH(CH_{3})_{2}] was not sufficiently intense to be observed above background chemical noise. Other structurally significant fragment ions were also present including an ion at m/z 351, corresponding to a D_{2}/E_{2}-isoprostane, and an ion at m/z 283, corresponding to the carboxylate anion of stearic acid. Collision-induced dissociation on m/z 843 resulted in the formation of a series of structurally relevant daughter ions (Fig. 7). In particular, the ions at m/z 283 and 351 confirmed the presence of stearic acid and a D_{2}/E_{2}-isoprostane, respectively. The prominent ion at m/z 208 represents the phosphatidylcholine backbone resulting from the loss of the D_{2}/E_{2}-isoprostane and stearate. Collision-induced dissociation on m/z 351 produced daughter ions at m/z 333 (M-H_{2}O), 315 (M-2H_{2}O), 307, 279 (M-C_{2}H_{4}CO_{2}), 265, 236, 222, 181, 168, 137, 97, and 79. Ions at m/z 333, 315, and 279 are also major fragment ions in the decomposition spectra of authentic PGD_{2} and PGE_{2} (27). Because of the limited amount of material that was analyzed, it difficult to be certain whether all of the other lower mass ions listed were decomposition ions of m/z 351 or background noise. However, analogous to the previously reported decomposition spectra of m/z 353, representing the F_{2}-isoprostane moiety of F_{2}-isoprostane-containing species of phosphatidylcholine, some of these lower mass ions likely arise from positional isomers of the isoprostane in the mixture (25). These data, therefore, provided further confirmation of the presence of a D_{2}/E_{2}-isoprostane esterified to phosphatidylcholine.

Assessment of the Biological Activity of the E_{2}-isoprostane 8-Epi-PGE_{2} in the Kidney—We had shown previously that the F_{2}-isoprostane, 8-epi-PGF_{2α}, exerts potent biological activity in the kidney. Specifically it has been found to be an extremely potent renal vasoconstrictor (5). Recently we have obtained compelling evidence that 8-epi-PGF_{2α} is, in fact, one of the more abundant F_{2}-isoprostanes that are produced in vivo (28). The intermediate endoperoxide that undergoes reduction to yield 8-epi-PGF_{2α} is 8-epi-PGG_{2}. Since D/E-ring isoprostanes arise from rearrangement of the intermediate endoperoxides, 8-epi-PGG_{2} would also be expected to undergo rearrangement to form 8-epi-PGE_{2}. In this regard, we have been able to obtain preliminary evidence that 8-epi-PGE_{2} is, in fact, one of the E-type isoprostanes that is produced in vivo by demonstrating that the major O-methoxylme isomer of synthetic 8-epi-PGE_{2} cochromatographs perfectly on capillary GC with the major peak (*) in the upper chromatogram in Fig. 2 (data not shown). The minor O-methoxylme isomer cochromatograms in the middle of the unresolved series of peaks eluting approximately 25 s earlier. Since we had shown previously that 8-epi-PGF_{2α} is a potent vasoconstrictor of renal vasculature, it was of interest, therefore, to explore whether 8-epi-PGE_{2} also exerted biological activity in the kidney.

When infused into the renal artery, 8-epi-PGE_{2} decreased both GFR and RPF in a dose-dependent manner (Fig. 8) with no effect on arterial blood pressure. A significant decrease compared with vehicle alone was identified at every dose used in the study and, at 4 μg/kg/min, both parameters decreased by 80%. Simultaneous intravenous administration of SQ29548 at 3 mg/kg/h completely abolished this effect at the intrarenal arterial infusion rate of both 2 and 4 μg of 8-epi-PGE_{2}/kg/min with no effect on arterial blood pressure.
Formation of $D_2/E_2$-isoprostanes

**DISCUSSION**

These studies report the discovery that isoprostanes with D/E-type prostane rings are formed in vivo. Analogous to the formation of $F_2$-isoprostanes, $D_2/E_2$-isoprostanes are also formed in situ esterified to phospholipids and released in free form, presumably by a phospholipase(s). The mechanism involved in the formation of $D_2/E_2$-isoprostanes is outlined in Fig. 9. This mechanism is identical to that outlined previously for the formation of $F_2$-isoprostanes through the formation of the bicyclic endoperoxide intermediates (11). In the formation of $D_2/E_2$-isoprostanes, however, the endoperoxides undergo rearrangement to form D/E-ring compounds rather than reduction to form F-ring compounds. Analogous to the formation of $F_2$-isoprostanes, four regioisomers of $D_2/E_2$-isoprostanes are formed, each of which can theoretically be comprised of eight racemic diastereomers. It is also conceivable that the endoperoxides might be stabilized in the hydrophobic environment when esterified to phospholipids and may exist for prolonged periods of time before rearranging to $D_2/E_2$-isoprostanes. However, the finding of high concentrations of $D_2/E_2$-isoprostanes free in the circulation of rats following administration of CCl₄ establishes that the endoperoxides do undergo rearrangement in vivo to form $D_2/E_2$-isoprostanes.

It has been shown previously that autoxidation of unsatur-
FIG. 8. Dose-dependent reduction in GFR (panel A) and RPF (panel B) caused by intrarenal arterial infusion of 8-epi-PGE₂ at rates of 1, 2, and 4 µg/kg/min. □, GFR in vehicle-infused animal; ■, GFR in 8-epi-PGE₂-infused animal; △, RPF in vehicle-infused animal; ◼, RPF in 8-epi-PGE₂-infused animal. *p < 0.05; **p < 0.01 for vehicle infused animal.

FIG. 9. Outline of the mechanism involved in the formation of D₂/E₂-isoprostanes. Four regioisomers of both D₂-isoprostanes and E₂-isoprostanes are produced, each of which can theoretically be comprised of eight racemic diastereomers. For simplicity, stereochromical orientation is not indicated in the figure.

Atated fatty acids can result in the formation of compounds with a cyclopentane ring (29). Interestingly, it was found that the side chains of these compounds are almost exclusively oriented cis which is in contrast to prostaglandins formed enzymatically by the cyclooxygenase in which the side chains are exclusively oriented trans (29). Based on this information, 8-epi prostaglandins would be expected to be formed as isoprostanes. In this regard, recently we obtained evidence that 8-epi-PGF₂α is, in fact, one of the E₂-isoprostanes that is produced in abundance in vivo (28). Therefore, it would be expected that 8-epi-PGG₂α, the endoperoxide intermediate that is reduced to form 8-epi-PGF₂α, also undergoes rearrangement to form 8-epi-PGE₂. Thus, we examined whether this E₂-isoprostane exhibited biological activity. Since 8-epi-PGF₂α is a very potent renal vasoconstrictor (5), we thought it of interest to investigate the effects of 8-epi-PGE₂ on renal hemodynamics. 8-Epi-PGE₂ was also found to be a potent renal vasoconstrictor, albeit somewhat less potent than 8-epi-PGF₂α (5). This is an important finding because it suggests that the formation of the D₂/E₂-isoprostanes is not simply a phenomenon of biochemical curiosity, but one that may also have biological relevance. The finding that 8-epi-PGE₂ is a vasoconstrictor of renal vasculature is somewhat unexpected since PGE₂ derived via the cyclooxygenase is a vasodilator (30). In contrast, both PGF₂α derived via the cyclooxygenase and the E₂-isoprostane 8-epi-PGF₂α are vasoconstrictors (5). Thus, one might anticipate that 8-epi-PGE₂ and 8-epi-PGF₂α would also have opposing biological effects on vascular smooth muscle. However, this may be an oversimplistic expectation based on the structure/function relationships of prostaglandin molecules. We reported previously that the renal vasoconstricting actions of 8-epi-PGF₂α could be abrogated by SQ29548, a thromboxane receptor antagonist, suggesting that the vascular actions of 8-epi-PGF₂α are mediated via an interaction with thromboxane receptors (16). This was an interest-
ing finding in that thromboxane receptors have not been implicated in the vasoconstricting activity of PGF2α. More recently, however, we reported that 8-epi-PGF2α appears to interact with a distinct novel receptor on vascular smooth muscle, which is apparently antagonized by SQ29548, which may be similar, but different, from the thromboxane receptor (31). Thus, simple inversion of the stereochemistry of the upper side chain of PGF2α may be a key determinant of receptor interaction. Of interest, we found that the vasoconstricting actions of 8-epi-PGF2α in the kidney could also be abrogated by SQ29548. Future studies investigating whether 8-epi-PGF2α also interacts with the same distinct receptor as 8-epi-PGF2α (owing to the fact that the stereochemistry of the upper side chain is inverted), or interacts with the thromboxane receptor, will be of considerable interest. In this regard, studies investigating further the spectrum of the biological activity of 8-epi-PGF2α and the biological actions of other D2/E2-isoprostanes will also be of interest and of potential importance since these compounds may participate as mediators in the pathophysiology of oxidant injury.

It should be noted that the quantities of D2/E2-isoprostanes that are formed in vivo are only slightly less than the amounts of F2-isoprostanes formed. Since the levels of F2-isoprostanes in normal human biological fluids are approximately an order of magnitude higher than cyclooxygenase derived prostaglandins (5), the amounts of D2/E2-isoprostanes which are produced in vivo are not trivial. At present, enzymes and/or nonenzymatic substances that may be involved in the reduction of the isoprostane endoperoxide intermediates in vivo to F-ring isoprostanes remain to be identified. Nonetheless, understanding factors regulating the production of D/E-ring isoprostanes in relation to F-ring isoprostanes might be important since changes in the relative abundances of these different isoprostanes may have different biological consequences owing to potential variations in their biological actions.

It should also be mentioned that there are potentially important biological ramifications associated with the formation of D2/E2-isoprostanes esterified to phospholipids. We reported previously that molecular modeling of phospholipids with F2-isoprostanes esterified at the sn-2 position revealed them to be extremely distorted molecules (7). Thus, the formation of isoprostane-containing phospholipids in settings of oxidant stress may have deleterious effects on membrane fluidity and integrity, well recognized sequelae of oxidant injury. Since we have now discovered that D2/E2-isoprostanes are also formed esterified to phospholipids in only slightly less abundance compared with F2-isoprostanes, the total quantities of isoprostane-containing phospholipids that may be formed in settings of free radical injury are substantially greater than thought previously.

We demonstrated that D2/E2-isoprostane-containing phospholipids are substrates for bee venom phospholipase in vitro. We also were able to detect free D2/E2-isoprostanes in the circulation of rats treated with CCl4 to induce lipid peroxidation. Presumably the free compounds arose primarily from hydrolysis of isoprostane-containing phospholipids. The type(s) of mammalian phospholipase responsible for hydrolysis of D2/E2-isoprostanes from phospholipids has not yet been identified. However, understanding how this process is regulated is of importance. As discussed above, isoprostane-containing phospholipids are very distorted molecules that should have profound effects on the integrity and fluidity of cellular membranes. However, we have demonstrated that once released in free form, D2/E2-isoprostanes are capable of exerting biological activity. How these two processes are balanced, therefore, will determine the overall net biological ramifications associated with the formation of isoprostanes in vivo.

In summary, we report the discovery that D2/E2-isoprostanes capable of exerting potent biological activity are formed in vivo as products of nonenzymatic free radical-catalyzed lipid peroxidation. Analogous to the formation of F2-isoprostanes, D2/E2-isoprostanes are formed in situ esterified to phospholipids and subsequently released in free form. Further understanding the biological consequences of the formation of these novel compounds and factors that can influence their formation may provide valuable insights into the pathophysiology of oxidant injury.

Acknowledgment—We appreciate the technical assistance of Brian J. Nobes.

REFERENCES
1. Halliwell, B., and Gutteridge, J. M. C. (1990) Methods Enzymol. 186, 1–85
2. Southern, P. A., and Peace, G. (1989) Mayo Clin. Proc. 63, 590–608
3. Ames, B. N. (1983) Science 221, 1256–1254
4. Harman, D. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 7124–7128
5. Morrow, J. D., Hill, K. E., Burk, R. F., Nammour, T. M., Badr, K. F., and Roberts, L. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6885–6887
6. Morrow, J. D., Awad, J. A., Kato, T., Takahashi, K., Badr, K. F., Roberts, L. J., and Burk, R. F. (1992) J. Clin. Invest. 90, 2502–2507
7. Morrow, J. D., Awad, J. A., Boss, H. J., Blair, I. A., and Roberts, L. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10721–10725
8. Taber, D. F., and Hoerrner, R. J. (1992) J. Org. Chem. 57, 441–447
9. Wendelborn, D. F., Morrow, J. D., and Roberts, L. J., II (1990) Methods Enzymol. 187, 51–62
10. Parsons, W. G., Ill, and Roberts, L. J., II (1988) J. Immunol. 141, 2413–2419
11. Morrow, J. D., Harris, T. M., and Roberts, L. J., II (1990) Anal. Biochem. 164, 1–10
12. Burk, R. F., and Lane, M. J. (1979) Toxicol. Appl. Pharmacol. 50, 467–475
13. Jackson, E. K. (1989) J. Pharmacol. Exp. Ther. 260, 9–21
14. Radin, N. S. (1969) Methods Enzymol. 14, 245–248
15. Hughes, H., Smith, C. V., Harning, E. C., and Mitchell, J. R. (1983) Anal. Biochem. 130, 431–436
16. Takahashi, K., Nammour, T. M., Fukunaga, M., Ebert, J., Morrow, J. D., Roberts, L. J., and Badr, K. F. (1992) J. Clin. Invest. 90, 136–141
17. Hamberg, M., and Samuelsson, B. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 899–903
18. Pace-Asciia, G., and Wolfe, L. S. (1971) J. Chromatogr. 62, 129–133
19. Roberts, L. J. (1987) in CRC Handbook of Ricosanoids: Prostaglandins and Related Compounds, Vol. I: Chemical and Biochemical Aspects, part A (Willis, A., ed) pp. 233–294, CRC Press, Boca Raton, FL
20. Awad, J. A., Morrow, J. D., and Roberts, L. J. (1993) J. Biol. Chem. 268, 4161–4169
21. Ellegren, J. S., and Zimmerman, R. L. (1987) J. Lipid Res. 28, 1016–1018
22. Hanans, C., and Perrin, L. J. (1991) J. Oil Chem. Soc. 68, 804–807
23. Breath, A. R., Ingram, C. D., and Harris, T. M. (1987) Biochemistry 26, 5465–5471
24. Pace-Asciia, C. R. (1989) Prostaglandin Thromboplast Leukotriene Res. 18, 251, 274
25. Kaygios-Harrison, K. A., Rose, D. M., Murphy, R. C., Morrow, J. D., and Roberts, L. J., II (1993) J. Lipid Res. 34, 1229–1235
26. Jensen, N. J. K., Tomer, K. B., and Gros, M. L. (1986) Lipids 21, 580–588
27. Zirrei, J. A., Davoli, E., Bettaazzoli, L., Gros, M. L., and Murphy, R. C. (1990) J. Am. Soc. Mass Spectrom. 1, 225–235
28. Morrow, J. D., Badr, K. F., and Roberts, L. J., II (1993) Biochim. Biophys. Acta, in press
29. O'Connor, D. E., Mihelich, E. D., and Coleman, M. C. (1981) J. Am. Chem. Soc. 103, 223–224
30. Dunn, M. J., and Hoed, V. L. (1977) J. Am. Chem. Soc. 103, F169–F184
31. Fukunaga, M., Makita, N., Roberts, L. J., Morrow, J. D., Takahashi, K., and Badr, K. F. (1993) Am. J. Physiol. 264, C1619–C1624