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Biologically active oxidized lipids (phytoprostanes) in the plant diet and parenteral lipid nutrition

KATHRIN KARG[1,†], VERENA M. DIRSCH[2,†], ANGELIKA M. VOLLMAR[2], JEAN-LUC CRACOWSKI[3], FRANÇOIS LAPORTE[4], & MARTIN J. MUELLER[1]

[1]*Julius-von-Sachs-Institute for Biosciences, Pharmaceutical Biology, University of Wuerzburg, Julius-von-Sachs-Platz 2, D-97082 Wuerzburg, Germany,* [2]*Department of Pharmacy, Butenandtstrasse 5-13, D-8 1377 Munich, Germany,* [3]*Inserm 03 Clinical Research Center, Grenoble University Hospital, Grenoble, France, and* [4]*Department of Biochemistry, Grenoble University Hospital, Grenoble, France*

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Abstract

Phytoprostanes (PP) are autoxidation products of α-linolenate that are present in all plant tissues. Several classes of PP with a prostaglandin (PG) F_1-, E_1-, A_1- and B_1-like structure were identified and quantified by gas chromatography-mass spectrometry in vegetable oils and parenteral nutrition (intralipid). High levels of PP (0.09 up to 99 mg/l) were found even in apparently fresh vegetable oils. After oral consumption of olive or soybean oil, PPF_1 were absorbed, found to circulate in plasma in conjugated form and excreted in free form into urine. Evidence is emerging that certain PP, such as the PPE_1, may modulate the function of immune cells in a PG-like fashion. Here, we show that PPA_1- and deoxy-PPJ_1 display potent anti-inflammatory and apoptosis inducing activities similar to PGA_1 and deoxy-PGJ_2. Results of this study indicate that PP are novel, biologically active lipids in plant nutrition.

Keywords: *Isoprostanes, phytoprostanes, lipid peroxidation, intralipid, soybean oil, olive oil*

Introduction

A series of isomeric prostaglandins (isoprostanes) can be formed by free radical catalyzed oxidation and cyclisation of polyunsaturated fatty acids. In man, long chain fatty acids (C20 and C22) such as arachidonic acid and docosahexaenoic acid are precursors of isoprostanes (IsoP) and neuroprostanes, respectively. In addition, a C18 fatty acid, α-linolenate present in human plasma at very low levels (<2% of total fatty acids) is a precursor of dinor IsoP also termed phytoprostanes (PP) [1]. Since α-linolenate is a major polyunsaturated fatty acid in plants, PP can typically be found in all plant parts [2]. The non-enzymatic, biochemical mechanism of fatty acid oxidation and cyclization is the same in all isoprostanoid pathways. The first product formed is a cyclic fatty acid with a prostaglandin (PG) H ring system. PGH-like compounds are chemically unstable and rapidly degrade to racemic isoprostanoids with a PGD, PGE and PGF ring system. The D- and E-ring classes of isoprostanoids may further dehydrate and isomerize to racemic deoxy-J- as well as A- and B-ring isoprostanoids, respectively. Thus, several structurally closely

Correspondence: M. J. Mueller, Julius-von-Sachs-Institute for Biosciences, University of Wuerzburg, Julius-von-Sachs-Platz 2, D-97082 Wuerzburg, Germany. Tel: 49 931 888 6160. Fax: 49 931 888 6182. E-mail: martin.mueller@biozentrum.uni-wuerzburg.de

†These authors contributed equally to this work. Present address V. D.: Department of Pharmacognosy, Althanstrasse 14, A-1090 Vienna, Austria.

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related classes each comprising a great variety of isomers are formed in mammals and plants *in vivo* (Figure 1).

IsoP and PP have been shown to represent extremely reliable markers of oxidative stress in mammals and plants *in vivo* [3–5]. In animals, several IsoP exert potent biological activities already in the nanomolar concentration range. Biological activities comprise vasoconstriction, platelet activation and aggregation, smooth muscle contraction of bronchi as well as anti-inflammatory and apoptosis-inducing activities in different tissues. IsoP are often discussed as mediators of oxidative stress in the pathophysiology of chronic diseases such as atherosclerosis, diabetes, asthma and others [3]. On the other hand, isoprostanes may also have beneficial effects depending on the site of generation and the context, i.e. acute inflammation (see discussion).

In plants, several PP have been shown to activate plant defense and detoxification responses [5–7]. However, little is known about the biological properties of PP that structurally resemble PGs and IsoP in mammalian systems (Figure 1). Previously, it has been shown that E_1-phytoprostanes (PPE_1) that occur abundantly in pollen of certain species such as birch display PGE_2-like activities, inhibit dendritic cell IL-12 production and augment Th2 cell polarization [8]. Since high levels of α-linolenate are present in several vegetable oils used as cooking oils, high concentrations of several phytoprostane classes are expected to occur in cooking oils, and after oral consumption of the oils in the human gastro-intestinal tract. Moreover, levels of oxidized lipids may further dramatically increase when oils become autoxidized. Thus, humans can potentially be exposed to extremely high levels of PG-like PP in normal and especially in partially autoxidized cooking oils. Therefore, actual phytoprostane levels and their potential PG-like effects should be studied in detail. Interestingly, vegetable

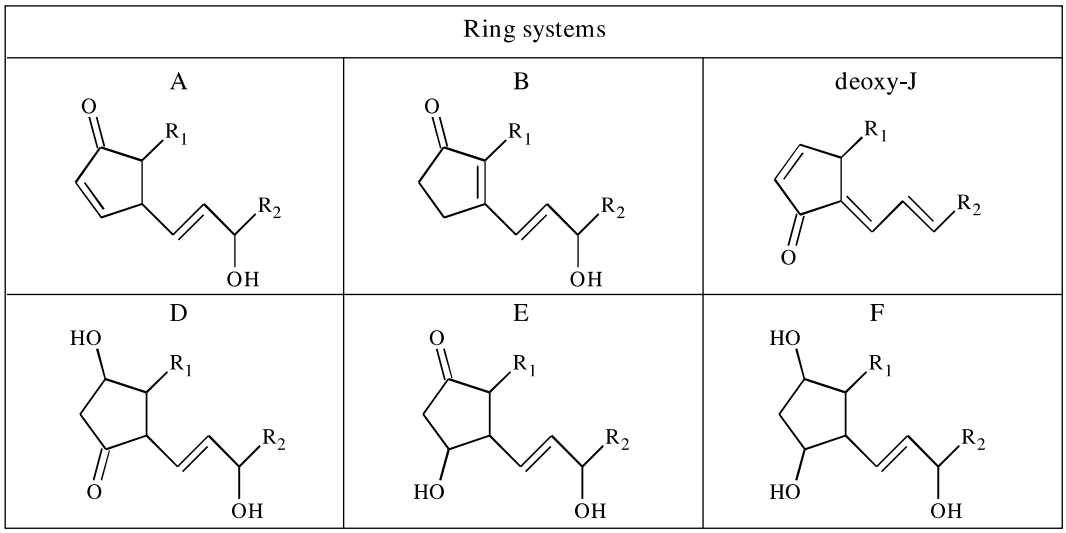

Figure 1. Selected isoprostanoid and PG structures discussed in the article. Isoprostanoids and PGs are composed of a ring system (abbreviated with the letter shown in the table) and two side chains. PGs and IsoP (racemic) differ in their stereochemistry (for simplicity not indicated). Details on the nomenclature can be found in several detailed reviews [2,46,47].

diet rich in polyunsaturated fatty acids (and especially α-linolenic acid) lowers the risk of cardiovasular diseases such as atherosclerosis and myocardial infarction and also reduces the levels of inflammatory markers and adhesion molecules [9]. However, the underlying mechanism(s) are not known.

Here, we show that at least four classes of PP occur in several cooking oils in significant amounts. Moreover, some PP, $dPPJ_1$ and PPA_1, display anti-inflammatory and apoptosis inducing activities in several established bioassay systems. We also show that PPF_1, belonging to one of the phytoprostane classes, are absorbed after oral consumption of cooking oils and excreted into the urine.

Materials and methods

Phytoprostanes

PP were prepared by autoxidation of α-linolenate under normal atmosphere or oxygen-18 gas (internal standards) and purified as described [6,10–12]. PP were dissolved in dimethyl sulfoxide (DMSO) and further diluted in medium. Final DMSO concentration in the cell supernatant was $<0.1\%$, a concentration shown not to interfere with the bioassay systems used.

Materials

Vegetable oils were purchased from local markets in Germany and France and analyzed immediately after opening of the bottles. Some oils were marked with superscript letters to differentiate different oil qualities in the results section. The following products were used: Linseed oil (crude oil, Carl Roth GmbH, Germany), soybean oil[a] (cold pressed, native oil, Vitaquell, Germany), soybean oil[b] (cold pressed, native oil, Emile Noel, France), soybean oil[c] (refined, Brändle vita, P. Brändle GmbH, Germany), soybean margarine "sojola" (partially hydrated oil, Vandermoortele GmbH, Germany), olive oil[a] (cold pressed, native oil, Brändle vita, P. Brändle GmbH, Germany), olive oil[b] (cold pressed, native oil, Lesieur, France), rapeseed oil[a] (cold pressed, native oil, Vitaquell, Germany), rapeseed oil (refined oil, Tegut, Germany), walnut oil (cold pressed, native oil, Brändle vita, P. Brändle GmbH, Germany), grape seed oil (refined oil, Lesieur, France), intralipid (infusion solution, 30% (w/v) soybean oil, Fresenius-Kabi, Germany).

Plasmids used

pNF-κBluc, (Stratagene, Heidelberg, Germany) and pSV-β-gal (Promega, Mannheim, Germany) as transfection control were used.

PPF_1 extraction from urine and blood

For analysis of free PPF_1, urine (10 ml) or plasma (15 ml) samples were thawed before $[^{18}O_3]PPF_1$ (50 ng, internal standard) was added. Urine was acidified with 1 M citric acid to pH 3 and immediately extracted with 20 ml of ethyl acetate containing 5 mg butylated hydroxytoluene (antioxidant) and 50 mg of triphenylphosphine (peroxide reducing agent). Free PPF_1 in the organic phase were purified, derivatized and analyzed by gas chromatography-negative ion chemical ionization-mass spectrometry (GC-NCI-MS) as described [4].

Total PPF_1 (free and esterified) were determined after alkaline hydrolysis of plasma samples (15 ml). Plasma samples were mixed with 15 ml of methanol containing 50 ng of $[^{18}O]_3PPF_1$, 5 mg of butylated hydroxytoluene and 50 mg of triphenylphosphine, and hydrolysed after addition of KOH (1.5 g) for 1 h at 80°C. Thereafter, methanol was removed with a rotary evaporator. The remaining water phase was acidified to pH 3 with 1 M citric acid and extracted with ethyl acetate. Free PPF_1 were determined as described above.

Hydrolysis of vegetable oils

Triglycerides were hydrolysed either with KOH or with porcine pancreatic lipase. For alkaline hydrolysis, 100 mg of oil was dissolved in 2 ml ethanolic NaOH solution (1 M). After addition of internal standard, the solution was degassed by bubbling argon through the mixture for 2 min. The solution was incubated under an argon atmosphere for 1 h in the dark at 80°C. Thereafter, 10 ml of water was added and the mixture was acidified with 1.3 ml of 6 M HCl and brought to pH 3 with 1 M citric acid. PP were extracted with ethyl acetate containing triphenylphosphine and butylated hydroxytoluene.

For enzymatic hydrolysis, 100 mg of oil was suspended in 20 ml of 1 M Tris-buffer (pH 8), 2 ml $CaCl_2$ (2.2% w/v) and 5 ml deoxycholic acid (0.05% w/v). After addition of 20 mg of porcine pancreas lipase (3580 U, type II, crude from Sigma), the vial was flushed with argon, sealed and incubated on a rotary shaker for 1 h at room temperature in the dark. Thereafter, the mixture was acidified with citric acid to pH 3 and extracted with ethyl acetate as described above. Extracts were analysed for PP as described below.

Phytoprostane analysis in vegetable oils

Plant oils (100 mg) and 50 ng of each appropriate internal standard (PGA_1, PGB_1, $[^{18}O_3]PPE_1$ and $[^{18}O_3]PPF_1$) were dissolved in 1 ml of chloroform. For analysis of total (free and esterified) PPA_1 and PPB_1, the sample obtained from enzymatic hydrolysis

(dissolved in chloroform, see above) was applied to a silica (SiOH) solid phase extraction (SPE) column. Alternatively for analysis of free PPA_1/PPB_1, 100 mg of oil dissolved in 1 ml of chloroform was directly loaded on the column. After loading, the column was washed with 6 ml of hexane/diethyl ether/acetic acid (66:33:1, v/v). PPA_1/PPB_1 were eluted with 9 ml of diethyl ether/acetic acid (98:2, v/v). The eluate was brought to dryness under a stream of nitrogen and derivatized with 50 µl of methoxymation reagent (50 mg methoxyamine hydrochloride in 1 ml of dimethylformamide) for 2 h at 60°C. Subsequently, the sample was partitioned between 2 ml of water (pH 3) and 5 ml of diethyl ether. The organic phase was dried under a stream of nitrogen and esterified with 10 µl of pentafluorobenzyl (PFB) bromide and 10 µl of *N*,*N*-diisopropylamine in 200 µl of chloroform (1 h at 40°C). Thereafter the sample was taken to dryness, silylated with 50 µl of *N*,*O*-bis(trimethylsilyl) trifluoracetamide (BSTFA) in 200 µl of chloroform (1 h at 40°C) and taken to dryness. The sample was reconstituted in 500 µl of chloroform and applied to a SiOH-SPE column. The column was eluted with hexane/diethyl ether (1:2, v/v). The eluate was taken to dryness and reconstituted in 20 µl of hexane for GC-MS analysis.

For analysis of PPE_1 and PPF_1, the sample obtained from hydrolysis (for determination of free and esterified PPE_1 and PPF_1, see above) or 100 mg oil dissolved in 1 ml of chloroform (for determination of free PPE_1 and PPF_1) were applied to a SiOH-SPE column. The column was washed with 6 ml of diethyl ether/acetic acid (98:2, v/v). The wash fraction containing PPA_1 and PPB_1 was discarded. Subsequently, the column was eluted with 9 ml of diethyl ether/methanol/acetic acid (88:10:2, v/v) to obtain PPE_1 and PPF_1. The sample was taken to dryness under a stream of nitrogen and reconstituted in 100 µl of methanol and 70 µl of 1 M aqueous KOH. The solution was incubated for 5 min at room temperature to convert PPE_1 into PPB_1 (note: during dehydratisation and isomerisation $[^{18}O_3]PPE_1$ are converted to $[^{18}O]PPB_1$). The sample was diluted with 3 ml of water and the pH was adjusted with 1 M citric acid to pH 3. PPB_1 and PPF_1 were extracted with diethyl ether, taken to dryness, reconstituted in 500 µl of chloroform and applied to a SiOH-SPE column. The column was eluted with 6 ml of hexane/diethyl ether/acetic acid (33:66:1, v/v) to obtain a fraction containing PPB_1 (derivatives of PPE_1). Subsequently, the column was eluted with diethyl ether/methanol/acetic acid (88:10:2, v/v) to obtain a fraction containing PPF_1. Both fractions were taken to dryness and processed separately. PPB_1 were derivatized to their corresponding PFB esters and trimethylsilyl (TMS) ethers and analysed by GC-NCI-MS as described [10]. PPF_1 were hydrogenated, derivatized to their corresponding PFB-esters and TMS-ethers and analysed by GC-NCI-MS as described [4].

Study design, blood and urine sampling

The study was designed as an open-label trial and was approved by the instutitional Review Board of Grenoble University Hospital, France. Twelve healthy male subjects ages 18–35 years participated in the study (Table III). Following a screening visit and written informed consent, they were advised to reduce consumption of plant oils three days preceding the study. On the study visit, volunteers came in a fasting state at 8 a.m. to the Inserm Clinical Research Center. A peripheral vein catheter was placed, and a first blood sample was taken. Then, 100 ml of a vegetable oil was mixed with standardized mashed potatoes and salt to facilitate consumption, and every volunteer was asked to eat the whole plate. The three vegetable oils used in the study were olive oil[b], soybean oil[b] and grape seed oil that were obtained from a local supermarket. One new bottle was used for every subject, the remaining oil was used for phytoprostane analysis. Each volunteer was randomized to one of the three oils, i.e. four subjects were enrolled in each group. All the subjects stayed in the Clinical Research center for 8 h, in a fasting state except the intervention. They were free to drink water.

Blood samples (10 ml) for the determination of PP were collected into EDTA containers at 0, 0.25, 0.5, 1, 1.5, 2, 3,4, 8 and 24 h postdose and immediately centrifuged at 1900*g* at 4°C for 5 min. Plasma samples were then immediately stored at −80°C (to prevent autoxidation *in vitro*) until analysis. Morning urine was collected before consumption of vegetable oils to determine baseline phytoprostane levels. Collection periods for urine after consumption of vegetable oils were 0–4, 4–8 and 8–24 h postdose. Urine was kept frozen at <-20°C in polypropylene tubes until measurement. Since urine is devoid of PUVA's, $[^{18}O_3]PPF_1$ can not be formed during storage *in vitro*. PPF_1 are chemically stable under neutral and alkaline conditions.

Cell culture

Human leukemia Jurkat T cells (clone J16, kindly provided by Dr P. Krammer, DKFZ Heidelberg, Germany) and RAW 264.7 mouse macrophages (American Type Culture Collection, ATCC, TIB 71), were cultivated as described [13,14]. Cells were maintained at 37°C, 5% CO_2 and used for experiments between passage 5 and 20. The human embryonic kidney cell line 293 (HEK 293; DSMZ-German collection of microorganisms and cell cultures, ACC 305) was grown in DMEM (Biowitthaker, Heidelberg, Germany) supplemented with

10% FCS (Biochrom KG, Berlin, Germany) and 2 mM glutamine (Merck, Munich, Germany). Cells were split 1:5–10 when reaching ~85–90% confluence using 0.05% trypsin/0.02% EDTA in PBS.

Cell viability assay (MTT assay)

To exclude the effects measuring iNOS-dependent nitrite accumulation due to cytotoxicity, cell viability was measured immediately after nitrite quantification in cell culture supernatants. Cell respiration, an indicator of cell viability, was determined by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan as described previously [14].

iNOS-dependent nitrite accumulation

RAW 264.7 macrophages were seeded in 96 well plates (8×10^4/200 μl), cultured for two days and then incubated with or without lipopolysaccharide (1 μg/ml) in the absence or presence of test compounds at the indicated concentrations (0–160 μM) for 20 h. As an indicator of NO synthesis, nitrite concentration was assessed in the supernatant of RAW 264.7 macrophages by the Griess reaction [15] as described earlier [16]. Experiments were performed three times in triplicate.

Detection and quantification of apoptosis

Apoptosis was judged by cell morphology and the visualization of apoptotic nuclei after staining with Hoechst 33342 (Sigma, Deisenhofen, Germany) by fluorescence microscopy. The rate of apoptotic cell death was quantified by determining DNA fragmentation according to Nicoletti et al. [17]. Briefly, cells were incubated overnight in a hypotonic buffer (1% sodium citrate, 0.1% Triton X-100, 50 μg/ml propidium iodide) and analyzed by flow cytometry on a FACScalibur (Becton Dickinson) using CellQuest software. Nuclei to the left of the "G1-peak" showing a sub-diploid DNA content were considered apoptotic.

Luciferase reporter-gene assay

HEK 293 cells were seeded at a concentration of 5×10^5 cells/60 mm dish. On the following day cells were transfected with the pNF-κBluc and pRSV-β-gal plasmid using the Ca^{2+}-phosphate method. Transfected cells were seeded in 24-well plates at a concentration of 1×10^5 cells/well and grown for additional 16 h. Then cells were pre-incubated with vehicle, test compounds or positive controls for 2 h and subsequently stimulated with 1 ng/ml TNF-α for 6 h. Cells were washed with PBS, lysed with *passive lysis buffer* (Promega, Heidelberg, Germany) and NF-κB activity was measured with the Luciferase Assay System from Promega (Heidelberg, Germany) according to the manufacturers instructions using an AutoLumat plus luminometer (Berthold, Bad Wildbad, Germany).

Statistics

All data are expressed as mean ± SEM, unless otherwise stated. Statistical analysis was performed by ANOVA followed by a Dunnett multiple comparison test. $P < 0.05$ was considered statistically significant. Analysis of data was performed using the software GraphPad PRISM®, Version 3.02 (GraphPad Software Inc., USA).

Results

Phytoprostane levels in vegetable oils and in parenteral lipid emulsions (intralipid)

Several classes of free and esterified PP are formed by autoxidation of free linolenate and linolenate esterified in triglycerides in vegetable oils. PPF_1 are the predominant PP formed in non-aqueous solvents while in the presence of water PPE_1 that may degrade upon prolonged storage to PPA_1 and finally to PPB_1, predominate [4]. Similar to F_2-IsoP in mammals, PPF_1 (comprising 32 isomers) are reliable markers of oxidative degradation of plant lipids [11]. Total PPF_1 (esterified and free) levels were determined after alkaline hydrolysis of the oils. Free PPF_1 were measured by GC-negative ion chemical ionization-MS using $[^{18}O_3]PPF_1$ as internal standard. As expected, the isomer pattern of PPF_1 produced by autoxidation of free linolenate (oxygen-18 labeled standards) and the pattern of esterified PPF_1 isomers in the oils was identical (Figure 2). As shown in Table I, relative high levels of total PPF_1 were found in all oils. Linseed oil which contains the highest amount of linolenate (60% of all fatty acids) also displayed highest PPF_1 levels, i.e. 13.8 mg/l. However, the second highest total PPF_1 levels were determined in a sample of olive oil (4.8 mg/l) and the lowest levels were found in grape seed oil (0.09 mg/l) although both oils contain only very little linolenate (1%).

In addition to PPF_1, levels of free and esterified PPE_1, PPA_1 and PPB_1 were analyzed by GC-negative ion chemical ionization-MS using $[^{18}O_3]PPE_1$, PGA_2 and PGB_2 as internal standards. Representative GC-MS chromatograms of PP are shown in Figure 2. As expected for autoxidation products, type I and type II isomers (Figure 1) of PPE_1, PPA_1 and PPB_1 were of equal abundance. As shown in Table II, the predominant phytoprostane class in all oils except for linseed oil was PPE_1. Highest levels of total PPE_1 were found in one soybean oil sample (22 mg/l) and in linseed oil (4.4 mg/l). Highest levels of the

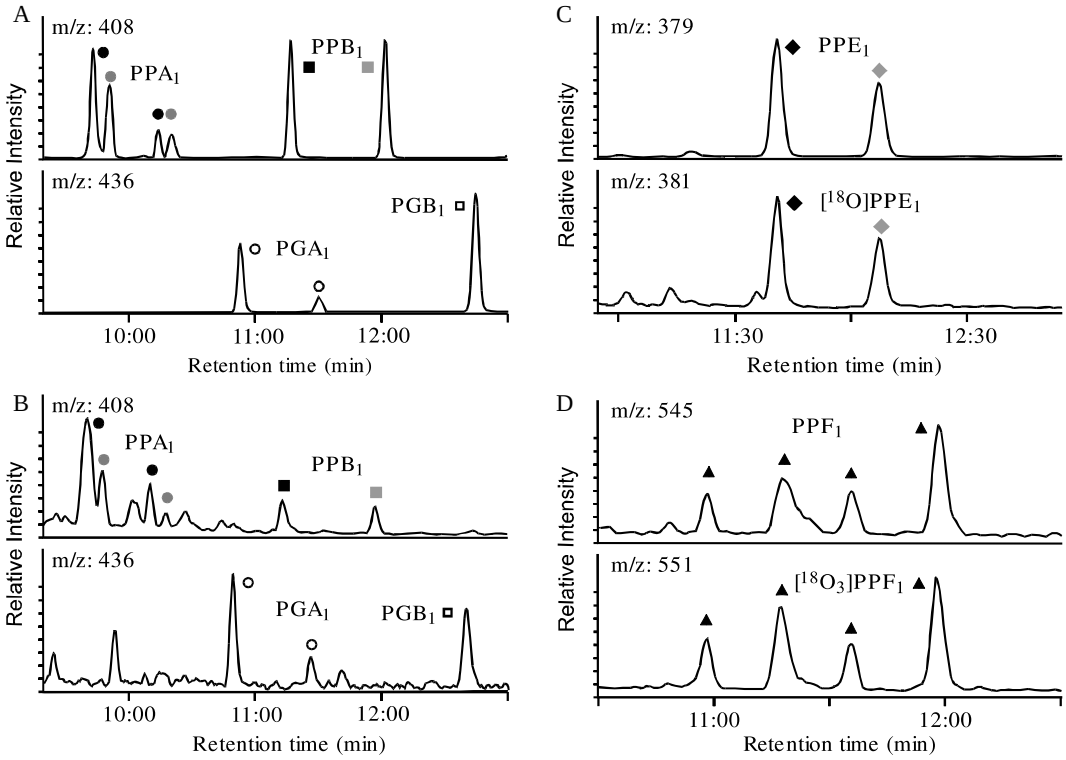

Figure 2. GC-NCI-MS analysis of phytoprostane derivatives in linseed oil. Authentic reference compounds (PPA_1-I, •; PPA_1-II, ●; PPB_1-I, ■; PPB_1-II, ■; PGA_1, ○; PGB_1, □) were converted into their methoxime, TMS, PFB derivatives and measured in the selected ion monitoring mode (A). Levels of total PPA_1 and PPB_1 were determined after digestion with pancreatic lipase as methoxime, TMS, PFB derivatives using PGA_1 and PGB_1 as internal standard (B). Levels of total PPE_1 (PPE_1-I, ◆; PPE_1-II, ◆) were determined after lipase digestion of the oil, purification and conversion into TMS, PFB, PPB_1 derivatives; oxygen-18 labelled PPE_1 were used as internal standards (C). Levels of total PPF_1 (▲) in lipase digested oil were measured after hydrogenation and formation of TMS, PFB derivatives; [$^{18}O_3$]PPF_1 were used as internal standards (D).

cyclopentenone PP PPA_1 (6.8 mg/l) and PPB_1 (2.4 mg/l) were found in the linseed oil.

Notably, vegetable oils stored in open bottles at room temperature are exposed to air and prone to autoxidation. Several markers of lipid peroxidation increase dramatically (more than 10-fold) before the oil becomes apparently rancid and is discarded [18]. For instance, storage of 50 ml of fresh, refined soybean oil with low levels of free PPF_1 (170 μg/l) and esterified PPF_1 (4.09 mg/l) in an open bottle for 18 days at 50°C yielded a 20-fold increase of free PPF_1 (3.5 mg/l) and a 5-fold increase in esterified PPF_1 (21.0 mg/l). Thus,

Table I. F_1-Phytoprostane levels in vegetable oils and products thereof determined after alkaline hydrolysis of oils or without digestion (free PP).

	Total PPF_1 (mg/l)	Free PPF_1 (mg/l)	Esterified PPF_1 (mg/l)	Free PPF_1 (%)
Cold pressed, native oils				
Linseed oil (crude)	13.83 ± 0.98	5.53 ± 1.04	8.27 ± 1.42	40
Olive oil[a]	2.53 ± 0.186	0.82 ± 0.06	1.74 ± 0.19	32
Olive oil[b]	4.84 ± 0.42	3.39 ± 0.52	1.5 ± 0.67	69
Soybean oil[a]	2.91 ± 0.03	1.83 ± 0.09	1.12 ± 0.10	62
Soybean oil[b]	1.32 ± 0.09	1.08 ± 0.06	0.26 ± 0.11	81
Rapeseed oil[a]	0.32 ± 0.09	0.31 ± 0.06	0.01 ± 0.11	97
Processed/refined oils				
Soybean oil[c]	4.00 ± 0.78	0.48 ± 0.1	12.76 ± 2.6	4
Soybean margarine	1.2 ± 0.27	0.44 ± 0.2	3.53 ± 1.0	11
Intralipid (oil fraction)	2.38 ± 0.1	0	2.38 ± 0.1	0
Rapeseed oil[b]	0.15 ± 0.01	0.16 ± 0.01	0.34 ± 0.03	33
Walnut oil	0.62 ± 0.03	0.2 ± 0.08	1.84 ± 0.1	10
Grape seed oil	0.09 ± 0.01	0	0.09 ± 0.03	0

Levels of esterified PPF_1 were calculated (esterified PPF_1 = total PPF_1 − free PPF_1). Superscript letters indicate differents oils (see material and methods). Values are means ± SD ($n = 3$).

Table II. Phytoprostane levels in vegetable oils and products thereof determined after hydrolysis of oils by pancreatic lipase (total PP) or without digestion (free PP).

	PPA_1 [MW 308] (μmol/l)	PPB_1 [MW 308] (μmol/l)	PPE_1 [MW 326] (μmol/l)	PPF_1 [MW 328] (μmol/l)
Total phytoprostanes				
Linseed oil (crude)	21.94 ± 0.42	7.77 ± 1.83	13.59 ± 0.66	33.54 ± 5.09
Olive oil[a] (native)	0.27 ± 0.10	0.12 ± 0.05	3.41 ± 1.89	3.51 ± 0.83
Soybean oil[a] (native)	4.10 ± 1.47	1.98 ± 0.72	11.57 ± 1.11	5.67 ± 0.40
Rapeseed oil[a] (native)	1.15 ± 0.44	0.32 ± 0.08	3.41 ± 0.39	1.07 ± 0.03
Soybean oil[c]	2.28 ± 0.7	0.53 ± 0.18	66.50 ± 0.16	11.85 ± 0.01
Rapeseed oil[b]	0.29 ± 0.09	0.07 ± 0.05	4.44 ± 1.74	0.45 ± 0.04
Walnut oil	0.88 ± 0.36	0.25 ± 0.02	12.53 ± 1.35	1.80 ± 0.08
Intralipid (oil fraction)	2.70 ± 0.53	0.97 ± 0.33	5.06 ± 0.66	1.07 ± 0.17
Free phytoprostanes				
Linseed oil (crude)	2.27 ± 0.42	0.76 ± 0.16	4.61 ± 0.88	16.86 ± 3.11
Olive oil[a] (native)	0.02 ± 0.00	0.01 ± 0.01	0.75 ± 0.28	2.47 ± 0.22
Soybean oil[a] (native)	0.42 ± 0.04	0.15 ± 0.02	7.45 ± 0.35	5.51 ± 0.29
Rapeseed oil[a] (native)	0.21 ± 0.06	0.03 ± 0.01	0.52 ± 0.07	0.94 ± 0.20
Soybean oil[c]	0.02 ± 0.02	0.03 ± 0.00	1.17 ± 0.77	0.44 ± 0.44
Rapeseed oil[b]	0.02 ± 0.02	0.02 ± 0.01	n. d.	0.15 ± 0.01
Walnut oil	0.07 ± 0.01	0.09 ± 0.03	n. d.	0.18 ± 0.07
Intralipid (oil fraction)	0.10 ± 0.26	0.07 ± 0.03	2.53 ± 0.73	n. d.

Superscript letters indicate different oils (see material and methods). Values are means ± SD ($n = 3$).

phytoprostane levels in vegetable oils stored in households may be usually much higher than in the fresh oils shown in Tables I and II.

PPF_1, PPE_1, PPA_1 and PPB_1 are also present in intralipid (Table II). The total amount of PP (in most part esterified in triglycerides) in the intralipid sample analyzed was 96 μg/100 ml (calculated from Table II).

Release of phytoprostanes from oxidized triglycerides by pancreatic lipase

After oral consumption of vegetable oils, free and esterified PP could potentially decompose at low gastric pH values. However, 95–98% of PPF_1, PPE_1, PPA_1 or PPB_1 could be recovered after incubation in 0.1 M HCl for 3 h at 37°C (data not shown), indicating that phytoprostane-containing triglycerides may enter the intestine in intact form.

Linolenate occurs predominately the *sn*-2 position in plant triglycerides and, thus, PP are expected to be enriched in the *sn*-2 position [18]. Pancreatic lipase has a high preference for the *sn*-1 and *sn*-3 position and, thus, hydrolysis at the *sn*-2 position becomes apparent only after long incubation times. *In vitro*, porcine pancreatic lipase efficiently released all classes of PP from triglycerides (Table II). When comparing the release of PPF_1 from oils by alkaline hydrolysis (assumed to be complete, Table I) and lipase (Table II), we found that porcine pancreatic lipase releases 40–95% of all esterified PPF_1 after an 1 h incubation time. Hence, phytoprostane-containing triglycerides can be hydrolized by pancreatic lipase to yield a mixture of 2-monoacylglycerol/2-phytoprostanoylglycerol and free fatty acids/PP. Since free PP may accumulate to high levels in the intestine, it was of interest if PP other than PPE_1 [8] also display PG-like activities.

Cyclopentenone phytoprostanes PPA_1 and deoxy-PPJ_1 downregulate NF-κB signalling in HEK 293 cells and RAW 264.7 macrophages

In mammalian cell systems, it has consistently been shown that cyclopentenone PGs and IsoP with an A-, J- or deoxy-J-ring structure display anti-inflammatory, apoptosis-inducing and antiviral properties [19]. The anti-inflammatory properties of cyclopentenones are at least in part due to inhibition of NF-κB signalling [20,21]. In order to examine whether cyclopentenone PP are able to inhibit the activation of the transcription factor NF-κB we employed PPA_1 (1:1 mixture of type I and II), PPB_1-I and II as well as deoxy-PPJ_1-I and -II to a NF-κB-driven luciferase reporter gene assay and compared their activity with that of the PGs PGA_1 and deoxy-PGJ_2. HEK 293 cells transfected with the pNF-κBluc plasmid were exposed for 2 h to these compounds as well as to the proteasome inhibitor MG 132 (10 μM) as a control. Subsequently, cells were activated with 1 ng/ml TNF-α for 6 h and transactivation activity of NF-κB was quantified by measuring luciferase activity. As shown in Figure 3, PPA_1 dose-dependently (20–80 μM) inhibited NF-κB transactivation to a comparable extent as PGA_1. The positive control MG 132 (10 μM) inhibited TNF-α-induced NF-κB transactivation completely. $dPPJ_1$-I and -II showed also a dose-dependent effect even at lower concentrations (10–40 μM) that correlated very well with the effect of $dPGJ_2$ (Figure 3). PPB_1 were less effective showing a significant effect only for PPB_1-II at a concentration as high as 160 μM.

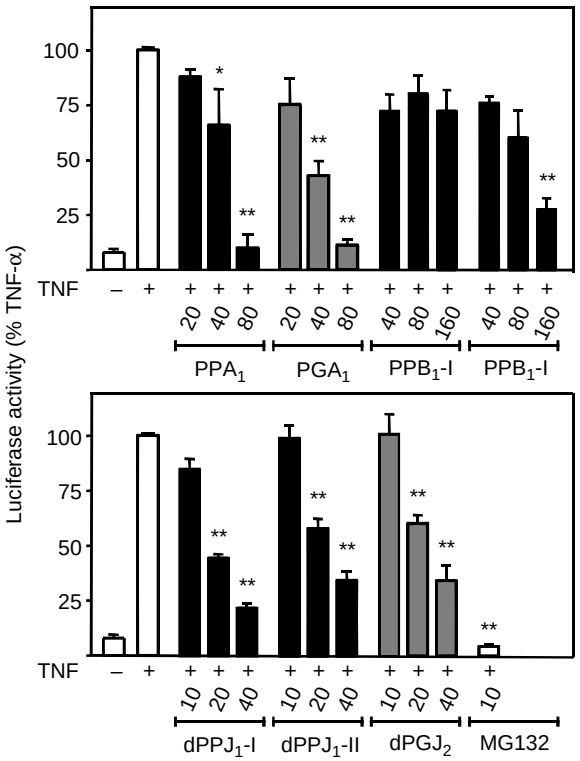

Figure 3. PPA_1 and $dPPJ_1$ inhibit TNF-α-induced NF-kB activation. HEK 293 cells were transiently transfected with an NF-κB luciferase reporter plasmid and pre-treated with test compounds at the concentrations indicated (μM) or MG132 (10 μM, positive control) for 2 h, then stimulated with 1 ng/ml TNF-α for 6 h. Data is presented as % luciferase activity of cells treated with TNF-α alone (mean ± SEM, $n = 3$).

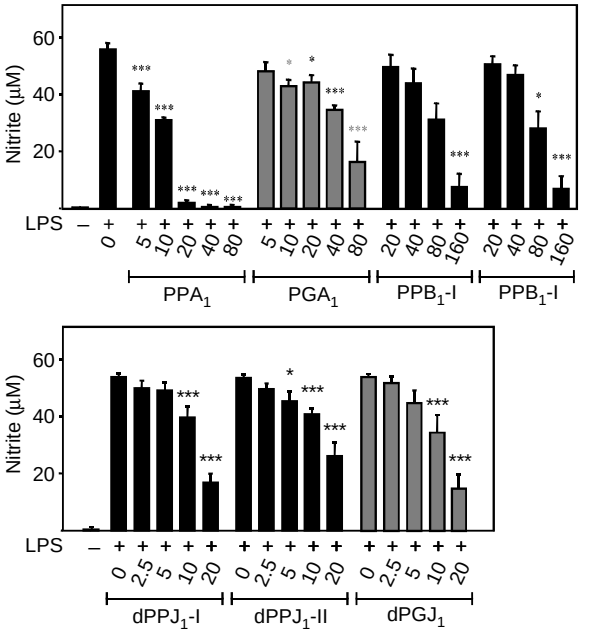

Figure 4. PPA_1 and $dPPJ_1$ inhibit NO formation in LPS-stimulated RAW 264.7 macrophafes. RAW 264.7 murine macrophages were incubated with test compounds at the indicated concentrations (μM) and LPS (1 μg/ml) for 20 h. As indicator for NO synthesis, nitrite, a stable breakdown product of NO, was determined. Bars represent means ± SEM ($n = 3$).

In murine RAW264.7 macrophages, transactivation of the transcription factor NFκB is required to trigger inducible nitric oxide synthase (iNOS) dependent NO formation in response to bacterial LPS challenge [22]. Therefore, as a second line of evidence for the impact of PP on NF-κB-regulated pathways, we investigated whether PP are able to affect iNOS-dependent nitric oxide production. RAW264.7 cells were incubated with PP or PGs and activated with LPS (1 μg/ml). Accumulated (20 h) nitrite was quantified by the Griess reaction. As can be seen in Figure 4, PPA_1 were the most potent NO synthesis inhibitors among the cyclopentenones tested. IC_{50} values of PPA_1, $dPPJ_1$-I, $dPPJ_1$-II and $dPGJ_1$ were in the range of 10–20 μM while the IC_{50} values for PGA_1 and PPB_1 was >40 μM. The phytoprostane effects measured in LPS-activated murine RAW cells were slightly more pronounced than that obtained from TNF-activated transfected human HEK 293 cells.

In order to exclude that the inhibitory effects observed on NF-κB-driven gene transactivation and nitrite accumulation were due to cytotoxicity of PP, cell viability was judged by the MTT assay (RAW 264.7 cells) and the β-galactosidase activity assay (pRSV-β-gal transfected HEK 293 cells), respectively. No cytotoxicity was detected in response to PP in the concentration ranges measured compared to cells activated with TNF-α and LPS, respectively (data not shown).

Cyclopentenone phytoprostanes PPA_1 and $dPPJ_1$ induce apoptosis in Jurkat T cells

There are reports that PGs of the A- and J-series are able to induce apoptosis in several cell types, among them SH-SY5Y human neuroblastoma, but also human umbilical endothelial cells (HUVEC) [23–25]. We therefore examined whether plant-derived PP are able to induce apoptosis in leukemia Jurkat T-cells, a well established cell model for studying apoptosis, and compared the effect with that measured for PGA_1 and $dPGJ_2$ in the same cell model. Apoptosis was judged by cell morphology and visualisation of apoptic nuclei after staining with Hoechst 33342 by fluorescence microscopy. The rate of apoptotic cell death was quantified by flow cytometric analysis of DNA fragmentation (see experimental procedures for details). As can be seen in Figure 5, PPA_1-I induced apoptosis in the same concentration range (10–40 μM) as PGA_1 although to a higher degree (60 vs. 33% apoptosis). In contrast, isomers of PPA_1-I, PPA_1-II, were ineffective up to a concentration of 40 μM and showed only moderate apoptosis at 80 μM. In contrast, PPB_1-I and -II which are also isomers of PPA_1 were completely inactive up to a concentration of 80 μM. $dPPJ_1$-I, $dPPJ_1$-II and $dPGJ_1$ were equally active and triggered apoptosis in a concentration range of 20–40 μM.

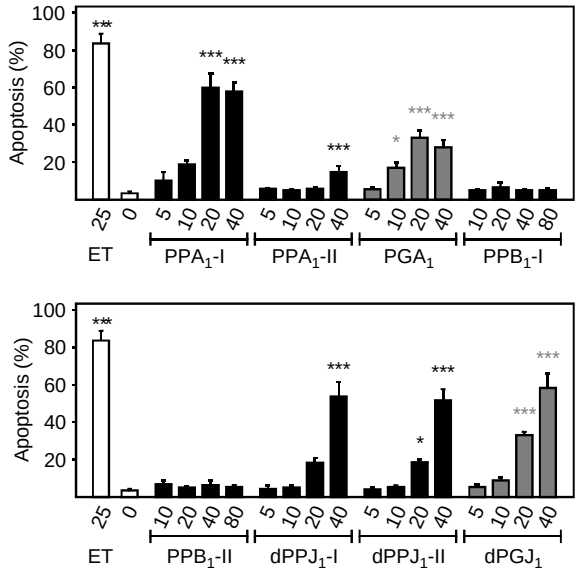

Figure 5. PPA_1 and $dPPJ_1$ induce apoptosis in leukemia Jurkat T-cells. Cells were incubated with test compounds at the concentrations indicated (μM) or etoposide (25 μg/ml, positive control) and analysed after 12 h by flow cytometry. Nuclei to the left of the "G1-peak" showing a sub-diploid DNA content were considered apoptotic. Bars represent means ± SEM ($n = 3$).

Phytoprostane levels in human blood after oral consumption of plant oils

Typically, PGs and IsoP are rapidly absorbed in the gastro-intestinal tract, metabolized and excreted [26]. However, isoprostanoids with the E-, A- and J-ring system are metabolically highly unstable and, thus far, have not been detected in free form in normal human plasma and urine. In contrast, F-ring isoprostanoids are chemically highly stable, are metabolized more slowly and can readily be detected in human plasma and urine. In order to investigate the absorption and excretion of PP in man, we therefore focused on PPF_1. Three different oils (olive oil[b], soybean oil[b] and grapeseed oil, see details on the origin of the oils and study design in the material and method section) were each orally administered (100 ml) to four apparently healthy subjects. Blood samples (2 ml) were taken 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 8 and 24 h postdose. Clinical characteristics of the volunteers are given in Table III. PPF_1 were analyzed from plasma obtained from the blood samples. Total PPF_1 amounts administered with the oils (calculated from Table I) were 484 μg (1.5 μmol) PPF_1 in olive oil[b], 132 μg (0.4 μmol) PPF_1 in soybean oil[b] and 9 μg (0.03 μmol) in grape seed oil. Levels of free PPF_1 in plasma samples (2 ml) were below the limit of detection (100 pg/ml). Therefore, all plasma samples of each subject were pooled and analyzed for free and total PPF_1. Free PPF_1 were barely detectable and still below the limit of quantification in samples of all twelve subjects of the study.

In order to prove if PP are present in conjugated form, blood samples were subjected to alkaline hydrolysis and subsequently analyzed for PPF_1 (Figure 6). After hydrolysis, PPF_1 could be determined in all pooled plasma samples from subjects that have consumed olive oil (0.4 ± 0.08 ng/ml) and in one subject that has taken soybean oil (0.32 ng/ml). Plasma levels of PPF_1 were below the limit of detection before consumption of vegetable oils as well as after consumption of grape seed oil that contained low levels of PPF_1. Results suggest that PP circulate in human plasma in conjugated form after consumption of oils with high PPF_1 levels such as the olive oil. Release of PPF_1 from conjugates by alkaline hydrolysis suggests that PPF_1 was present in plasma in esterified form. The chemical structure of the conjugate is not known.

Excretion of F_1-phytoprostanes into urine after oral consumption of plant oils

Before consumption of vegetable oils, PPF_1 could not be detected in urine. After oral consumption of

Table III. Characteristics of the 12 male volunteers enrolled in the clinical study.

Randomization number	Age (years)	Body mass index	Syst./diast. Arterial pressure (mm Hg)	Heart rate (beat/min)	Allocated oil (100 ml)	Plasma total cholesterol (g/l)	Plasma tri-glycerides (g/l)
1	21	19.5	120/70	59	Olive	1.62	0.58
2	19	21.5	119/80	64	Soybean	1.32	0.50
3	18	23.3	120/90	68	Grape seed	2.03	0.88
4	23	22.9	123/70	68	Grape seed	1.62	0.56
5	21	20.0	120/70	69	Olive	1.34	0.55
6	21	20.2	120/70	75	Soybean	1.44	0.31
7	19	23.1	120/70	59	Grape seed	1.56	0.61
8	21	25.3	100/60	50	Olive	1.40	0.36
9	21	24.7	122/70	59	Soybean	2.20	1.57
10	21	23.4	110/60	60	Grape seed	1.92	1.12
11	24	23.5	105/60	60	Soybean	1.41	0.60
12	34	29.4	120/70	70	Olive	1.82	0.72

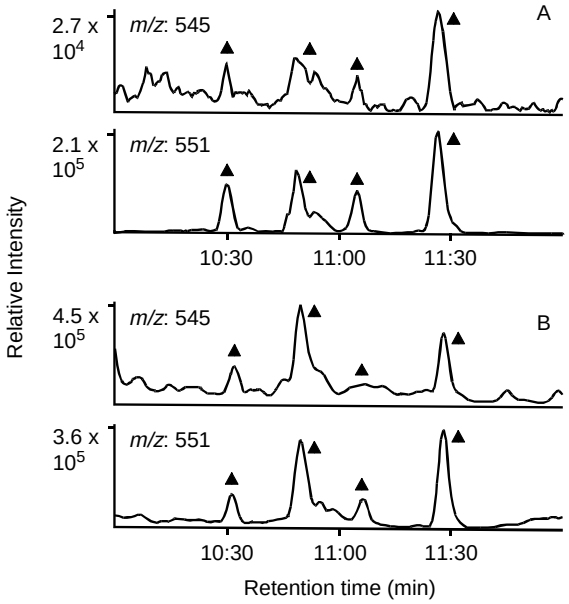

Figure 6. GC-NCI-MS analysis of PPF_1 derivatives in plasma (A) and urine (B) after consumption of olive oil. Endogenous PPF_1 (▲, ion recording at m/z 545) were measured as hydrogenated TMS, PFB derivatives using [$^{18}O_3$]PPF_1 (▲, ion recording at m/z 551) as internal standards. Free and esterified PPF_1 were determined in pooled plasma samples (0–24 h postdose) after alkaline hydrolysis (A). Free endogenous PPF_1 were quantified from a urine collection (4–8 h postdose) without hydrolysis (B). Endogenous PPF_1 levels were below the limit of detection in plasma and urine before consumption of the oil.

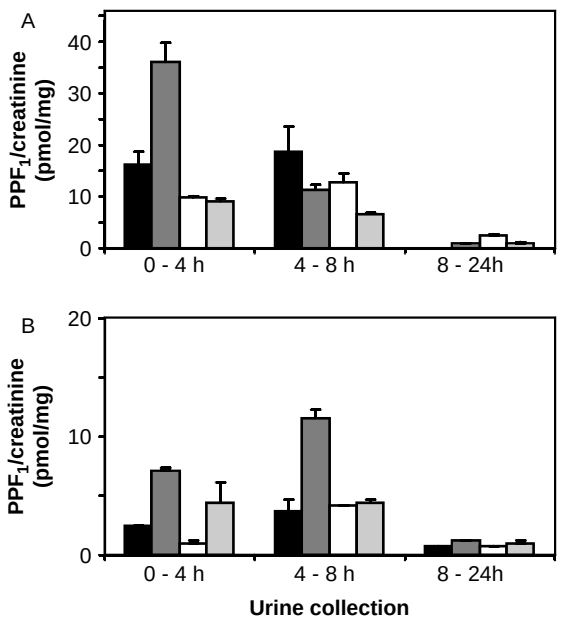

Figure 7. Urinary levels of PPF_1 after consumption of vegetable oils. Levels of free PPF_1 were determined in urine collections of four subjects after consumption of 100 ml olive oil (A) or 100 ml soybean oil (B). Endogenous PPF_1 levels were below the limit of detection in urine before consumption of vegetable oil. Bars of individuals are encoded with different colors. Values are the mean ± SD ($n = 3–4$).

olive oil and soybean oil, non-conjugated, free PPF_1 were clearly detectable in the urine collections 4, 8 and 12 h postdose (Figures 6 and 7). After oral consumption of olive oil, PPF_1 levels (pmol/mg creatinine, mean ± SD, $n = 4$) in urine were 17.8 ± 12.7 pmol/mg (0–4 h), 12.3 ± 5.0 (4–8 h) and 1.5 ± 0.9 (8–12 h). After oral consumption of 484 μg of PPF_1 (in 100 ml of olive oil), 1.75 μg PPF_1 or 0.36% were excreted into urine within 12 h. Intake of soybean oil resulted in PPF_1 levels of 3.8 ± 2.7 pmol/mg (0–4 h), 6.0 ± 3.7 (4–8 h) and 0.9 ± 0.2 (8–12 h). After oral consumption of 132 μg of PPF_1 (in 100 ml of soybean oil), 0.75 μg PPF_1 or 0.57% were excreted into urine within 12 h. After consumption of 100 ml of grape seed oil containing 9 μg of PPF_1, PPF_1 could not be quantified in urine samples at any time point indicating that PPF_1 levels at baseline and after consumption of vegetable oils with low PPF_1 amounts are at least very low (< 5 pg/ml). Notably, racemic PPF_1 of type I and II comprise in total 32 isomers (Figure 1) which produce typical patterns of peaks in GC-MS chromatograms (Figure 2). The isomer pattern of PPF_1 isolated from plasma and urine largely resembles the isomer pattern of the oxygen-18 labelled standard produced by linolenate autoxidation *in vitro* (Figure 6).

Discussion

Autoxidation of α-linolenate *in vitro* and *in vivo* may yield not only a large number, but also high amounts of PP. For instance, 6–11 g of PPF_1 and PPE_1 can be formed per kg of linolenate (free or esterified) by autoxidation under normal atmosphere at room temperature *in vitro*. Here, we show that PPF_1, PPE_1, PPA_1 and PPB_1 are present in up to milligram quantities in 100 ml of fresh vegetable oils. Levels of PP are highly variable due to differences in the linolenate/antioxidant content and the processing history of certain plant oils. During storage, phytoprostane levels may further increase by more than an order of magnitude.

In most oils, PPE_1 is the most abundant phytoprostane class (Table II). Previously, it has been shown that PPE_1 potently modulate dendritic cell maturation and inhibit interleukin 12 production in response to lipopolysaccharide (LPS) in concentrations ranging from 300 nM to 3 μM in a PGE_2-like fashion [8]. Notably, PPE_1 occur in soybean oil in a concentration of 5–72.5 μM (162–2440 μg/100 ml). These levels are remarkably high when compared to the oral dosage (200 μg) of a metabolically stable PGE_1 derivative, misoprostol, used for prevention of gastric ulcers. However, the pharmacological activities of PPE_1 on gastric mucosa (i.e. metabolism and PGE_2 receptor activation) have not been studied yet.

Vegetable oils also contain relatively large quantities of free and esterified cyclopentenone PP that can be released pancreatic lipase. Moreover, it is well known that prostanoids of the E- [27] and D-series [28] rapidly dehydrate to A- and J-ring cyclopentenone prostanoids in the presence of albumin [29]. Cyclopentenone PGs and IsoP with an A- or deoxy-J-ring system have consistently been shown to exhibit a wide variety of biological actions, including anti-inflammatory, anti-viral, and both cytotoxic and cytoprotective activities [19,21]. In this work we show that the cyclopentenone PP, PPA_1 and $dPPJ_1$, display anti-inflammatory activities in the same concentration range (10–50 μM) as PGA_1 and deoxy-PGJ_2 on HEK 293 cells and RAW264.7 macrophages. Notably, concentrations of PPA_1 even in fresh vegetable oils (up to 22 μM) are higher than cyclopentenone prostanoids in any other biological material so far measured.

Typically, IsoP and PGs are rapidly absorbed, metabolized, excreted into urine and do not accumulate in blood of the systemic circulation. In several previous studies, it was shown that the diet does not influence F_2-isoprostane levels in blood and urine [30–32] since isoprostane levels found in food products were theoretically far too low (< 1 ng/g) to alter significantly endogenous F_2-isoprostane levels [32]. Notably, F_1-phytoprostane levels in fresh vegetable oils (up to 12.5 μg/g, Table I) are several orders of magnitude higher than F_2-isoprostane levels in lipid-rich diet (0.06–1.3 ng/g) [32], rat liver (6 ng/g) [33] or human plasma (5–40 pg/ml) [34]. However, levels of free PPF_1 were still below the limit of detection in human blood even after oral consumption of 100 ml vegetable oils which is most likely due to rapid metabolism of PPF_1.

The half life of a F_2-isoprostane (F-ring isoprostane in Figure 1) in rabbit plasma was reported to be below 4 min and metabolites of the F_2-isoprostane were excreted into urine within 40 min [34]. In man, the main metabolite of 15-F_{2t}-isoprostane, a F_1-dinor isoprostane [35] (Figure 1), found in urine is a potent vasoconstrictor and an isomer of PPF_1 [36]. After oral consumption of olive and soybean oil, PPF_1 levels in urine increased from undetectable levels at zero time to 17.8 and 3.8 pmol/mg of creatinine (0–4 h collection period), respectively, and exceeded the reported whole body formation and excretion of a major F_2-isoprostane metabolite, F_1-dinor isoprostane (1.2 pmol/mg of creatinine) [37]. However, only a small fraction (0.36–0.57%) of the totally administered PPF_1 was recovered in the urine indicating incomplete absorption and/or rapid metabolism of PPF_1.

A somewhat surprising finding of the study is the fact that PPF_1 could be determined in esterified form in human blood after oral consumption of olive or soybean oil. There is no evidence that free PGF_2 or F_2-IsoP can be esterified into complex lipids or conjugated to glucuronic acid or glutathione. It is also unlikely that significant linolenate autoxidation occurred *in vivo* since urine and plasma PPF_1 levels correlated with the PPF_1 but not with the linolenate concentration of the oils. Alternatively, it is feasible that 2-phytoprostanoylglycerols (produced by pancreatic lipase hydrolysis of triglycerides) are absorbed and incorporated into ühospholipids of lipoprotein particles. Esterified IsoP [35] and PP are protected from enzymatic metabolism/renal elimination and may be released at the major sites of fat consumption.

In intensive care medicine, large amounts (up to 100 ml) of intralipid are administered intravenously. The administered lipid micelles in intralipid presumably harbor by far the highest concentration of E-ring (1652 ng/g) and A-ring (832 ng/g) isoprostanoids in the human body that greatly exceeds levels of endogenous D/E-ring (0.9 ng/g) and A/J-ring (5 ng/g) IsoP present, for instance, esterified in rat liver [38,39]. Even under extreme conditions of oxidative stress such as severe CCl_4 intoxication, maximum levels of endogenous cyclopentenones in rat liver (122 ng/g) [39] are well below the PPA_1 levels in intralipid. Numerous studies have indicated that parenteral soybean- or olive-oil based lipid emulsions (inevitably containing PP and other oxidized lipids) may compromise several immune function of phagocytes, however, the underlying mechanisms remain to be clarified [40,41]. There is evidence that oxidized phospholipids can modulate maturation and function of dendritic cells, inhibit inflammation and protect mice from lethal endotoxin shock in response to LPS [42]. Apparently, a great variety of oxidized lipids including PP attenuate acute inflammatory processes via different mechanisms [43–45].

Over the last 25 years, the effects of consumption of vegetable oils, vegetables and legumes rich in polyunsaturated fatty acids (Mediterranean diet) on the immune system, the cardiovascular system and in the prevention of cancers have been characterized using *in vitro* as well as *in vivo* animal and human studies. In this study, we have identified novel PG-like compounds in vegetable oils that may contribute to the beneficial effects of Mediterranean diet.

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