Evidence for the Formation of Dinor Isoprostanes E$_1$ from α-Linolenic Acid in Plants

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The free radical oxidation of arachidonic acid is known to generate complex metabolites, termed isoprostanes, that share structural features of prostaglandins and exert potent receptor-mediated biological activities. In the present study, we show that α-linolenic acid can undergo a similar oxidation process, resulting in a series of isomeric dinor isoprostanes E$_1$. E-ring dinor isoprostane formation from linolenate was found to be catalyzed by soybean lipoxygenase. The main enzymatic products were 13- and 9-hydroperoxylinolenate but in addition, two dinor isoprostane E$_1$ regioisomers were formed with a yield of 0.31%.

Identification and quantification of two dinor isoprostane E$_1$ regioisomers in plant cell cultures was achieved by a negative chemical ionization gas chromatography-mass spectrometry method using [$^{18}$O]dinor isoprostanes E$_1$ as internal standards. Endogenous levels of these compounds were determined in four taxonomically distant plant species and found to be in the range of 4.5 to 60.9 ng/g of dry weight.

Thus analogous pathways in animals and plants exist, each leading to a family of prostaglandin-like compounds derived from polyunsaturated fatty acids. It remains to be shown whether the dinor isoprostanes exert biological activities in plants as has been demonstrated for their C20 congeners in mammals.

In 1990, a series of prostaglandin (PG)$^1$ F2-like compounds termed isoprostanes F$_{2g}$ (iPF$_{2g}$) was discovered to be produced in abundance in humans by a free radical mechanism independent of the cyclooxygenase enzyme from arachidonic acid (1). Formation of these prostanooids proceeds through bicyclic endoperoxide intermediates that are reduced in vivo to isoprostanes F$_{2g}$. In addition, the unstable bicyclic endoperoxide intermediates (2) may also readily rearrange in an aqueous environment to isothromboxane (3) and isoprostanes of the D$_2$ and E$_2$ type (4). Four regioisomers for each of the D$_2$ E$_2$ and F$_2$ isoprostanes are formed from arachidonic acid, each of which is comprised of 16 stereoisomers (5, 6). Interest in these molecules stems not only from the fact that they have been shown to provide a valuable index of free radical-induced lipid peroxidation in vitro and in vivo but also from the finding that isoprostanes E$_2$ and F$_2$ exert potent biological activities in mammals (6).

As early as the 1960s, it was shown by Nutgeren et al. (7) that prostaglandin-like compounds could be formed by autoxidation of 8,11,14-eicosatrienoic acid. Some years later Pryor et al. (8) autoxidized methyl linolenate and obtained mass spectra evidence for the formation of PGF-type products after reduction of the autoxidation mixture. Porter and co-workers prepared a single hydroperoxy fatty acid from γ-linolenic acid and allowed it to react in the presence of oxygen and a free radical source. After reduction, GC-MS analysis indicated the presence of a number of PGF-like compounds, one of which was identical with an authentic sample of dinor-PGF$_{1a}$ (9).

The mechanism of formation of dinor isoprostanes involves initial hydrogen abstraction from one of the two bisallylic methylene groups of α-linolenic acid at carbons 11 and 14. Two bicyclic endoperoxide regioisomeric families, each comprising 16 members, result from the subsequent reaction of the initial linolenate free radical with two oxygen molecules, where each of the isomers has a cyclopentane ring containing a cis-1,3-endoperoxide moiety. Two alkyl chains extend from the cyclopentane ring, one containing the original carboxylic acid moiety, and the second chain containing the methyl terminus (see Fig. 1).

The stereochemical aspects of the formation of bicyclic endoperoxides from hydroperoxy methyl α- and γ-linolenate have been studied, and it was found that the side chains of the bicyclic endoperoxides are almost exclusively oriented cis similar to the C20 isoprostanes (10, 11). This is in contrast to prostaglandins formed by cyclooxygenase in which the side chains are exclusively oriented trans.

The bicyclic endoperoxide moiety of the molecules easily rearranges in the presence of water to E- and D-ring compounds having a hydroperoxy group in the side chain, which in turn may be reduced in vitro or in vivo to dinor isoprostanes E$_1$ and D$_1$ (see Fig. 1; see Ref. 12 for a discussion of the nomenclature).

In the present study, we demonstrate that α-linolenic acid autoxidizes readily via the 12- and 13-hydroperoxyradical intermediate to dinor isoprostanes of the E-type in aqueous buffers, a reaction that can be catalyzed by soybean lipoxygenase. Dinor isoprostanes were prepared in the mg scale and characterized by mass spectroscopy and NMR. Analytical methods were developed that allowed us to quantitate the formation of the two dinor isoprostane E$_1$ (dinor-iPE$_1$) regioisomers in vitro by HPLC and GC-MS and to demonstrate that plants form dinor-iPE$_1$ in vivo.

EXPERIMENTAL PROCEDURES

Materials—Soybean lipoxygenase (L-8383, Type-I-S, 43,000 units/mg) was from Sigma. α-Linolenic acid (puriss. p.a.) was purchased from Fluka. Silica solid phase extraction columns (500 mg) were from Ma-

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The abbreviations used are: PG, prostaglandin; iP, isoprostane; HPLC, high pressure liquid chromatography; GC-MS, gas chromatography-mass spectrometry; LOX, lipoxygenase; BHT, 2,6-di-tert-butyl-4-methylphenol; NCI, negative ion chemical ionization; PFB, pentfluorobenzyl; TMS, trimethylsilyl; EI, electron impact; HPOTE, hydroperoxycocadecatrienoic acid.
chery and Nagel, Duren, Germany. Silica gel 60 (particle size 63–100 μm) for column chromatography was from Merck. Oxygen-18O gas (99.1 atom % 18O) was obtained from Isotech, Miamisburg, OH. Plant suspension cultures were obtained from the departmental culture collection and grown as described (13).

Preparation of 18O-Labeled and Unlabeled Dinor Isoprostanes E—Linolenic acid (6 g) was dissolved in chloroform, loaded on a silica column (10 g), and eluted with hexane/diethyl ether (9:1, v/v) to remove any contaminating oxygenated fatty acids. The eluate was taken to dryness in a 500-ml round bottom flask and dissolved in 12 ml of methanol/water (1:1, v/v). In the case of the labeling experiment, the flask was evaporated in vacuo and filled with 500 ml of methanol, spiked with 5 μg of PGE1 as internal standard. After stirring the mixture for 7 days at room temperature, the autoxidation reaction was stopped by the addition of 6 g of triphenylphosphine in 50 ml of chloroform. After 30 min of incubation time, 12 ml of saturated NaCl solution was added. The organic phase was evaporated in vacuo, reconstituted in 60 ml of chloroform, and loaded on a silica column (30 g of silica gel 60). The remaining linolenic acid, monohydroperoxides, and triphenylphosphine were eluted with 600 ml of diethyl ether/acetone/acidic acid (99:1, v/v) and discarded. A crude mixture of [18O]dinor-iPE-s was obtained with 3% 18O atoms was obtained by eluting the column with 500 ml of diethyl ether/acetone/acidic acid (7:3:0.1, v/v). The eluate containing an isomeric mixture of [18O]dinor-iPE-s was quantitated by HPLC (see below) and used without further purification. High-resolution EI mass spectra from the dinor-iPE derivatives were obtained on a Finnigan MAT 95Q mass spectrometer. For electron impact ionization (EI) GC-MS, dinor isoprostanes B1, B2, and B3 were methylated in ethanol with an excess of ethereal diazomethane and silylated with N,O-bis(trimethylsilyl)trifluoroacetamide as described (15). Quantitation was performed by comparison of the peak areas of unlabeled and labeled dinor-iPB1, after correction for the fraction (1.5%) of unlabeled dinor-iPB1 derived from the internal standard.

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The $^{[18O]}$dinor isoprostanes $B_1$ yielded the following fragment pattern: type I, EI-MS (70 eV): $m/z = 396 [M^{+}]$, 367 $[M^{+} - \text{C}_2\text{H}_4]$; 337 $[M^{+} - \text{C}_2\text{H}_3 - (\text{C}_8\text{O})]$; type II, EI-MS (70 eV): $m/z = 396 [M^{+}]$, 381 $[M^{+} - \text{CH}_3]$; 367 $[M^{+} - \text{C}_2\text{H}_3]$; 365 $[M^{+} - \text{OCH}_3]$. The $^{[18O]}$dinor isoprostanes $B_1$ yield the following fragment pattern: type I, EI-MS (70 eV): $m/z = 396 [M^{+}]$, 367 $[M^{+} - \text{C}_2\text{H}_4]$; 337 $[M^{+} - \text{C}_2\text{H}_3 - (\text{C}_8\text{O})]$; type II, EI-MS (70 eV): $m/z = 396 [M^{+}]$, 381 $[M^{+} - \text{CH}_3]$; 367 $[M^{+} - \text{C}_2\text{H}_3]$; 365 $[M^{+} - \text{OCH}_3]$. The other two $^{18O}$ atoms originally present in dinor-iPE$_1$ were lost during the base-catalyzed dehydration process.

Lipoxigenase-catalyzed Formation of Two Series of Dinor Isoprostane $E_1$ Regioisomers from $\alpha$-Linolenic Acid—We first analyzed linolenic acid for the presence of any autoxidized material. Interestingly, on a molar basis linolenic acid (puriss. p.a., stored under argon at $-20^\circ$C) was found to be contaminated with 0.72% hydroperoxy compounds and 0.02% dinor isoprostanes $E_1$ type I and type II in a ratio of 1.0:8. A ratio of dinor-iPE$_1$ type I/type II of 1.0:0.75 ± 0.2 is typically found when linolenic acid is autoxidized in water/methanol or without any solvent (see Fig. 3). Therefore, the dinor isoprostane $E_1$ concentration was checked in every batch of linolenic acid before use, and the results were corrected accordingly. Alternatively, linolenic acid was purified by silica column chromatography (see above) before use.

To assess the time course of the formation of linolenic acid oxidation products generated by the action of LOX, linolenic acid (60 mg, 216 $\mu$mol) was dissolved in 1.2 ml of n-octane and incubated with 48 mg of soybean lipoxigenase in 600 ml of 100 mM sodium borate buffer, pH 7.5. The incubation mixture was sonicated for 1 min and stirred at room temperature. The linolenic acid hydroperoxide (HPOTE) concentration increased linearly over the first 2 h of incubation and then reached a plateau (see Fig. 4). Two h after onset of the reaction, an aliquot of the mixture was analyzed for hydroperoxy fatty acids by straight phase-HPLC and GC-MS as described under “Experimental Procedures.” The main compounds formed were 13- and 9-HPOTE in a ratio of 9:1. Only minor amounts of the 12- and 16-hydroperoxy isomers (<2%, see “Experimental Procedures” for details) were found, whereas 10- and 15-hydroperoxides were not detectable with HPLC.

In addition, dinor isoprostanes $E_1$ were quantitated by HPLC as their $B_1$-type derivatives. The time course of product formation is shown in Fig. 4. Synthesis of linolenic acid hydroperoxides and dinor isoprostanes $E_1$ occurred almost in parallel. Both theoretically possible dinor-iPE$_1$ regioisomers were formed: type II compounds, which are derived from 13-HPOTE radicals, and type I compounds, which must have been formed via the 12-HPOTE radical (see Fig. 1). The ratio of type I to type II regioisomers detected at different time points after the addition of LOX linearly increased from 1:0.80 at zero time (=$E_1$-dinor isoprostane contaminants of the starting material) to 1:1.5 after 5 h.

To explore the effect of LOX on dinor isoprostane $E_1$ formation in more detail, linolenic acid (5 mg, 18 $\mu$mol) dissolved in 100 $\mu$l of n-octane was incubated in 50 ml of borate buffer, pH 7.5, for 5 h in the presence and absence of LOX (4 mg) and analyzed for conjugated diene, HPOTE, and dinor-iPE$_1$ formation. Without LOX or with heat-inactivated LOX, only minute amounts of these products were formed (Fig. 5). Inclusion of the radical scavenger, BHT, resulted in a significant increase of conjugated diene compounds and hydroperoxides formed within 5 h (±61%) but efficiently suppressed the formation of dinor-iPE$_1$,$_8$ (−78%), indicating that dinor-iPE$_1$ synthesis involves free radicals. To further ascertain that LOX catalytic activity rather than a LOX product is responsible for the generation of dinor-iPE$_1$s, we incubated linolenic acid (18 $\mu$mol) with 13-hydroperoxylinolenate (9 $\mu$mol) and t-butyldihydroperoxide (18 $\mu$mol) in two separate experiments. The 13-HPOTE used for the assay was produced by LOX-catalyzed oxidation of linolenic acid, purified by reversed phase-HPLC, and quantitated before incubation with linolenic acid. However, both types of hydroperoxides had no significant effect on the autoxidation of $\alpha$-linolenic acid as indicated by only minor formation of conjugated dienes, hydroperoxides, and dinor-iPE$_1$s, suggesting that LOX activity is required to form significant amounts of dinor isoprostanes (Fig. 5).

In all these experiments, n-octane has been added to facilitate the dispersion of $\alpha$-linolenic acid and to increase the yield of prostaglandin-like cyclization products. However, omission of octane in the standard incubation mixture still resulted in the formation of dinor-iPE$_1$s but reduced the yield to 39%. Thus, a hydrophobic environment, i.e. n-octane droplets, increased the formation of dinor isoprostanes but was not strictly required. Increasing the pH of the borate buffer in the standard incubation to pH 9.6 also decreased the yield of dinor-iPE$_1$s (49%), which may be because of the base-catalyzed conversion of $E_1$ into $B_1$-type compounds, which will not be detected in our assay system.

Presence of Dinor Isoprostanes $E_1$ in Plants—Although the HPLC analysis method described above is rapid and allows the detection of 100 ng of dinor-iPE$_1$ in linolenate autoxidation mixtures, the sensitivity and specificity is not high enough to analyze these compounds in plants. For high sensitivity detection of these compounds, a NCI GC-MS method was employed. To identify unesterified dinor isoprostanes $E_1$ in plant cell cultures, cells of four taxonomically distant plant species were collected and shock frozen with liquid $N_2$. Dinor-iPE$_1$s were extracted, purified, converted into the corresponding dinor-
iPBs, and quantified by NCI GC-MS as outlined under "Experimental Procedures." Nicotiana tabacum (Solanaceae), Glycerine max (Fabaceae), Rauwolfia serpentina (Apocynaceae), and Agrostis tenuis (Poaceae) contained dinor isoprostanes E₁ of the type I and type II in the range of 4.5 - 61 ng/g of dry weight (Table I). A representative NCI GC-MS chromatogram (type I and type II in the range of 4.5 - 61 ng/g of dry weight) is shown in Fig. 6. Because of the low concentrations of unesterified dinor isoprostanes E₁ in plants, it was not possible to obtain EI mass spectra. To further ascertain the identity of these compounds, we prepared the dinor isoprostane ethers derivatives (Fig. 6). These derivatives showed the same GC-MS retention time as authentic reference compounds and yielded identical quantitative data as obtained with the TMS ether derivatives (Fig. 6).

**DISCUSSION**

Analysis of isoprostanes in vitro and in vivo generally requires a multistep purification procedure. In addition, quantification is difficult because of the presence of up to 16 theoretically possible stereoisomers for each regioisomer, which cannot all be separated by GC or HPLC procedures. The conversion of E-ring into B-ring compounds has been shown to facilitate the work-up procedure for GC-MS analysis leading to highly purified dinor-iPB₁ samples (see "Experimental Procedures" for details). Furthermore, by this procedure the 32 stereoisomers of dinor-iPE₁ are converted into 2 racemic dinor-iPB₁ compounds (Fig. 2). On GC and HPLC, the two dinor-iPB₁ peaks are well separated (Fig. 3 and 6) and represent an integrative measure for the type I and type II dinor-iPE₁ regioisomers, thereby enhancing the limit of detection considerably. In contrast to E-, D- and F-ring (dinar) isoprostanes, it is possible to detect B-ring compounds at a convenient wavelength (278 nm) that allows the straightforward quantification of these compounds by HPLC. In vitro synthesis of 18O-labeled and unlabeled dinor isoprostanes yielded mg quantities of these compounds, which were ideally suited as internal standards and reference compounds for GC-MS analysis.

We speculated that bicycloendoperoxides might be generated more easily and in a regiosomeric pure form from autoxidation of 13(S)-HPOTE, which is the main product produced from linolenic acid by many plant lipoxygenases. Therefore, we incubated linolenic acid with soybean lipooxygenase, which catalyzes the formation of predominately 13(S)- and 9(S)-HPOTE in a ratio of 9:1. Because 13-HPOTE but not 9-HPOTE can autoxidize to bicycloendoperoxides, we expected the formation of regiosommer II almost exclusively. Furthermore, the initially formed bicycloendoperoxides are prone to rapid rearrangement of the endoperoxide group to yield dinor isoprostanes E₁ in an aqueous environment with a t₁/₂ of less than 10 min as has been shown for the corresponding C₂₀ endoperoxide PGH₂. Surprisingly, we observed not only the formation of type II- but also of type I-dinar isoprostanes E₁ in lipoxygenase/linolenic acid incubations (Fig. 4 and 5). This result together with the finding that dinor-iPE₁ formation was strongly inhibited by BHT suggests that both regioisomers are formed predominately from linolenic acid by a free radical-catalyzed mechanism. However, some of the dinor isoprostane E₁ type II compounds were apparently formed from LOX-dependent free radical-catalyzed oxidation of 13-HPOTE, because the ratio of type II to type I dinor-iPE₁ increased from 0.75 (typical ratio observed after linolenate autoxidation) to 1.5 during the first...

**TABLE I**

| Plant            | Dinor-iPE₁ type I | Dinor-iPE₁ type II |
|------------------|-------------------|--------------------|
| N. tabacum       | 14.7 ± 2.6        | 16.9 ± 1.8         |
| G. max           | 5.1 ± 1.4         | 5.2 ± 1.2          |
| R. serpentina    | 26.2 ± 4.8        | 34.7 ± 1.8         |
| A. tenuis        | 2.6 ± 0.6         | 1.9 ± 0.6          |

**FIG. 2.** Base-catalyzed dehydration of dinor isoprostane E₁. Two regioisomers (type I and II) of dinor isoprostanes E₁ comprised of 32 stereoisomers can be converted to 2 racemic dinor isoprostanes B₁ (type I and II) by brief exposure (10 min, room temperature) to potassium hydroxide.

**FIG. 3.** Reversed-phase HPLC analysis of linolenate autoxidation products after base treatment. Aliquots of linolenate autoxidation mixtures were spiked with 5 μg of PGE₁ and worked up as described under "Experimental Procedures." Peaks I and II correspond to dinor isoprostane B₁, type I and II, respectively. The UV spectra of the compounds are shown as the inset.

**FIG. 4.** Time course of lipoxygenase-dependent oxidation of linolenate. The fraction of oxidized starting material (mol/mol) recovered as dinor isoprostanes E₁, type I (△) and II (●), conjugated dienes (○), and peroxides (◆) after different incubation periods was determined as outlined under "Experimental Procedures."
different treatments. In six separate experiments, linolenate (18 μmol) was incubated with LOX (4 mg), LOX (4 mg) together with BHT (180 μmol), heat-inactivated LOX (4 mg), 18/3-HPOTE (9 μmol), tert-butylhydroperoxide (BuOOH, 18 μmol), or without the additions, respectively. After 5 h of incubation, the yield (mol/mol) of dinor isoprostanes E1 type I (solid bars) and type II (open bars), conjugated dienes (solid bars), and peroxydes (open bars) was quantitated. Values are mean ±S.D. (n = 3).

2 h of incubation while LOX was active.

It is well known that (especially under low oxygen tension) many lipoxygenase isozymes can generate singlet oxygen and free alkoyl radicals that may leak out from the active site during the catalytic cycle, i.e. formation of 1-hydroperoxy-2(E),4(Z)-pentadiene systems from 1(Z),4(Z)-pentadiene structures, reviewed in Ref. 18. These radicals may attack the lipoxygenase leading to time-dependent suicide inhibition of the enzyme but are also responsible for so-called secondary reactions such as actinoid and chlorophyll bleaching in vivo (19). Thus, BHT may have a dual effect by scavenging free radicals generated by LOX anaerobic cycling, namely preventing suicide inactivation of LOX resulting in higher HPOTE formation and suppression of isoprostane F2 formation. Hydroperoxides such as 13-HPOTE and tert-butyl hydroperoxide have been shown to be inefficient inducers of linolenate autoxidation (Fig. 5). After 5 h of incubation while LOX was active.

FIG. 5. Formation of linolenate oxidation products after different treatments. In six separate experiments, linolenate (18 μmol) was incubated with LOX (4 mg), LOX (4 mg) together with BHT (180 μmol), heat-inactivated LOX (4 mg), 18/3-HPOTE (9 μmol), tert-butylhydroperoxide (BuOOH, 18 μmol), or without the additions, respectively. After 5 h of incubation, the yield (mol/mol) of dinor isoprostanes E1 type I (solid bars) and type II (open bars), conjugated dienes (solid bars), and peroxydes (open bars) was quantitated. Values are mean ±S.D. (n = 3).

A variety of peroxidation products is formed in plant cell cultures, such as 13-HPOTE and tert-butyl hydroperoxide, which can be detected in vivo with NMR and GC-MS measurements. The formation of these products is dependent on the oxygen tension and the presence of antioxidants such as BHT. Furthermore, the formation of these products appears to be inhibited by the presence of LOX, suggesting a role for LOX in the generation of reactive oxygen species.

Interestingly, synthesis of PGF2 from arachidonic acid by soybean LOX-2 was reported by Axelrod and co-workers in 1978 (20). However, the reaction mechanism leading to the formation of apparently several prostaglandin-like compounds has not been clarified. Because the stereochemistry of the PGF2-like product(s) has not been established, it is possible that not PGF2 but rather isoprostanes F2 were formed, some of which exhibit similar EII GC-MS properties as PGF2-ω2.

To investigate if dinor isoprostanes can be generated in vivo, we analyzed different plant cell cultures for the presence of dinor isoprostanes E1, Linolenic acid is a major constituent of plant membranes where it is predominately esterified in glycolipids, phospholipids and neutral lipids. However, in contrast to mammalian cells, only 0.25% of the total fatty acids are found unesterified as free acids in comparable high concentrations (about 8 μg/g of dry weight (21)). Analysis of four different plant species revealed that both theoretically possible dinor isoprostane E1 regiosomers (Fig. 6, Table I) are present as free acids at low concentrations. In analogy to the pathway in animals, it is likely that even higher levels of dinor isoprostanes are found esterified in membranes rather than unesterified in the cytosol and that dinor isoprostanes F1a and D1 may also be present in plants.

Isoprostanes, however, cannot generally be formed in plants because arachidonate is rarely found in significant quantities in higher plants (22). In contrast, dinor isoprostane formation from α-linolenate may not be significant in animals because of the relatively low concentration of this fatty acid in mammalian membranes.

Thus, parallel pathways in plants and animals are operative, each leading to a series of characteristic prostaglandin-like compounds. Dinor isoprostanes may be suitable markers of lipid peroxidation in plants or plant products similar to the isoprostanes, which have been demonstrated to represent specific and reliable markers of oxidant stress in the animal kingdom. In the future, it will be exciting to study if there is also a functional analogy between the mammalian isoprostane and the plant dinor isoprostane pathway. These are fundamental issues to be addressed in the future.

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