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Non-cyclooxygenase-derived prostanoids (F_2-isoprostanes) are formed *in situ* on phospholipids

(eicosanoids/lipids/oxidative stress/peroxidation/free radicals)

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ABSTRACT **We recently reported the discovery of a series of bioactive prostaglandin F_2-like compounds (F_2-isoprostanes) that are produced *in vivo* by free radical-catalyzed peroxidation of arachidonic acid independent of the cyclooxygenase enzyme. Inasmuch as phospholipids readily undergo peroxidation, we examined the possibility that F_2-isoprostanes may be formed *in situ* on phospholipids. Initial support for this hypothesis was obtained by the finding that levels of free F_2-isoprostanes measured after hydrolysis of lipids extracted from livers of rats treated with CCl_4 to induce lipid peroxidation were more than 100-fold higher than levels in untreated animals. Further, increased levels of lipid-associated F_2-isoprostanes in livers of CCl_4-treated rats preceded the appearance of free compounds in the circulation, suggesting that the free compounds arose from hydrolysis of peroxidized lipids. This concept was supported by demonstrating that free F_2-isoprostanes were released after incubation of lipid extracts with bee venom phospholipase A_2 *in vitro*. When these lipid extracts were analyzed by HPLC, fractions that yielded large quantities of free F_2-isoprostanes after hydrolysis eluted at a much more polar retention volume than nonoxidized phosphatidylcholine. Analysis of these polar lipids by fast atom bombardment mass spectrometry established that they were F_2-isoprostane-containing species of phosphatidylcholine. Thus, unlike cyclooxygenase-derived prostanoids, F_2-isoprostanes are initially formed *in situ* on phospholipids, from which they are subsequently released preformed, presumably by phospholipases. Molecular modeling of F_2-isoprostane-containing phospholipids reveals them to be remarkably distorted molecules. Thus, the formation of these phospholipid species in lipid bilayers may contribute in an important way to alterations in fluidity and integrity of cellular membranes, well-known sequelae of oxidant injury.**

Free radicals derived from oxygen are thought to play an important role in the pathophysiology of an increasingly wide variety of human diseases (1–4). Nonetheless, much remains to be understood about mechanisms of oxidant injury *in vivo*. Polyunsaturated fatty acid-containing lipids are recognized targets of oxidant damage, readily undergoing peroxidation upon exposure to free radicals. Peroxidation of lipids can greatly alter the physicochemical properties of membrane lipid bilayers, resulting in severe cellular dysfunction. In addition, a variety of lipid byproducts are produced as a consequence of lipid peroxidation, some of which can exert adverse biological effects (5–7).

Recently, we reported the discovery that a series of prostaglandin (PG)-like compounds capable of exerting potent biological activity are produced *in vivo* in humans as products of free radical-catalyzed peroxidation of arachidonic acid (8). The notable aspect of this discovery was that the formation of these prostanoids occurs independent of the catalytic activity of the cyclooxygenase enzyme, which had been considered obligatory for endogenous prostanoid biosynthesis. Circulating levels of these compounds were shown to increase dramatically in animal models of free radical injury (8). Interestingly, the levels of these prostanoids in normal human plasma and urine are one or two orders of magnitude higher than those of prostaglandins produced by the cyclooxygenase enzyme. Formation of these compounds proceeds through intermediates composed of four positional peroxyl radical isomers of arachidonic acid which undergo endocyclization to yield bicyclic endoperoxide PGG_2-like compounds. The PGG_2-like compounds are then reduced to PGF_2-like compounds (9). In conjunction with the Committee of Eicosanoid Nomenclature constituted by the Joint Commission on Biochemical Nomenclature, a facile nomenclature for the individual compounds produced by this mechanism is being developed, based on the general term "isoprostanes" and the type of prostane ring they contain.

Unsaturated fatty acids in phospholipids readily undergo peroxidation upon exposure to free radicals. Peroxidation of arachidonoyl phospholipids would result in the formation of the positional peroxyl isomers of arachidonic acid that are intermediates leading to the formation of isoprostanes (9). Therefore, we investigated the possibility that isoprostanes may be formed completely *in situ* on phospholipids.

EXPERIMENTAL PROCEDURES

Analysis of F_2-Isoprostanes. Purification, derivatization, and analysis of F_2-isoprostanes by gas chromatography/mass spectrometry were performed as described (8, 9).

Animal Model of Free Radical-Induced Lipid Peroxidation. Endogenous lipid peroxidation was induced in rats by administration of CCl_4 as described (10). At various time points, animals were sacrificed and the livers were removed, frozen in liquid N_2, and either processed immediately or stored at −70°C. We have previously determined that formation of F_2-isoprostanes by autoxidation of lipids *in vitro* is completely inhibited at −70°C.

Extraction, Purification, and Hydrolysis of Lipids. Lipids from livers were extracted (11), subjected to alkaline hydrolysis, and subsequently analyzed for F_2-isoprostanes as described (9). Depending on the experiment, 0.005% butylated hydroxytoluene or 0.5% triphenylphosphine was added to lipid extracts during the extraction procedure.

Release of F_2-Isoprostanes from Lipid Extracts by *Apis mellifera* Venom Phospholipase A_2. Lipid extracts of livers of CCl_4-treated rats (containing approximately 1 μmol of phospholipid) were allowed to react with *Apis mellifera* venom phospholipase A_2 (approximately 200 μg) (Sigma) as described (12).

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Abbreviation: PG, prostaglandin.

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As a positive control for enzymatic activity, phosphatidylcholine containing [^{3}H]arachidonate in the *sn*-2 position (New England Nuclear) was added to the incubation mixture and the percent of radiolabeled arachidonate released was determined. After 2 hr, the diethyl ether was evaporated and free arachidonic acid and phospholipids were extracted from ½ of the incubation mixture at pH 7 with $CHCl_3$/methanol (2:1, vol/vol) (13). The organic layer was then evaporated under a stream of N_2 and the residue was subjected to TLC (silica gel 60A LK6D, Whatman) using a solvent system of petroleum ether/diethyl ether/acetic acid (90:10:1, vol/vol). Free arachidonic acid migrates with an R_f of 0.18 and phospholipids remain at the origin. The ratio of free arachidonic acid to esterified phospholipid arachidonate was determined by comparing the amount of radioactivity present between R_f 0.15–0.25 to that present at the origin.

Free F_2-isoprostanes were extracted from the remaining ½ of the reaction mixture with an equal volume of ethyl acetate twice after decreasing the pH to 3 with 1 M HCl. After evaporation of the organic layer under N_2, the residue was resuspended in 0.5 ml of methanol to which 10 ml of pH 3 water was added. Free F_2-isoprostanes were then reextracted by using C_{18} Sep-paks (Waters), purified by TLC, and quantified by mass spectrometry as described (8). The percent of total F_2-isoprostanes released by bee venom phospholipase A_2 was calculated by determining the ratio of the amount of free F_2-isoprostanes released by enzymatic hydrolysis to the amount released by chemical hydrolysis of an aliquot (approximately 1 μmol) of the same lipid extract.

Microsomal CoA Incubation Experiments. In these experiments, esterification of tritiated $PGF_{2\alpha}$ into lysophosphatidylcholine by a rat liver microsomal system (14) was compared to that of tritiated arachidonic acid. Free and esterified arachidonate were extracted (14) and the ratio was determined as described for the bee venom experiments discussed above. The ratio of free to incorporated $PGF_{2\alpha}$ was determined as for arachidonic acid except that extracts were chromatographed in a solvent system of acetone/hexane/acetic acid (75:25:1, vol/vol). In this solvent system, phospholipids remain at the origin and $PGF_{2\alpha}$ has a R_f of 0.30.

Analysis of Lipid Extracts from Livers of CCl_4-Treated Rats by Normal-Phase HPLC. Normal-phase HPLC analysis of lipid extracts was performed on a 25 cm × 4.6 mm Econosil SI column with 5-μm particles (Alltech Associates), using an isocratic solvent system of hexane/isopropyl alcohol/water (4:6:1, vol/vol) at a flow rate of 1 ml/min. UV absorbance was monitored continuously at 205 nm. Aliquots of fractions eluted were subjected to alkaline hydrolysis, after which free F_2-isoprostanes were quantified as described previously.

Analysis of Phospholipids by Fast Atom Bombardment Mass Spectrometry. Fast atom bombardment mass spectra were obtained on a VG 70/250-HF double-focusing mass spectrometer (VG Instruments, Danvers, MA). A saddle field gun was used at 8 kV with xenon as reactant gas at a filament current of 1.2 mA. Negative-ion and positive-ion mass spectra were obtained with a matrix of triethanolamine/dimethyl sulfoxide (1:1, vol/vol) at accelerating voltages of 5.5 and 6.0 kV, respectively.

RESULTS

Indirect Evidence for Free Radical-Induced Formation of F_2-Isoprostane-Containing Lipids *in Vivo*. Initial evidence supporting the hypothesis that F_2-isoprostanes may be present as the acyl groups of lipids *in vivo* was obtained by comparing levels of free F_2-isoprostanes in saponified lipid extracts of livers from CCl_4-treated rats and untreated rats. The liver is the major target organ of CCl_4 toxicity owing to its high content of cytochrome P450, which metabolizes CCl_4 to trichloromethyl radicals (15). Levels of free F_2-isoprostanes measured after the hydrolysis of lipid extracts from livers of rats 2 hr after administration of CCl_4 (2 ml/kg) were 825 ± 198 ng/g of liver (n = 5). These levels were 142-fold higher than levels measured after saponification of lipid extracts of livers of untreated rats (5.8 ± 1.2 ng/g of liver) (n = 5). Levels of free F_2-isoprostanes measured in lipid extracts that were not subjected to saponification were <2% of levels measured after saponification.

The following evidence was obtained confirming that the compounds detected after alkaline hydrolysis of the lipid extracts of liver were identical to F_2-isoprostanes previously identified in human plasma and urine: (*i*) analysis of the compounds by gas chromatography/mass spectrometry as a deuteriated silyl ether derivative indicated the presence of three hydroxyl groups; (*ii*) analysis after catalytic hydrogenation indicated the presence of two double bonds; (*iii*) all compounds formed cyclic boronate derivatives, indicating the presence of *cis* prostane ring hydroxyl groups; and finally (*iv*) analysis by electron ionization mass spectrometry revealed multiple compounds eluting from the capillary GC column over approximately a 40-sec period that yielded mass spectra essentially identical to those obtained previously for F_2-isoprostanes (data not shown) (8, 9).

The above data were consistent with the hypothesis that F_2-isoprostanes are present *in vivo* in ester linkages to lipids. However, we also considered the possibility that their formation may not have occurred actually *in vivo* but rather *ex vivo* during or after lipid extraction and saponification from peroxyl radical precursors of arachidonic acid present on oxidized lipids. To test this possibility, lipids were treated during extraction prior to hydrolysis with triphenylphosphine (5 mg/ml) to reduce peroxyl groups to hydroxyl groups, which are incapable of cyclizing to form the prostane ring. If the formation of the F_2-isoprostanes that were detected occurred *ex vivo* from peroxidized arachidonic acid precursors present in the extracted lipids, then treatment of the lipids with triphenylphosphine during extraction to reduce peroxyl groups on arachidonic acid to hydroxyl groups would markedly decrease the levels of free isoprostanes measured after saponification. However, the levels of free F_2-isoprostanes measured after hydrolysis of untreated lipid extracts of rat livers obtained 1 hr after administration of CCl_4 (1 ml/kg) (370 ± 121 ng/g of liver, n = 5) were not suppressed by pretreatment with triphenylphosphine (353 ± 70 ng/g, n = 5). Further, the presence of the free radical scavenger butylated hydroxytoluene (BHT; 0.005%) did not reduce levels of F_2-isoprostanes measured (371 ± 137 ng/g, n = 5). Since BHT markedly suppresses the formation of free isoprostanes by autoxidation *in vitro* (9), this result provided additional evidence that the formation of the isoprostanes detected did not occur *ex vivo*.

We then examined the time course of the increase in F_2-isoprostanes measured in hydrolyzed lipid extracts from livers of CCl_4-treated rats in relation to the appearance of increased levels of free compounds in the circulation. Whereas lipid-associated levels of F_2-isoprostanes in the liver were found to increase very rapidly, reaching approximately half maximum as early as 15 min, the appearance of increased quantities of free compounds in the circulation was delayed considerably (Fig. 1). This finding was consistent with the concept that these compounds are initially formed *in situ* on arachidonoyl-containing lipids and subsequently released in free form into the circulation.

Evidence supporting the above hypothesis was obtained by demonstrating that incubation of lipid extracts with phospholipase A_2 *in vitro* results in the release of free F_2-isoprostanes. Incubation of lipid extracts from livers of CCl_4-treated rats with phospholipase A_2 *in vitro* for 2 hr resulted in a release of free F_2-isoprostanes that was 48% ± 11% of that from chemical saponification (n = 5). As a control

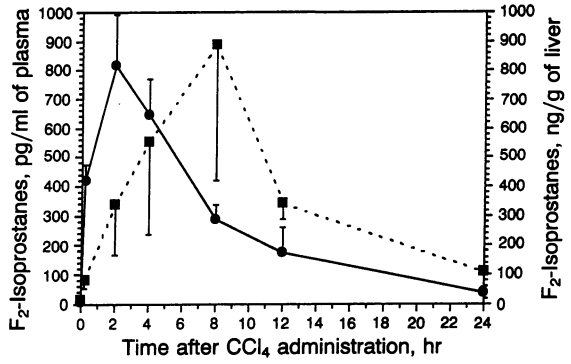

FIG. 1. Time course of appearance of lipid-associated F_2-isoprostanes in liver (●) in comparison with the appearance of free compounds in the circulation (■) of rats after intragastric administration of CCl_4 (1 ml/kg). Each time point represents the mean ± SD of levels measured in at least four animals.

for enzymatic activity, the extent of hydrolysis of [^{3}H]arachidonate from [^{3}H]arachidonoyl phosphatidylcholine was determined; it was 95% ± 10% (n = 5). The relevance of the finding that F_2-isoprostane phospholipids were hydrolyzed somewhat less efficiently than arachidonic acid phospholipids under the conditions used *in vitro* may be difficult to interpret in relation to the situation *in vivo*. This is because the orientation of substrate to the phospholipase A_2 is an important determinant of the activity of the enzyme (16) and the orientation of F_2-isoprostane-containing phosphatidylcholine in the conditions used *in vitro*, because of its enhanced polarity, may be quite different from the orientation of arachidonoyl phosphatidylcholine. However, these results do indicate that isoprostane-containing phospholipids can indeed serve as substrates for phospholipase A_2.

Evidence That F_2-Isoprostanes Are Not Formed from Free Arachidonic Acid and Incorporated into Lysophospholipids. Although the above findings suggested strongly that F_2-isoprostanes exist in ester linkages to lipids *in vivo*, it could not be concluded from these results that they are actually formed *in situ* from esterified arachidonic acid. Alternatively, they might be formed from free arachidonic acid and subsequently incorporated into lysophospholipids. To investigate this possibility, we examined the ability of a rat liver microsomal CoA-dependent esterification system to incorporate $PGF_{2\alpha}$ into lysophosphatidylcholine. Under *in vitro* conditions in which arachidonic acid was quantitatively incorporated into lysophosphatidylcholine (96% ± 3%, n = 4), essentially no incorporation of $PGF_{2\alpha}$ was detected (1.6% ± 0.9%, n = 4).

Analysis of Lipid Extracts by Normal-Phase HPLC. Collectively, the results obtained up to this point provided substantial indirect evidence that the formation of F_2-isoprostanes *in vivo* occurs *in situ* on lipids. We then attempted to obtain direct physical evidence for the existence of F_2-isoprostane-containing phospholipids from analysis of lipids extracted from livers of CCl_4-treated rats by fast atom bombardment mass spectrometry after purification by normal-phase HPLC. Lipid extracts were subjected to normal-phase HPLC analysis using a solvent system which separates phosphatidylcholine from other phospholipids and neutral lipids (17–19). To detect fractions that contained lipid species containing F_2-isoprostanes, aliquots of fractions collected were combined and saponified, and free F_2-isoprostanes were quantified by gas chromatography/mass spectrometry. Fractions in which the majority of free F_2-isoprostanes were detected after hydrolysis (68% of the total) eluted at a much more polar retention volume (35–50 ml) than nonoxidized phosphatidylcholine, which eluted between 18 and 25 ml (Fig. 2). This is consistent with the predicted more polar characteristics of phosphatidylcholine species containing F_2-isoprostanes. In addition, fractions also were eluted between 5 and 10 min in which free isoprostanes were detected after saponification. Although these latter fractions were not further analyzed, they were thought likely to represent phospholipid species other than phosphatidylcholine and/or neutral lipids that contain esterified F_2-isoprostanes.

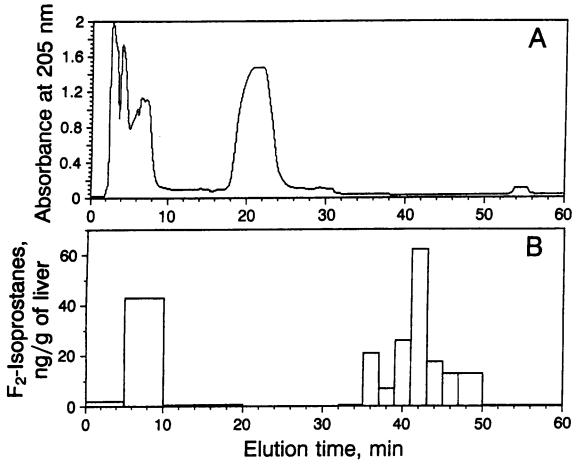

FIG. 2. Normal-phase HPLC chromatogram obtained from the analysis of lipid extracts from livers of CCl_4-treated rats. The isocratic solvent system of hexane/isopropyl alcohol/water (4:6:1, vol/vol) was used at a flow rate of 1 ml/min. UV absorbance was continually monitored at 205 nm. The large UV peak eluted between 18 and 26 ml in chromatogram *A* represents nonoxidized phosphatidylcholine, while the peaks eluted near the solvent front between 2 and 9 ml represent less polar phospholipids and neutral lipids. In chromatogram *B* is shown the quantities of free F_2-isoprostanes measured after alkaline hydrolysis of aliquots of collected fractions that were pooled as indicated by the widths of the bars.

Analysis of the Polar F_2-Isoprostane-Containing Lipids by Fast Atom Bombardment Mass Spectrometry. The polar lipids described above that eluted from the HPLC column between 35 and 50 ml were then subjected to analysis by negative-ion fast atom bombardment mass spectrometry. The characteristics of phosphatidylcholine analyzed by negative-ion fast atom bombardment mass spectrometry have been detailed (20, 21). The negative-ion fast atom bombardment mass spectrum of phosphatidylcholine is characterized by high-mass ions representing losses of 15, 60, and 86 Da from the molecular ion. These ions are generated from fragmentations at different sites in the choline moiety. In addition, assignment of the substituents at the *sn*-1 and *sn*-2 positions can be made by the presence of intense ions representing the carboxylate anions of the fatty acids. The two most abundant fatty acids at the *sn*-1 position in rat liver phosphatidylcholine are palmitate and stearate. The mass spectra obtained from analysis of the polar F_2-isoprostane-containing lipids purified by HPLC (Fig. 3) revealed high-mass negative ions predicted for F_2-isoprostane-containing phosphatidylcholine at M − 15 $(M - CH_3)^-$, M − 60 $[M - HN(CH_3)_3]^-$, and M − 86 $[M - HN(CH_3)_3C_2H_2]^-$ for both 1-palmitoyl 2-F_2-isoprostane phosphatidylcholine and 1-stearoyl 2-F_2-isoprostane phosphatidylcholine. As predicted, prominent ions are present at m/z 255 and 283, representing the carboxylate anions of palmitate and stearate, respectively, from the *sn*-1 position. In addition, and most importantly, there is also a prominent ion at m/z 353, the predicted ion representing the carboxylate anion of F_2-isoprostane from the *sn*-2 position.

Analysis by positive-ion fast atom bombardment mass spectrometry further confirmed the structure and molecular weight of these F_2-isoprostane-containing species of phosphatidylcholine. In addition to a prominent ion at m/z 184 from the phosphocholine moiety, prominent ions were also

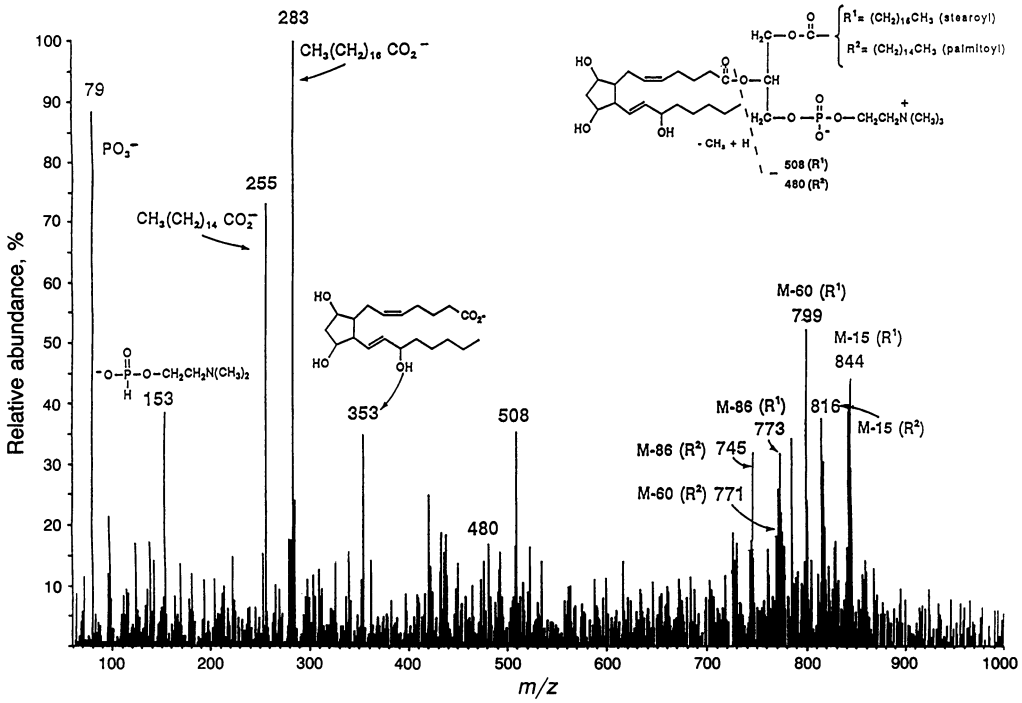

FIG. 3. Negative-ion fast atom bombardment mass spectrum obtained from analysis of polar lipids eluted between 33 and 50 ml in Fig. 2*B*.

present at *m/z* 854 and 882, representing the $[M + Na]^+$ ions of 1-palmitoyl 2-F_2-isoprostane phosphatidylcholine and 1-stearoyl 2-F_2-isoprostane phosphatidylcholine, respectively.

These species of phosphatidylcholine have also been subjected to further analysis by tandem mass spectrometry. Analysis of the collision-induced dissociation of the negative ions generated by fast atom bombardment mass spectrometry firmly established the identity of these lipids as phosphatidylcholine with palmitate and stearate at the *sn*-1 position and F_2-isoprostane at the *sn*-2 position (J.D.M., L.J.R., and R. C. Murphy, unpublished results).

DISCUSSION

These studies provide direct evidence that F_2-isoprostanes exist in ester linkages of phospholipids *in vivo*. Evidence was also obtained indicating that these compounds are not incorporated preformed into lysophospholipids but are formed *in situ* by free radical-catalyzed peroxidation of arachidonic acid in phospholipids. We have previously shown that F_2-isoprostanes can be formed from free arachidonic acid *in vitro* (9). However, this is unlikely to be a major contributor to the formation of these compounds *in vivo* because the vast majority of arachidonic acid is present *in vivo* in ester linkages of phospholipids. In addition, it has been shown that arachidonic acid in phospholipids is much more readily oxidized than unesterified arachidonic acid (22). For these reasons, F_2-isoprostanes are likely formed predominantly *in situ* on phospholipids, and free isoprostanes detected in biological fluids *in vivo* likely derive from the hydrolysis of preformed compounds from phospholipids. The results of the time course experiment presented in Fig. 1 support this hypothesis in that increases in levels of free compounds in the circulation lag behind increases in F_2-isoprostanes esterified in lipids in the liver.

These findings introduce the following concept: In direct contrast to cyclooxygenase-derived prostaglandins, which are only formed *de novo* from free arachidonic acid and are not stored, isoprostanes are formed from esterified arachidonic acid, exist in a storage depot as acyl moieties of phospholipids, and are released preformed. After their release, isoprostanes are capable of exerting biological activity. We have previously shown that one of the isoprostanes that can be produced in abundance, 8-epi-$PGF_{2\alpha}$, is an extremely potent renal vasoconstrictor (8). Recently we found that markedly increased circulating levels of F_2-isoprostanes are present in patients with hepatorenal syndrome, a disorder in which the deterioration in renal function has been attributed to renal vasoconstriction (23). We have also shown that 8-epi-$PGF_{2\alpha}$ is a potent pulmonary artery vasoconstrictor (24) and that the vasoconstricting actions of 8-epi-$PGF_{2\alpha}$ in both the renal and pulmonary vascular beds are mediated through thromboxane/endoperoxide receptors (24, 25). Interestingly, whereas 8-epi-$PGF_{2\alpha}$ is a potent agonist of vascular thromboxane/endoperoxide receptors, we have found that it acts primarily as an antagonist of platelet thromboxane/endoperoxide receptors (26).

Among the known and sometimes devastating consequences of oxidant injury are alterations in the physicochemical properties and function of cellular membranes. This has been attributed to an accumulation of products of lipid peroxidation in lipid bilayers (27). Molecular modeling of phosphatidylcholine with the F_2-isoprostane 8-epi-$PGF_{2\alpha}$ at the *sn*-2 position reveals it to be a remarkably kinked molecule (Fig. 4). Thus, in addition to the fact that these compounds can exert bioactivity once they are released from phospholipids, the formation of isoprostane-containing phospholipid species in settings of oxidant stress would be predicted to have profound effects on the fluidity and integrity of cellular membranes and thus may contribute in an important way to these recognized sequelae of oxidant injury that can lead to severe cellular dysfunction or death.

Therefore, the biological significance of the findings reported herein potentially encompasses both the formation of these compounds on phospholipids, which can alter the physicochemical properties of cell membranes, and the fact that once released, these compounds can exert biological activity in target tissues. How these two processes are balanced will influence the overall biological consequences which ensue as a result of the formation of these compounds in settings of oxidant injury. Presumably central to the

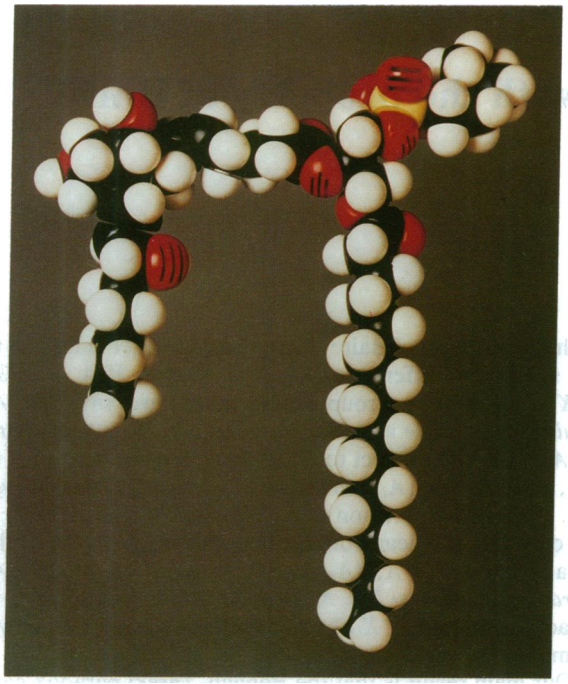

FIG. 4. Space-filling molecular model of F_2-isoprostane-containing phosphatidylcholine with palmitic acid at the *sn*-1 position and the F_2-isoprostane 8-epi-$PGF_{2\alpha}$ at the *sn*-2 position.

regulation of the levels of esterified versus free isoprostanes are phospholipases. There are conflicting views on the role of phospholipase A_2 in oxidant injury. Whereas it has been thought that the removal of oxidized fatty acids by phospholipase A_2 is important in the repair of oxidized membranes, it has been reported that inhibitors of phospholipase A_2 can be protective in some settings of oxidant injury (28, 29). With regards to the formation of isoprostanes during oxidant stress, it is possible to envision that either the effects resulting from their formation in lipid bilayers or the biological consequences of their activity in the free state may be more detrimental, depending on the situation. In addition, quantification of F_2-isoprostanes is emerging as a valuable tool to assess endogenous lipid peroxidation and oxidant status *in vivo* in humans (30). Depending on the balance of formation on phospholipids and their release in free form, it may be important to quantify levels of both free compounds and compounds esterified to lipids in tissues of interest to accurately assess total production of isoprostanes. Thus, further understanding of the role of phospholipases in oxidant injury and specifically in relation to their activity towards isoprostane-containing phospholipids will be important.

In this context, the type(s) of phospholipase A_2 important in hydrolyzing F_2-isoprostanes *in vivo* is at present not known. Previous studies have shown that oxidized fatty acids esterified in phospholipids are preferential substrates for low molecular weight phospholipase A_2 (27). In this regard, we demonstrated that F_2-isoprostane-containing phospholipids are substrates for the low molecular weight phospholipase A_2 in bee venom, which is related to mammalian low molecular phospholipases (31). In contrast, the high molecular weight phospholipase A_2 recently characterized by Clark *et al.* (32) exhibits a marked specificity for hydrolyzing arachidonic acid linkages compared with those of other unsaturated fatty acids at the *sn*-2 position. On the other hand, platelet-activating factor acetylhydrolase exhibits only low activity for hydrolysis of esters of nonoxidized long chain fatty acids but will hydrolyze esters of longer chain fatty acids that have been oxidized (33). Thus, future studies examining the activity of these various endogenous phospholipases for isoprostane-containing phospholipids will be of relevance.

In summary, we report the finding that F_2-isoprostanes are formed *in situ* on phospholipids *in vivo* and subsequently released preformed, presumably by phospholipase A_2. Further understanding of factors important in regulating the formation and hydrolysis of isoprostane-containing phospholipids should provide valuable insights into key fundamental processes involved in the pathogenesis of oxidant injury.

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