Analysis of Oxidative Stress and Wound-Inducible Dinor Isoprostanes F_1 (Phytoprostanes F_1) in Plants[1]

Ruth Imbusch and Martin J. Mueller*

Julius-von-Sachs-Institute, Pharmaceutical Biology, University of Wuerzburg, Julius-von-Sachs-Platz 2, D–97082 Wuerzburg, Germany

Isoprostanes F_2 are arachidonate autoxidation products in mammals that have been shown to be induced during several human disorders associated with enhanced free-radical generation. Isoprostanes F_2 represent not only extremely reliable markers of oxidative stress in vivo, but they also exert potent biological effects. Therefore, it has been postulated that isoprostanoids are mediators of oxidant injury in vivo. Higher plants, however, do not synthesize arachidonic acid or isoprostanes. Here we show that a series of isoprostane F_2 analogs termed phytoprostanes F_1 (previously dinor isoprostanes F_1) are formed by an analogous pathway from α-linolenate in plants. High-performance liquid chromatography and gas chromatography-mass spectrometry methods using $[^{18}O]_3$phytoprostanes F_1 as internal standard have been developed to quantify phytoprostanes F_1. In fresh peppermint (*Mentha piperita*) leaves, phytoprostanes F_1 were found in free form (76 ng/g of dry weight) and at about 150-fold higher levels esterified in lipids. It is notable that these levels of phytoprostanes F_1 are more than two orders of magnitude higher than the basal levels of isoprostanes F_2 in mammalian tissues. Furthermore, wounding, as well as butyl hydroperoxide or cupric acetate stress triggered a dramatic increase of free and esterified phytoprostanes F_1. Thus phytoprostanes F_1 may represent a sensitive measure of oxidative damage in plants similar to isoprostanes in mammals. However, one of the most exciting issues to be clarified is the possibility that linolenate-derived phytoprostanes F_1 exert biological activities in plants and/or animals.

Free radical-catalyzed oxidation of arachidonate has been shown to lead to a complex isomeric mixture of prostaglandin-like compounds termed isoprostanes. Isoprostanes have attracted considerable attention since it has been shown that isoprostanes are formed in mammals at concentrations that exceed the levels of enzymatically formed prostaglandins by at least an order of magnitude (Morrow and Roberts, 1997). Furthermore, isoprostanes are dramatically induced by oxidative stress (Lawson et al., 1999) and have been shown to be biologically active in the low nanomolar range. Therefore, it has been postulated that isoprostanes represent mediators of oxidative cell injury (Rokach et al., 1997a). Since isoprostanes F_2 represent chemically stable end products of lipid peroxidation, they have found to be extremely reliable and sensitive markers of oxidative stress in vivo (Morrow et al., 1999).

Higher plants generally do not synthesize the precursor arachidonate required for isoprostane formation, but rather utilize α-linolenic acid (the predominant polyunsaturated fatty acid in plants) for the formation of isoprostanoids and prostaglandin-like mediators of the jasmonate-type (Mueller, 1998). Linolenic acid (C18:3) can undergo free radical-catalyzed oxidation reactions similar to arachidonate (C20:4; Pryor et al., 1976; O'Connor et al., 1984) to yield C18 isoprostanoids (Fig. 1). Because the isoprostanoids derived from linolenate are two methylene groups shorter than those from C20 acids we have previously termed them dinor isoprostanes (Parchmann and Mueller, 1998; Mueller, 1998). However, C18 compounds derived from β-oxidation of isoprostanes (C20) in animals are also termed dinor isoprostanes, but differ from the plant dinor isoprostanes by the length of the two side chains (Chiabrando et al., 1999). To avoid confusion of terminology we suggest to term the plant prostanoids phytoprostanes.

We have recently elucidated the phytoprostane pathway in plants and have established that a group of prostaglandin/isoprostane E_1- and F_1-like compounds is formed in plant cells in vivo (Parchmann and Mueller, 1998; Imbusch and Mueller, 2000). The phytoprostane pathway theoretically yields several classes of phytoprostanes denoted with letters that specify the prostaglandin ring system according to the prostaglandin/isoprostane nomenclature (Fig. 1; Rokach et al., 1997b; Mueller, 1998). Each class represents a complex isomeric mixture that consists of two regioisomers (type I and type II). In addition, each regioisomer is theoretically comprised of 16 stereomers. In analogy to the mammalian isoprostane pathway, it is to be expected that phytoprostanes F_1 (PPF_1) are among the most abundant isoprostanoids in plant tissues. Due to the isomeric complexity of PPF_1, identification and quantitation of this class of compounds is technically difficult.

[1] This work was supported by the Sonderforschungsbereich 369 of the Deutsche Forschungsgemeinschaft, Bonn.

* Corresponding author; e-mail Martin.Mueller@botanik.uni-wuerzburg.de; fax 49–931–888–6182.

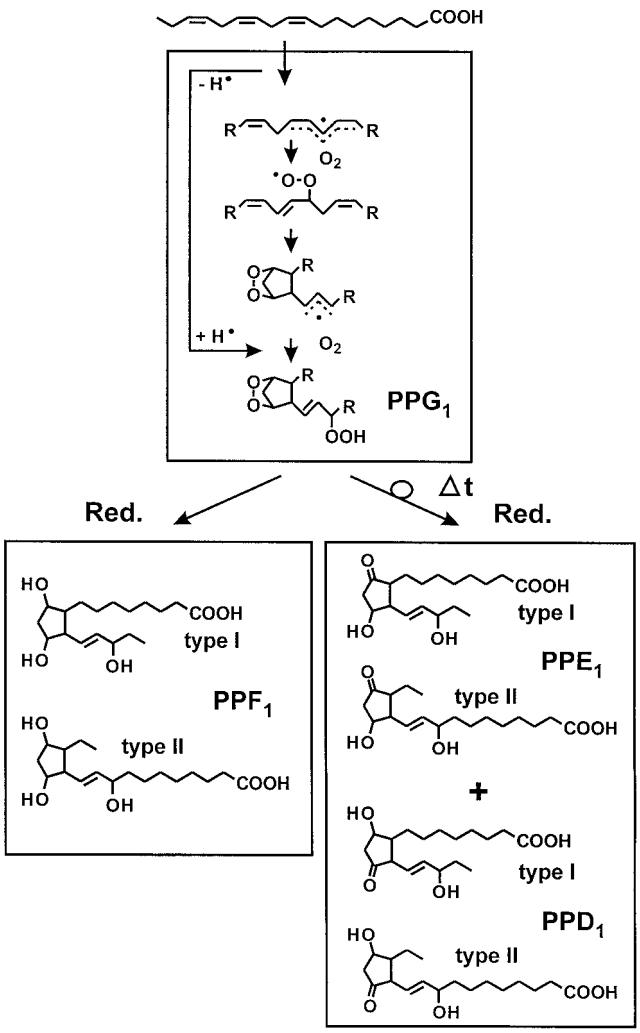

Figure 1. Free radical-catalyzed formation of phytoprostanes from linolenic acid. Hydrogen abstraction from one of the two bisallylic methylene groups of linolenic acid yields pentadienyl radicals that are prone to oxygenation and cyclization. Because the methyl and the carboxy chain (abbreviated with R) at the triene unit of linolenic acid are mechanistically equivalent, two regioisomeric PPG_1 can be generated. The two regioisomers of the unstable PPG_1 (each theoretically comprised of 16 stereomers) are precursors of type I and type II PPF_1 (formed by reduction of both peroxy groups), as well as of PPE_1 and PPD_1 (formed by rearrangement of the endoperoxy group followed by reduction of the hydroperoxy group).

Prostanoid analysis in plant tissues is further complicated by the occurrence of acyclic trihydroxylated fatty acid derivatives (Fig. 2) that have the same M_r as the cyclic trihydroxylated fatty acid isomers of the PPF_1-type. Acyclic triols show similar chromatographic mobilities as prostanoids and may erroneously be identified as prostaglandins in various prostaglandin immuno- and bioassays (Hamberg and Gardner, 1992). Various acyclic triols may be formed via the peroxygenase or epoxy alcohol synthase pathway in plants (Panossian et al., 1983; Hamberg and Hamberg, 1996; Hamberg, 1999). In addition, nonenzymatic formation of several acyclic triols by autoxidation (O'Connor et al., 1981) and photooxidation (Frankel, 1998) may not only take place in vitro, but also in vivo. Preparative and analytical methods were developed to specifically analyze and quantitate PPF_1 in vitro and in vivo. We show that PPF_1 are major products of the isoprostanoid pathway in plants (Fig. 1). Furthermore, it is demonstrated that PPF_1 accumulate dramatically in oxidatively stressed plant cell cultures and in wounded plants.

RESULTS

Preparation of PPE_1 and PPF_1

PPE_1 and PPF_1 were produced by autoxidation of α-linolenate in two different solvent systems. Bicyclic endoperoxides with the prostaglandin G-ring system are proposed to be common short-lived intermediates in the biosynthesis of phytoprostanes that readily rearrange to PPD_1 and PPE_1 in protic solvents (Fig. 1). Since endoperoxides are much less prone to spontaneous rearrangement in aprotic solvents, PPF_1 may favorably be formed in aprotic solvents by reduction of endoperoxides (Fig. 1). To produce PPF_1, linolenate (5 g) was dissolved in 50 mL of carbon tetrachloride and stirred under air for 10 d. In an alternate manner for optimized synthesis of PPE_1, a mixture of methanol/water (1:1, v/v) was chosen as solvent. Phytoprostanes were quantified by gas chromatography-mass spectrometry (GC-MS) as described below. Yields of PPF_1 were 2.3 mg/g of linolenate in the protic solvent and 11.7 mg/g in the aprotic solvent

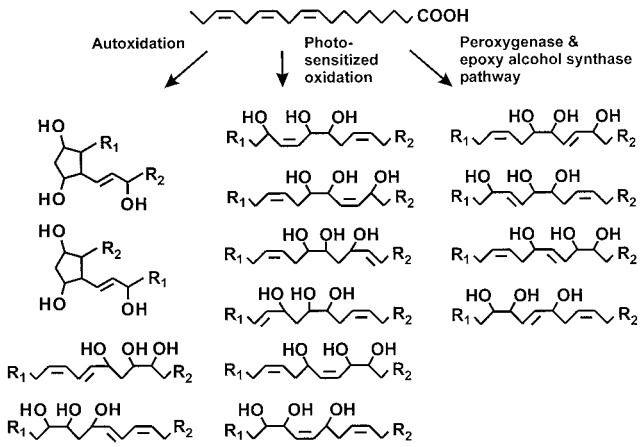

Figure 2. Formation of cyclic and acyclic trihydroxylated linolenic acid derivatives. Each cyclic triol structure (PPF_1) shown is composed of 16 isomers. Acyclic triol structures formed by either autoxidation or photooxidation represent eight isomers. Under the conditions of photooxidation in vivo and in most in vitro systems, autoxidation cannot be prevented. Thus autoxidation products such as phytoprostanes cannot be used to differentiate between autoxidation and photooxidation. Acyclic triols produced via the peroxygenase or epoxy alcohol synthase pathway are single, enantiomerically pure isomers. The residues R_1 and R_2 represent CH_3 and $(CH_2)_6COOH$, respectively.

Table I. *Relative formation of phytoprostanes F_1 and E_1 in vivo and in vitro*

Levels of free and esterified phytoprostanes in peppermint leaves (micrograms per gram of dry wt.), as well as levels of phytoprostanes in autoxidized linolenate samples (micrograms per gram of linolenic acid) were determined by GC-MS. Analysis were performed in triplicate. Values are means ± SD (n = 3).

Sample	PPF_1	PPE_1	Ratio of PPF_1 to PPE_1
		μg/g	
Fresh leaves			
Free	0.076 ± 0.004	0.037 ± 0.005	2:1
Esterified	11.24 ± 1.52	0.13 ± 0.004	86:1
Autoxidized linolenate			
Protic solvent	2,300	5,600	0.41:1
Aprotic solvent	11,700	300	39:1

(Table I). The protic solvent favored formation of PPE_1 (2.4-fold excess over PPF_1), whereas the aprotic solvent strongly favored PPF_1 formation (39-fold excess over PPE_1).

Purification, Identification, and Quantification of PPF_1

Autoxidation of linolenic acid as described above yields a complex mixture of peroxidized compounds. Endoperoxy and hydroperoxy groups of the reaction products were reduced to the corresponding hydroxy compounds by incubation of the autoxidation mixture with triphenylphosphine (1 g/g linolenic acid) for 30 min. A purification method was developed using tritiated prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) as tracer to optimize and calculate the recovery of prostaglandin F-like compounds. The reduced linolenate autoxidation mixture (equivalent to 300 mg of fatty acid) was purified by silica solid phase extraction on columns containing 500 mg of silica (see "Materials and Methods" for details). The separation efficiency was checked by scintillation counting of the radioactivity in different fractions. Recovery of $PGF_{2\alpha}$ in the sample was 98% ± 1.6%. In addition, the absence of phytoprostanes in the washing fluids and the presence in the eluate was verified by thin layer chromatography (TLC) analysis using PPF_1 prepared by $NaBH_4$ reduction of authentic PPE_1 (Parchmann and Mueller, 1998) as reference compounds.

The mixture of PPF_1, theoretically comprised of 32 isomers, was analyzed by HPLC. PPF_1 were partially resolved on a C18 reversed phase HPLC column (Fig. 3). GC-electron impact (EI)-MS analysis of the HPLC peaks after methylation and silylation of the samples showed that only peaks denoted with 1 to 4 eluted as single peaks from the GC column. PPF_1 regioisomers were identified by their EI spectra (Fig. 4, A and B). PPF_1 type I and type II reference spectra were recorded from PPF_1s obtained by $NaBH_4$ reduction of authentic PPE_1 type I and type II compounds (Parchmann and Mueller, 1998). HPLC peaks 1 and 3 contained PPF_1 type I isomers and HPLC peaks 2 and 4 contained type II isomers. HPLC peaks labeled with 5 to 7 (Fig. 3) contained mixtures of PPF_1 type I and II diastereomers.

High resolution MS revealed that PPF_1 regioisomers produce ions (Fig. 4, A and B) at *m/z* 217.1074 ($C_9H_{21}Si_2O_2$, trimethylsilyl [TMS]-*O*-CH=CH-CH=O^+-TMS) and 191.0928 ($C_7H_{19}Si_2O_2$, TMS-O^+=CH-*O*-TMS). These ions are indicative for F-ring prostanoids as they are generated by intramolecular rearrangement reactions of the cyclopentane F-ring system (Roberts et al., 1998). In addition, both regioisomers form ions at *m/z* 529.3207 ($C_{26}H_{53}Si_3O_5$, loss of C_2H_5), 468.3101 ($C_{25}H_{48}Si_2O_4$, loss of TMS-hydroxy [OH]), and 378.2578 ($C_{22}H_{38}SiO_3$, loss of 2 × TMS-OH). Fragment ions that are indicative for PPF_1 type I (Fig. 4A) were identified at *m/z* 439.2703 ($C_{23}H_{43}Si_2O_4$, loss

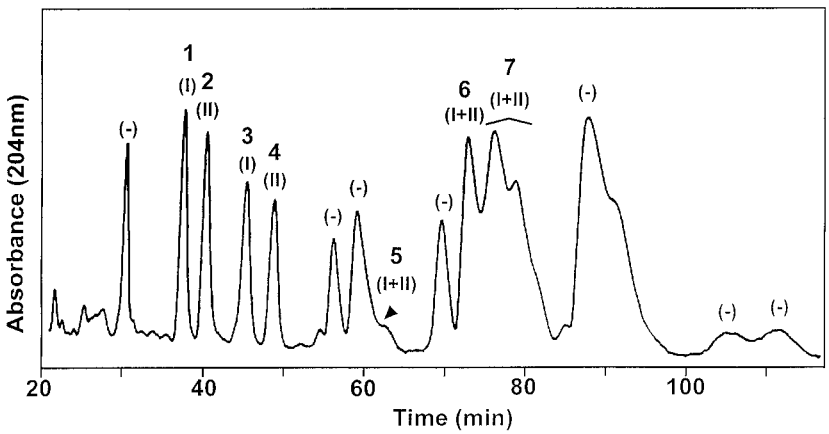

Figure 3. HPLC analysis of PPF_1. PPF_1 were identified in peaks labeled 1 to 7 and the regioisomeric composition (type I, type II, or both, as indicated in brackets) was established (see "Materials and Methods" section for details). Peaks labeled with (−) did not contain PPF_1.

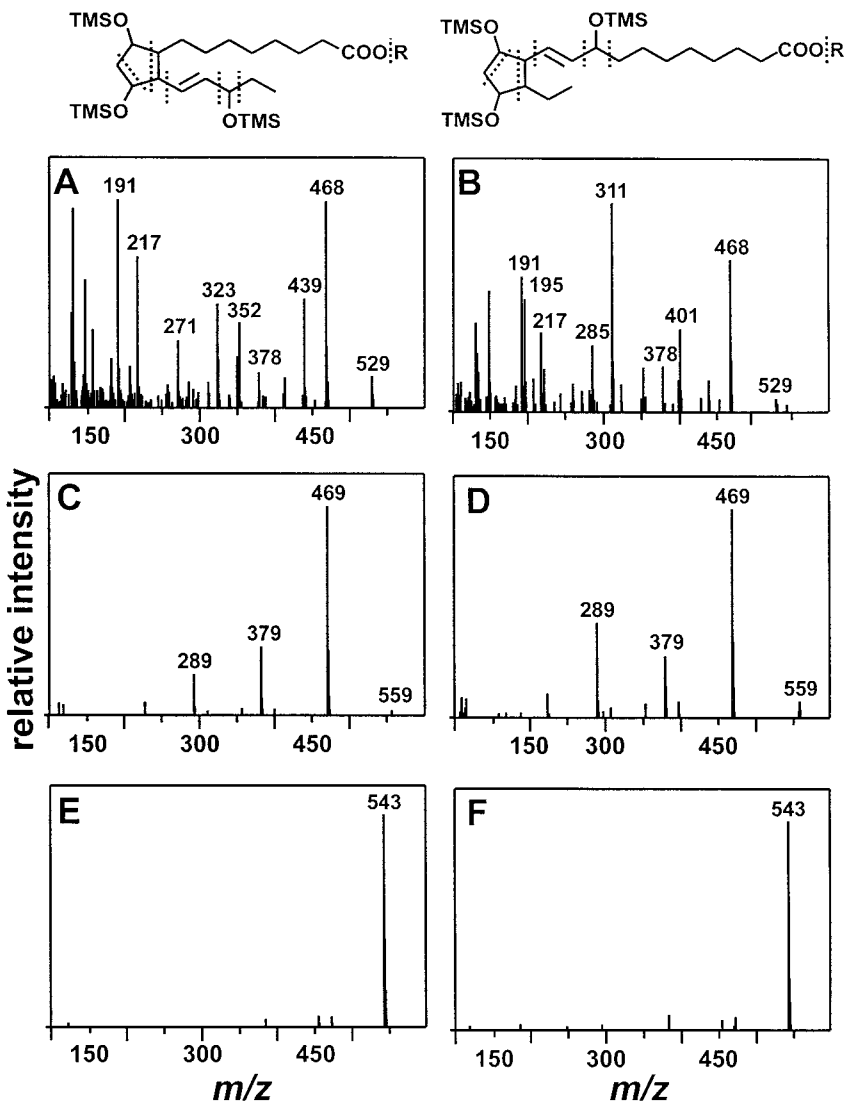

Figure 4. MS of PPF_1-type I and type II derivatives. PPF_1 were analyzed as their corresponding methyl ester, TMS ether derivatives in the EI (spectra A and B) as well as in the CI (spectra C and D) mode. PPF_1 were also analyzed as their PFB ester, TMS ether derivatives in the negative chemical ionization mode (spectra E and F). Type I compounds (A, C, and E) can only be differentiated from type II compounds (B, D, and F) by EI-MS. Characteristic cleavage points were indicated at the molecular formulae, see "Results" for details.

of C_2H_5 and TMS-OH), 323.2041 ($C_{18}H_{31}SiO$, loss of C_2H_5, CH_2CH-OTMS, and TMS-OH), and 131.0889 ($C_6H_{15}SiO$, $TMSO^+=C_3H_6$). Fragment ions indicative for PPF_1 type II compounds (Fig. 4B) are found at *m/z* 401.2342 [$C_{19}H_{41}Si_3O_3$, loss of $(CH_2)_7COOCH_3$], 311.1850 [$C_{16}H_{31}Si_2O_2$, loss of $(CH_2)_7COOCH_3$ and TMS-OH], and 195.1192 [$C_{11}H_{19}SiO$,loss of TMSO-$CH(CH_2)_7COOCH_3$ and TMS-OH]. Different isomers of each regioisomer could, however, not be differentiated by MS.

The methyl ester TMS ether derivatives of PPF_1 type I and II were also analyzed by positive chemical ionization-MS (Fig. 4, C and D). The predominant ions produced from type I and II compounds are the molecular ion $[M+H]^+$ at *m/z* 559 and three fragments at *m/z* 469, 379, and 289 derived from the sequential cleavage of TMS-OH from the three TMS ether groups of the molecules. The intensity of these ions varies greatly among different PPF_1 isomers. It is not possible to differentiate type I and II compounds by their chemical ionization spectrum.

PPF_1 were also analyzed as their corresponding pentafluorobenzyl (PFB)-ester, TMS derivatives by GC-MS in the negative ion chemical ionization (NICI) mode, which is an extremely sensitive detection method for prostanoids. NICI spectra of PPF_1 type I and II were identical, displaying only one major fragment ion at *m/z* 543 corresponding to the $[M\text{-}PFB]^-$ anion (Fig. 4, E and F).

Synthesis of Oxygen-18-Labeled PPF_1

GC-MS analysis requires an appropriate internal standard. In the case of PPF_1, which represents a mixture of 32 isomers, the use of a commercially available C20 homolog such as PGF_1 would be straightforward, but unlikely to be reliable since various PPF_1 isomers and PGF_1 probably would have

different chromatographic properties and ionization efficiencies. The most appropriate retention time and quantification standard was found to be an isotopically labeled PPF_1 isomer mixture, which was prepared by oxidation of linolenate in an oxygen-18 atmosphere. GC-MS analysis of oxygen-18 labeled PPF_1 revealed that each isomer contained three [^{18}O]hydroxy groups as predicted by the mechanism of formation (Fig. 1) and that the GC peak pattern matched exactly that of the unlabeled PPF_1 isomer mixture (data not shown). An unlabeled PPF_1 standard solution of known concentration (see "Materials and Methods") was used to quantify the mixture of $[^{18}O]_3PPF_1$ by GC-NICI-MS.

GC-NICI-MS of Phytoprostanes in Plant Material

The most sensitive method for quantifying prostanoids by GC-MS described in the literature involves analyzing the PFB ester, TMS ether derivatives in the NICI mode. A drawback of the method is, however, that various enzymatically and nonenzymatically formed acyclic, trihydroxylated derivatives of linolenate cannot be differentiated from PPF_1s since they may behave similar on GC-MS and would yield ions of identical mass (O'Connor et al., 1984). However, acyclic oxygenation products of linolenic acid could not be detected by GC-EI-MS analysis of purified linolenate autoxidation mixtures (see above). In contrast, PPF_1 samples purified from plant sources with the sample preparation procedure used for purification of PPF_1 from linolenate autoxidation mixtures revealed a peak pattern on GC that largely matched the pattern of the internal standard, but showed additional non-PPF_1 derived peaks at *m/z* 543. A representative example (analysis of dried peppermint [*Mentha piperita*] leaves) is shown in Figure 5A. Because phytoprostanes F_1 contain only one double bond, whereas all acyclic triols contain two double bonds (Fig. 2), hydrogenation of the samples increases the molecular mass of the compounds by 2 and 4, respectively. Thus PPF_1 can be selectively quantitated by GC-NICI-MS even in the presence of acyclic triols. Levels of free PPF_1 in dried peppermint leaves were found to be 20.01 ± 0.76 μg/g of dry weight. After hydrogenation, the peak pattern of PPF_1 formed within the plant (at *m/z* 545) matched almost perfectly the peak pattern of synthetic $[^{18}O]_3PPF_1$ at *m/z* 551 (Fig. 5B). The major peaks in the chromatogram of the internal standard have all been shown to represent mixtures of PPF_1 regioisomers of type I and type II by co-injection of different purified PPF_1 isomers. The presence of a family of PPF_1 isomers in dried peppermint leaves, which apparently has the same isomer distribution as PPF_1 from autoxidized linolenate suggests that PPF_1 have been formed by autoxidation of endogenous linolenate.

Assay Parameters and Validation

The recovery of PPF_1 in different steps of the sample preparation method was estimated by spiking 300 mg of lime tree flowers (dry powder) with [5,6,8,9,11,12,14,15-^{3}H]PGF_2 (specific activity of 179.00 Ci/mmol) prior to work-up. Recovery of radioactivity after various processing steps was determined by scintillation counting. Ether extraction (used for the measurement of free PPF_1) and modified Folch extraction/base hydrolysis (used for the determination of esterified plus free PPF_1), as well as the following silica solid phase extraction step were all performed with a recovery greater than 90%. After full sample preparation 60.57% ± 1.48% and 31.27% ± 8.97% of the

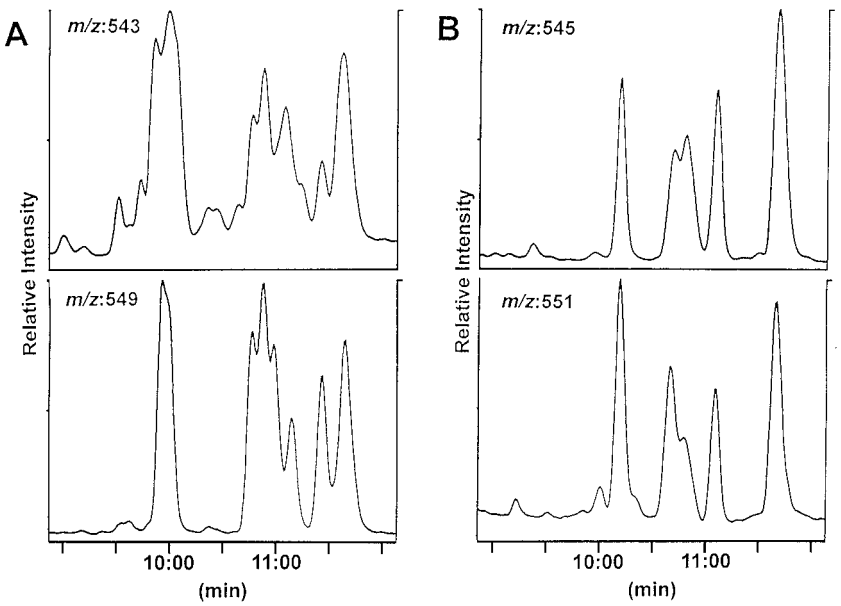

Figure 5. GC-NICI-MS analysis of PPF_1 in dried peppermint leaves. PPF_1 were determined as PFB ester, TMS ether derivatives without (A) and with (B) prior catalytic hydrogenation. Ion chromatograms at *m/z* 543 and 545 display endogenous PPF_1 (20.01 ± 0.76 μg/g of dry weight), whereas *m/z* 549 and 551 display the triply oxygen-18 labeled internal standard. For quantification, peaks in the chromatograms of sample B were integrated together and the endogenous level of PPF_1 was calculated from the ratio of the peak areas at *m/z* 545 to that at *m/z* 551.

radioactivity were recovered in samples containing free and total PPF_1, respectively.

The quantification of endogenous PPF_1 was performed by using $[^{18}O]_3PPF_1$ as an internal standard by comparison of the intensity of the peaks at *m/z* 545 co-eluting with the peaks of the internal standard at *m/z* 551. The lower limit of detection of PPF_1 (signal to noise ratio of 5:1) is in the range of 100 pg. Several procedures were performed to establish the accuracy of this assay. Precision was measured by analyzing aliquots of tobacco cells (170 mg of dry weight) obtained from a cell suspension culture 3 d after transfer to fresh medium. The mean of six replicate measurements of the ratio of *m/z* 545 to *m/z* 551 was found to be 1.07% ± 0.16% and corresponded to a concentration of 36.67 ± 5.77 ng/g of dry weight. Accuracy was assessed using aliquots of the same tissue. For this, 10 to 1,500 ng of unlabeled PPF_1 and 500 ng of $[^{18}O]_3PPF_1$ were added to eight sample aliquots that were re-assayed in triplicate. A linear relationship between added and found PPF_1 was documented (r = 0.9890). The amount of endogenous PPF_1 measured in the precision experiment was subtracted from the total measured and the accuracy of the assay to measure the added 10 to 1,500 ng of PPF_1 was calculated. The accuracy of PPF_1 determinations varied with the level of PPF_1 to be measured (Fig. 6).

PPE_1 and PPF_1 Are Formed in Plant Cells in Vivo

Levels of PPE_1 and PPF_1 in free and esterified form in peppermint leaves, chosen as a representative example, were determined. In fresh peppermint leaves, the concentrations of unesterified phytoprostanes were 76 ± 4 ng/g of dry weight of PPF_1 and 37 ± 5 ng/g of dry weight of PPE_1. Levels of esterified PPF_1 and PPE_1 (11,240 ± 1,520 and 130 ± 4 ng/g of dry weight, respectively) were one to two orders of magnitude higher than those of free phytoprostanes (Table I).

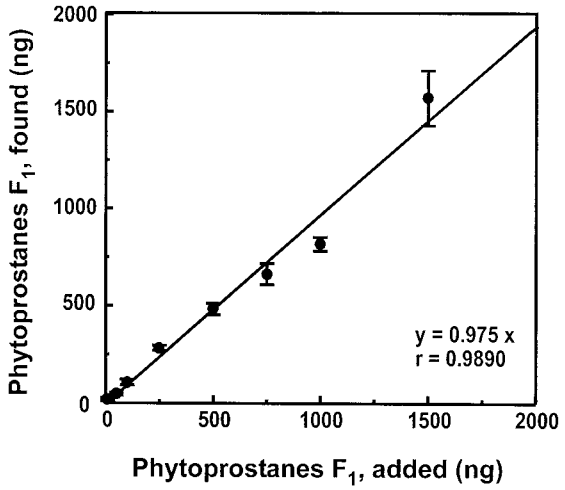

Figure 6. Accuracy and precision of the quantitative analysis of PPF_1. PPF_1 (1–1,500 ng) and $[^{18}O]_3PPF_1$ (500 ng, internal standard) were added to 170 mg (dry weight) of suspension culture cells of tobacco. PPF_1 were extracted and analyzed as described in "Material and Methods." Error bars indicate means ± SD (*n* = 3).

PPF_1 Levels Are Wound Inducible

To clarify if the large PPF_1 accumulation observed in harvested peppermint leaves (Fig. 5) is an early event triggered by wounding of living plants or occurs by autoxidation of lipids in dead plants we wounded fresh peppermint leaves with a nail stamp (12 nails/cm^2). Time-dependent PPF_1 accumulation was measured over 6 h after wounding (Fig. 7, E and F). The concentration of free PPF_1s increased and reached a maximum level of 192 ng/g of dry weight (more than 4-fold over control levels) within 1 h and declined thereafter. A second increase was observed after 3 h. Levels of esterified PPF_1 rose after wounding, reached a maximum level of 4,000 ng/g of dry weight (more than 5-fold over control levels) after 1.5 h and decreased thereafter. The PPF_1 isomer distribution was found to be similar to that of the synthetic $[^{18}O]_3PPF_1$ standard. Thus wounding appears to be accompanied by formation of free radicals, which trigger an appreciable synthesis of free and esterified PPF_1.

Although accumulation of PPF_1 in wounded plants does take place, levels of PPF_1 are well below the levels found in dried, stored plant powders, suggesting that massive PPF_1 formation takes place in dead plant material during storage.

Exogenous Addition of Butyl Hydroperoxide or Cupric Acetate Results in Dramatic Accumulation of PPF_1 in Plant Cells

To assess the effect of oxidative stress on PPF_1 levels in plant cells, California poppy (*Eschscholzia californica*) cell suspension cultures were treated with 10 mM *t*-butyl hydroperoxide. A dramatic increase of esterified PPF_1 followed by an increase of free PPF_1 was observed. Levels of esterified PPF_1 started to increase within 30 min and reached maximal levels (12 μg/g of dry weight) after 4 to 5 h (Fig. 7B). Levels of free PPF_1 increased after 3 h and reached a transient maximum (1.6 μg/g of dry weight) after 12 h (Fig. 7A).

In additional experiments, California poppy suspension cells were treated with 500 μM cupric acetate. A biphasic induction of PPF_1 levels was observed. Levels of esterified PPF_1 began to increase after a lag phase of 2 to 3 h and reached a first maximum (20 μg/g of dry weight) 6 h after copper(II) ion administration. Thereafter, PPF_1 levels declined and rose again after 14 h (Fig. 7D). Levels of free PPF_1 began to increase after a lag phase of about 4 h, reached a first maximum (2.8 μg/g of dry

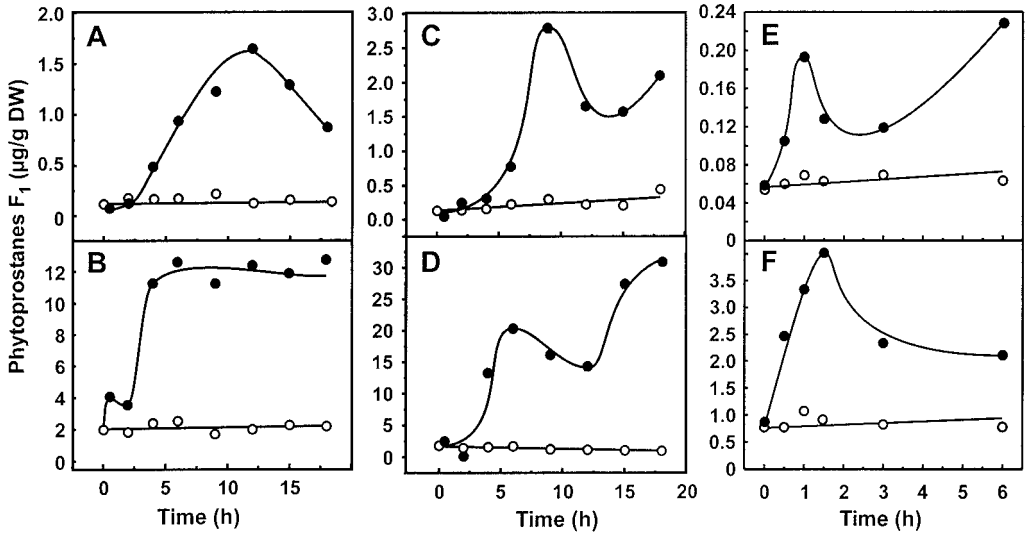

Figure 7. Induction of PPF_1 by oxidative stress and wounding. California poppy suspension cells were treated with 10 mM *t*-butyl hydroperoxide at zero time and levels of free (A) and esterified (B) PPF_1 (●) were monitored. The time course of free (C) and esterified (D) PPF_1 (●) in California poppy suspension cells was also monitored after application of 500 μM cupric acetate. The kinetic of free (E) and esterified (F) PPF_1s was measured in wounded peppermint leaves (●). Peppermint leaves were cut off at the stem and wounded with a nail stamp or left intact. Leaves were incubated in a chamber with a water-saturated atmosphere. Levels of PPF_1 (○) in untreated cells or leaves were determined in separate control experiments.

weight) after 9 h, declined thereafter, and increased again after 15 h (Fig. 7C).

DISCUSSION

Occurrence of Prostaglandin-Like Compounds in Plants

Since the discovery of prostaglandins in mammals, several reports have appeared describing the identification of prostanoids in plants and microorganisms (for review, see Bundy, 1985; Panossian, 1987; Hamberg and Gardner, 1992; Lamacka and Sajbidor, 1995). However, formation of prostaglandins in these organisms is difficult to rationalize since with the exception of some lower plants (algae, mosses, and ferns) and few filamentous fungi, the crucial substrate for prostaglandin synthesis, arachidonic acid, can generally not be biosynthesized by these organisms. Furthermore, prostaglandin-endoperoxide H synthase (cyclooxygenase) enzyme activity required for prostaglandin biosynthesis appears to be restricted to animals. A pathogen-inducible oxygenase with significant homology to mammalian cyclooxygenase-1 and 2 has recently been identified in tobacco (Sanz et al., 1998). However, the tobacco enzyme and its homologous enzyme from Arabidopsis have subsequently been shown to represent a novel α-dioxygenase, which catalyzes conversion of α-linolenic acid and other fatty acids into their 2(*R*)-hydroperoxy derivatives (Hamberg et al., 1999). Thus at present there exists no evidence for enzymatic formation of a prostaglandin H-like prostanoid precursor in plants. However, here we show that prostaglandin F_1-like PPF_1 can be generated from linolenic acid via a nonenzymatically formed prostaglandin H-like intermediate.

Analysis and Occurrence of PPF_1

For GC-NICI-MS analysis of PPF_1, an optimized sample preparation protocol has been established that largely removes acyclic trihydroxylated fatty acids. In addition, PPF_1 could be differentiated from remaining acyclic triols by MS after catalytic hydrogenation. Nonenzymatically formed PPF_1 yield a characteristic peak pattern in GC-NICI-MS chromatograms. The PPF_1 signature has been found in autoxidized linolenate samples, autoxidized plant drugs, in untreated cell cultures and plants, in copper(II) ion and peroxide stressed plant cell cultures, as well as in wounded plants.

We also demonstrated that PPF_1 occur in free and esterified form in plants. Esterified PPF_1 are likely located in membrane lipids as has been shown for isoprostanes F_2 in mammals (Morrow et al., 1992). It has been postulated that formation of esterified isoprostanes occurs by autoxidation of esterified arachidonate within the membrane where the majority of the fatty acid resides. Free isoprostanes in turn are thought to be generated predominantly by the action of lipases on preformed isoprostane esters in membrane lipids (Waugh et al., 1997). Because the majority of linolenate in plants occurs esterified (often more than 99% [Conconi et al., 1996]), a similar scenario may be assumed for the synthesis of plant phytoprostanes (Mueller, 1998).

In the species so far analyzed, levels of esterified PPF_1 were one to two orders of magnitude higher than free PPF_1. Furthermore, in butyl hydroperoxide or copper(II) ion treated cells, as well as in wounded plants, levels of esterified PPF_1 increased earlier and reached higher levels than free PPF_1 (Fig. 7), suggesting that reactive oxygen species (ROS) initiate PPF_1 synthesis in membrane lipids. However, if lipase-dependent release of PPF_1s from membranes occurs in plants remains to be shown.

In an alternate manner, it may be assumed that linolenate is liberated from membrane stores in various pathophysiological situations and may serve as precursor for free PPF_1. In the case of tissue injury by wounding, it has previously been shown that injury of tomato plants is accompanied by generation of ROS (Orozco-Cardenas and Ryan, 1999) and an increase of free linolenate (Conconi et al., 1996). Thus it is conceivable that ROS may catalyze not only formation of esterified PPF_1, but also of free PPF_1 from liberated linolenate. The data obtained so far are compatible with both concepts.

It is apparent that plants accumulate higher levels of PPF_1 than PPE_1. The preferential formation of PPF_1 is compatible with the concept that biosynthesis of phytoprostanes occurs in a hydrophobic environment such as a membrane, which would stabilize the endoperoxide intermediate and favor PPF_1, rather than PPE_1 formation (Fig. 1; Table I). In addition, nonenzymatic reduction of free and membrane-bound endoperoxide intermediates to F-ring prostanoids by glutathione appears to be more rapid than rearrangement of the endoperoxide intermediates to E-ring prostanoids, at least in mammals (Morrow et al., 1998).

Isoprostanoids as Marker of Oxidative Stress in Vivo

Perhaps the best established aspect of isoprostanoid research is the finding that isoprostanes can be used as a quantitative index of lipid peroxidation in humans. As they are chemically stable end products of lipid peroxidation they appear to be more reliable and accurate markers of oxidative stress than other oxidation products such as malondialdehyde, especially in vivo (Roberts and Morrow, 1997). F-ring isoprostanoids are generally preferred as markers since they are more abundant and stable than the other isoprostanoids. Increased isoprostane F_2 levels have been measured after intoxication and in several human disorders that are suspected to be associated with excessive generation of free radicals (Mueller, 1998; Lawson et al., 1999).

Here we show that PPF_1s accumulate in oxidatively stressed plant cells. Our preliminary findings suggest that PPF_1s may be useful as indicators of oxidative stress in plants. For instance, hydroperoxides and copper ions that are known to initiate lipid peroxidation (Frankel, 1998) dramatically elicit PPF_1 formation in vivo (Fig. 7).

Isoprostanoids May Deteriorate Membrane Integrity Leading to Cell Death

Formation of ROS, lipid peroxidation, loss of membrane integrity, and cell death are typical features of many abiotic stresses and the hypersensitive reaction of plants against incompatible microorganisms. ROS are thought to function as a signal that may induce defense gene activation and programmed cell death. In addition, excessive formation of ROS leads to cellular damage/cell death, primarily through damage to the photosystem II reaction center and to oxidation of membrane lipids (Bowler and Fluhr, 2000).

Esterified fatty acid oxidation products in membranes may disturb membrane integrity and induce plasma leakage. In this respect, esterified F-ring isoprostanoids have been postulated to participate in membrane leak development due to their remarkably kinked geometry, as well as the repulsion by the three newly created hydroxy groups and the adjacent lipophilic fatty acids in the membrane bilayer, leading to cell death (Morrow et al., 1992; Rokach et al., 1997a; Lawson et al., 1999). Other polyhydroxylated fatty acids that may be generated in addition to phytoprostanes by autoxidation of fatty acids in membranes have, to the best of our knowledge, not yet been quantified accurately.

A major source of oxygenated fatty acids in plants appears to be lipoxygenase acting on free fatty acids. For instance, fatty acid hydroperoxides were shown to increase from 25 to 335 $\mu g/g$ of fresh weight 30 h after application of cryptogein, an established inducer of hypersensitive reaction. It was estimated that 90% of the total fatty acid hydroperoxides were generated by lipoxygenase, whereas the remaining 10% represent membrane-bound fatty acid hydroperoxides (33.5 $\mu g/g$ of fresh weight) formed by free radical-catalyzed oxidation of membrane lipids simultaneously with the enzymatic process (Rusterucci et al., 1999). However, it is not known at which concentration different membrane-bound oxygenated fatty acid species become lethal to cells.

Biological Activity of Isoprostanoids

Several isoprostanes have been shown to have potent biological effects on mammalian tissues. For instance, isoprostanes F_2 act as broncho- and vasoconstrictors in the nanomolar concentration range, modulate platelet function, contract lymphatics, and may also induce mitogenesis (for review, see Mueller, 1998). It remains to be shown whether the structurally related inducible PPF_1 exert biological activity. However, orally administered prostanoids are slowly absorbed and rapidly metabolized and, thus may possibly not be effective in mammals.

It is notable that phytoprostanes are also structurally related to enzymatically formed linolenate metabolites of the jasmonate-type. Basal levels of phytoprostanes E_1 and F_1 are generally more than one

order of magnitude higher than jasmonic acid levels. However, if phytoprostanes play a role in plant stress physiology has not yet been addressed. This issue remains an exciting challenge for future research.

MATERIALS AND METHODS

α-Linolenic acid (puriss. p.a.) and *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) were purchased from Fluka (Neu-Ulm, Germany). Oxygen-$^{18}O_2$ gas (99.1 atom% ^{18}O) was obtained from Isotec (Miamisburg, OH). Silica solid phase extraction glass columns (500 mg) and silica TLC plates (Polygram Sil G/UV) were from Macherey and Nagel (Düren, Germany). Silica gel 60 (particle size 63–100 μm) for column chromatography was from Merck (Darmstadt, Germany). PFB bromide and *N,N*-diisopropylethylamine were from Sigma (Deisenhofen, Germany). [5,6,8,9,11,12,14,15-^{3}H]PGF_2 (specific activity of 179.00 Ci/mmol) was from NEN Life Science Products (Boston).

Preparation of PPF_1 and $[^{18}O]_3PPF_1$ by Linolenic Acid Autoxidation

Linolenic acid (5 g) was dissolved in hexane/diethylether (9:1, v/v), loaded on a silica column (10 g), and eluted with hexane/diethylether (9:1, v/v) to remove any preformed oxygenated fatty acids. The eluate was taken to dryness in a 500-mL round bottom flask and dissolved in 50 mL of carbon tetrachloride. The flask was evaporated in vacuo and filled with 500 mL of oxygen (or oxygen-18 gas, 99.1% atom ^{18}O in the labeling experiment). After stirring the mixture for 10 d at room temperature, the autoxidation mixture was reduced by the addition of 5 g of triphenylphosphine in 50 mL of chloroform and processed as described in "Purification of PPF_1."

Extraction of PPF_1 from Plant Material

For analysis of unesterified PPF_1, plant material (3 g of fresh weight or 0.3 g of dry weight) was suspended in 20 mL of cold brine containing 0.05% (w/v) of 2,6-di-*tert*-butyl-4-methylphenol, 20 mg of triphenylphosphine, and 0.2 mL of 1 M citric acid. $[^{18}O]_3PPF_1$s (500 ng) were added as an internal standard. After addition of 20 mL of diethyl ether the mixture was homogenized for 3 min with a high performance disperser (Ultra-Turrax T 25, IKA-Werk, Germany) at 24,000 rpm and centrifuged (2,000*g*, 10 min). The ether phase was removed, taken to dryness under a stream of nitrogen, and dissolved in chloroform.

For the determination of total PPF_1s (free and esterified), lipids were extracted using a modified Folch procedure. Plant material (1 g of fresh weight or 0.1 g of dry weight) was suspended in 18 mL of methanol/chloroform (2:1, v/v) containing 80 μL of 1 M citric acid, 0.115 mg of 2,6-di-*tert*-butyl-4-methylphenol, 20 mg of triphenylphosphine, and 500 ng of $[^{18}O]_3PPF_1$s. After homogenization of the mixture with a high performance disperser for 1 min, 18 mL of chloroform and 8 mL of a 0.88% (w/v) potassium chloride solution were added. The lower organic phase was separated and taken to dryness. The residue was reconstituted in 2 mL of methanol containing 0.1% (w/v) 2,6-di-*tert*-butyl-4-methylphenol and hydrolyzed after addition of 2 mL of 15% (w/v) KOH at 40°C for 1 h. The reaction was stopped by adding 4 mL of 1 M citric acid and 32 mL of water. Lipids were extracted with diethyl ether, taken to dryness, and reconstituted in 2 mL of chloroform.

Purification of PPF_1

Linolenic acid (5 g) autoxidation mixture was treated with triphenylphosphine (5 g) for 30 min. The mixture was taken to dryness, reconstituted in 50 mL of chloroform, and loaded on a silica column (4 × 21 cm, 30 g of silica gel 60). The column was washed with 600 mL of diethylether:acetic acid (99:1, v/v) and 300 mL of chloroform:acetone:acetic acid (80:20:1, v/v) to remove remaining linolenic acid, mono-oxygenated linolenate products, and triphenylphosphine. PPF_1 were chromatographed isocratically with diethylether:acetone:acetic acid (70:30:1, v/v). Sixty-milliliter fractions of the eluate were taken and aliquots were analyzed by TLC (see below) for the presence of PPF_1. PPF_1 eluted with 60 to 480 mL of eluent.

On an analytical scale, nonesterified PPF_1 from plant extracts or linolenate autoxidation mixtures were applied as a chloroform solution to a silica solid-phase extraction column (500 mg). The column was subsequently washed with 6 mL of diethyl ether:acetic acid (98:2, v/v) and 3 mL of chloroform:acetone:acetic acid (80:20:1, v/v). PPF_1 were eluted with 6 mL of diethyl ether:methanol:acetic acid (90:10:1, v/v), taken to dryness, and dissolved in 2 mL of methanol.

Preparation of PPF_1 Standard Solutions

A mixture of silica-purified PPF_1 was further purified on a reversed phase HPLC column (LiChrospher 100 RP18e, 5-μm particle size, 8 × 250 mm; Knauer, Berlin, Germany) eluted with acetonitrile:water:acetic acid (20:80:0.1, v/v) at a flow rate of 3.5 mL/min, and detected at 204 nm (Fig. 3). Fractions of the eluate were collected and PPF_1 isomers were identified by GC-EI-MS analysis after preparation of the corresponding methyl ester, TMS ether derivatives. PPF_1 isomers eluting under the peaks 1 to 4 (Fig. 3) were each mixed with 500 ng of prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) and esterified with 2,4′-dibromoacetophenone (100 μg) in 200 μL of acetonitrile containing 0.25% (w/v) diisopropylethylamine at 40°C for 90 min. The mixtures were taken to dryness, reconstituted in 2 mL of chloroform, and applied to a silica solid-phase extraction column. The column was washed with 3 mL of diethyl ether and the 4′-bromoacetophenone ester derivatives were eluted with 6 mL of diethylether:methanol (90:10, v/v). PPF_1 and $PGF_{1\alpha}$ derivatives were separated on a LiChrospher 100 RP18 endcapped column (5-μm particle size, 250 × 4 mm) eluted at a flow rate of 1 mL/min with a mixture of acetonitrile:water (40:60, v/v) for 15 min, followed by a gradient to 60% (v/v) acetonitrile over 15 min. The UV absorption of the 4′-bromoacetophenone moiety of the derivatives at 254

nm was used to detect the prostanoids and the amount of PPF_1 in each sample was calculated from the peak area of PPF_1 relative to that of $PGF_{1\alpha}$.

TLC Analysis of PPF_1

PPF_1 and $PGF_{1\alpha}$ were chromatographed on silica TLC plates developed with chloroform:methanol:water:acetic acid (86:14:0.8:1, v/v). F-ring prostanoids (5 μg) were detected by spraying the plate with 3% (w/v) of cupric acetate in 80% (v/v) phosphoric acid followed by heating at 60°C for 5 min. Prostaglandin F-like material yielded a characteristic violet color. $PGF_{1\alpha}$ migrated as a sharp band at R_F 0.51, whereas PPF_1 gave several bands in the R_F range of 0.37 to 0.78.

Analytical Derivatization Procedures for GC-MS Analysis

For GC-EI-MS, silica purified PPF_1 were converted to their corresponding methyl esters by treatment with an etheral solution of diazomethane for 5 min. The solution was taken to dryness and silylated with 50 μL of BSTFA in 200 μL of chloroform at 40°C for 30 min. The mixture was taken to dryness and dissolved in hexane for GC-MS analysis.

For routine GC- NICI-MS analysis, PPF_1 obtained from silica column purification were derivatized either directly or, in the case of plant material, after catalytic hydrogenation. For hydrogenation, PPF_1 were dissolved in methanol. Adam catalyst (20 mg) was added and hydrogen gas was bubbled through the sample for 10 min. The sample was filtered, taken to dryness, reconstituted in 200 μL of chloroform, and derivatized with 10 μL of PFB bromide and 10 μL of *N,N*-diethylisopropylamine at 60°C for 30 min. The mixture was taken to dryness, reconstituted in 2 mL of chloroform, and applied to a silica solid-phase extraction column. The column was washed with 3 mL of diethyl ether. PPF_1 PFB esters were eluted with 6 mL of diethyl ether:methanol (90:10, v/v). The sample was taken to dryness and TMS ether derivatives were prepared with BSTFA. For GC-MS analysis, the sample was dissolved in 20 μL of hexane and 1 μL was analyzed. Analyses of phytoprostanes E_1 (PPE_1) were performed as described (Parchmann and Mueller, 1998).

GC-MS

GC-NICI-MS was performed using a Varian 3400 gas chromatograph interfaced to a Finnigan MAT quadrupole SSQ 700 mass spectrometer. The MS source was set at 150°C and the electron energy at 70 eV. Isobutane was used as reactant gas when operating the instrument in the NICI mode. PPF_1 were analyzed on a 25 m, 0.25-μm film thickness Optima-5 column (Macherey and Nagel, Düren, Germany). The injector was set at 300°C.

For analysis of PFB ester, TMS ether derivatives of PPF_1, the column temperature was programmed from 175°C to 285°C at 30°C/min and 285°C to 300°C at 2.5°C/min. Methyl ester, TMS ether derivatives of PPF_1, were analyzed using a column temperature program from 140°C for 1 min, 140°C to 235°C at 20°C/min, 235°C to 250°C at 1°C/min, and 250°C to 300°C at 30°C/min.

Received February 22, 2000; accepted July 13, 2000.

LITERATURE CITED

Bowler C, Fluhr R (2000) The role of calcium and activated oxygens as signals for controlling cross tolerance. Trends Plant Sci **5:** 241–246

Bundy GL (1985) Non-mammalian sources of eicosanoids. *In* JE Pike, DR Morton, eds, Advances in Prostaglandin, Thromboxane, and Leukotriene Research, Vol 14. Raven Press, New York, pp 229–262

Chiabrando C, Valagussa A, Rivalta C, Durand T, Guy A, Zuccato E, Villa P, Rossi J-C, Fanelli R (1999) Identification and measurement of endogenous β-oxidation metabolites of 8-*epi*-prostaglandin $F_{2\alpha}$. J Biol Chem **274:** 1313–1319

Conconi A, Miquel M, Browse JA, Ryan CA (1996) Intracellular levels of free linolenic and linoleic acid increase in tomato leaves in response to wounding. Plant Physiol **111:** 797–803

Frankel EN (1998) Linolenate oxidation. *In* EN Frankel, ed, Lipid Oxidation. The Oily Press, Dundee, UK, pp 29–48

Hamberg M (1999) An epoxy alcohol synthase pathway in higher plants: biosynthesis of antifungal oxylipins in leaves of potato. Lipids **34:** 1131–1142

Hamberg M, Gardner HW (1992) Oxylipin pathway to jasmonates: biochemistry and biological significance. Biochim Biophys Acta **1165:** 1–18

Hamberg M, Hamberg G (1996) Peroxygenase-catalyzed fatty acid epoxidation in cereal seeds: sequential oxidation of linoleic acid into 9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*)-octadecenoic acid. Plant Physiol **110:** 807–815

Hamberg M, Sanz A, Castresana C (1999) α-Oxidation of fatty acids in higher plants: identification of a pathogen-inducible oxygenase (PIOX) as an α-dioxygenase and biosynthesis of 2-hydroperoxylinolenic acid. J Biol Chem **274:** 24503–24513

Imbusch R, Mueller MJ (2000) Formation of isoprostane F_2-like compounds (phytoprostanes F_1) from α-linolenic acid in plants. Free Radic Biol Med **28:** 720–726

Lamacka M, Sajbidor J (1995) The occurrence of prostaglandins and related compounds in lower organisms. Prostaglandins Leukot Essent Fatty Acids **52:** 357–364

Lawson JA, Rokach J, FitzGerald GA (1999) Isoprostanes: formation, analysis and use as indices of lipid peroxidation *in vivo*. J Biol Chem **274:** 24441–24444

Morrow JD, Awad JA, Boss HJ, Blair IA, Roberts LJ (1992) Non-cyclooxygenase-derived prostanoids (F_2-isoprostanes) are formed *in situ* in phospholipids. Proc Natl Acad Sci USA **89:** 10721–10725

Morrow JD, Chen J, Brame CJ, Yang J, Sanchez SC, Xu J, Zackert WE, Awad JA, Roberts LJ (1999) The isoprostanes: unique prostaglandin-like products of free-radical-initiated lipid peroxidation. Drug Metab Rev **31:** 117–139

Morrow JD, Roberts LJ (1997) The isoprostanes: unique bioreactive products of lipid peroxidation. Prog Lipid Res **36:** 1–21

Morrow JD, Roberts LJ, Daniel VC, Awad JA, Mirochnitchenko O, Swift LL, Burk RF (1998) Comparison of formation of D_2/E_2-isoprostanes and F_2-isoprostanes *in vitro* and *in vivo*-effect of oxygen tension and glutathione. Arch Biochem Biophys **353:** 160–171

Mueller MJ (1998) Radically novel prostaglandins in animals and plants: the isoprostanes. Chem Biol **5:** 323–333

O'Connor DE, Mihelich ED, Coleman MC (1981) Isolation and characterization of bicyclo endoperoxides derived from methyl linolenate. J Am Chem Soc **103:** 223–224

O'Connor DE, Mihelich ED, Coleman MC (1984) Stereochemical course of the autoxidative cyclization of lipid hydroperoxides to prostaglandin-like bicyclo endoperoxides. J Am Chem Soc **106:** 3577–3584

Orozco-Cardenas M, Ryan CA (1999) Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. Proc Natl Acad Sci USA **96:** 6553–6557

Panossian AG (1987) Search of prostaglandins and related compounds in plants. Prostaglandins **33:** 363–381

Panossian AG, Avetissian GM, Mnatsakanian VA, Batrakov SG, Vartanian SA, Gabrielian ES, Amroyan EA (1983) Unsaturated polyhydroxy acids having prostaglandin-like activity from *Bryonia alba*: II. Major components. Planta Med **47:** 17–25

Parchmann S, Mueller MJ (1998) Evidence for the formation of dinor isoprostanes E_1 from α-linolenic acid in plants. J Biol Chem **273:** 32650–32655

Pryor WA, Stanley JP, Blair E (1976) Autoxidation of polyunsaturated fatty acids: II. A suggested mechanism for the formation of TBA-reactive materials from prostaglandin-like endoperoxides. Lipids **11:** 370–379

Roberts LJ, Montine TJ, Markesbery WR, Tapper AR, Hardy P, Chemtob S, Dettbarn WD, Morrow JD (1998) Formation of isoprostane-like compounds (neuroprostanes) *in vivo* from docosahexaenoic acid. J Biol Chem **273:** 13605–13612

Roberts LJ, Morrow JD (1997) The generation and actions of isoprostanes. Biochim Biophys Acta **1345:** 121–135

Rokach J, Khanapure SP, Hwang S-W, Adiyaman M, Lawson JA, FitzGerald GA (1997a) The isoprostanes: a perspective. Prostaglandins **54:** 823–851

Rokach J, Khanapure SP, Hwang S-W, Adiyaman M, Lawson JA, FitzGerald GA (1997b) Nomenclature of isoprostanes: a proposal. Prostaglandins **54:** 853–873

Rusterucci C, Montillet J-L, Agnel J-P, Battesti C, Alonso B, Knoll A, Bessoule J-J, Etienne P, Suty L, Blein J-P, Triantaphylides C (1999) Involvement of lipoxygenase-dependent production of fatty acid hydroperoxides in the development of the hypersensitive cell death induced by cryptogein on tobacco leaves. J Biol Chem **274:** 36446–36455

Sanz A, Moreno JI, Castresana C (1998) PIOX, a new pathogen-induced oxygenase with homology to animal cyclooxygenase. Plant Cell **10:** 1523–1537

Waugh RJ, Morrow JD, Roberts LJ, Murphy RC (1997) Identification and relative quantitation of F_2-isoprostane regioisomers formed *in vivo* in the rat. Free Radic Biol Med **23:** 943–954