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In Vivo and in Vitro Lipid Peroxidation of Arachidonate Esters: The Effect of Fish Oil ω-3 Lipids on Product Distribution

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Abstract: The effect of lipid composition on the distribution of free radical oxidation products derived from arachidonic acid (20:4) esters has been studied in vitro and in vivo. Pro-inflammatory prostaglandin (PG) F_2-like compounds, termed F_2-isoprostanes (IsoPs), are produced in vivo and in vitro by the free radical-catalyzed peroxidation of arachidonic acid. Controlled free radical oxidation of mixtures of fatty acid esters in vitro showed that the formation of IsoPs from arachidonate is dramatically influenced by the presence of other fatty acid esters in the reaction mixture. Thus, three lipid mixtures containing the same arachidonate concentration but different amounts of other fatty esters (16:0; 18:1; 18:2; 20:5, and 22:6) were oxidized, and the product yields were determined by GC and LC/MS/MS analysis. The yield of F_2-IsoP formed after 1 h of oxidation was 18% (based on arachidonate consumed) for mixtures containing arachidonate as the only oxidizable PUFA, but yields of these biologically active compounds dropped to 6% in polyunsaturated fatty acid (PUFA) mixtures typical of those found in tissues of fish oil-fed animals. F_2-IsoP levels were also monitored in the livers of mice on diets supplemented with eicosapentaenoic acid (C20:5 ω-3; EPA), the PUFA most abundant in fish oil. While the level of arachidonic acid present in livers was not significantly different from that in control animals, levels of IsoPs in the liver were reduced in the EPA-fed mice compared to those in controls under conditions of oxidative stress (60 ± 25% reduction, $n = 5$) or at baseline (48 ± 14% reduction, $n = 5$). These results suggest that dietary ω-3 PUFAs may influence the formation of bioactive peroxidation products derived from ω-6 PUFAs by channeling the free radical pathway away from the F_2-IsoPs.

Introduction

Emerging evidence has implicated increased dietary intake of the ω-3 polyunsaturated fatty acids (PUFAs) present in fish oil in the prevention and treatment of a number of diseases in which environmental and lifestyle factors play a role. These include atherosclerotic cardiovascular disease,[1] neurodegenerative disorders,[2] and cancer,[3] among others. The mechanisms by which these PUFAs are protective are not understood although they possess anti-inflammatory properties.[4] Recent studies suggest that these anti-inflammatory effects and other biologically relevant properties of ω-3 fatty acids are due, in part, to the generation of various bioactive oxidation products.

Polyunsaturated lipids are particularly prone to react with molecular oxygen by mechanisms involving intermediate peroxyl free radicals and this process, *lipid peroxidation*, gives rise to a host of products.[5] Isoprostanes (IsoPs) are prostaglandin (PG)-like compounds that are formed from the peroxidation of arachidonic acid. Formation of these compounds proceeds by successive 5-*exo* radical cyclizations to give bicyclic endoperoxides that are reduced to PGF_2-like compounds, termed F_2-IsoPs.[6]

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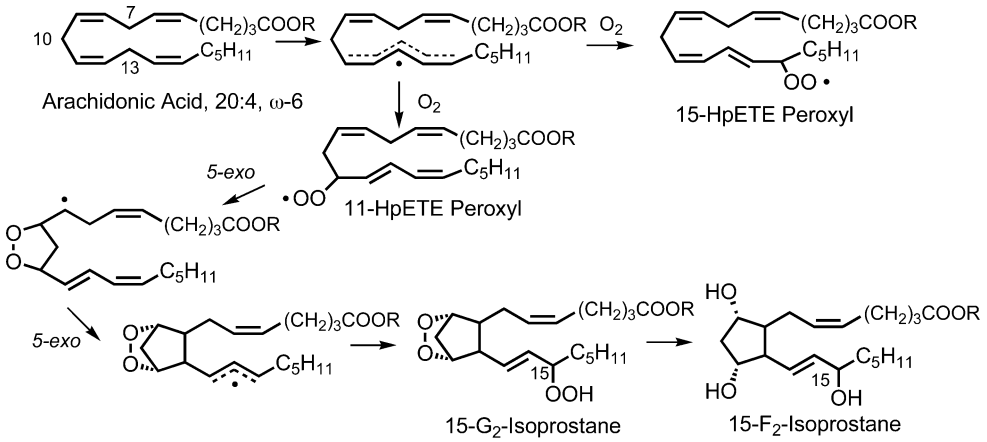
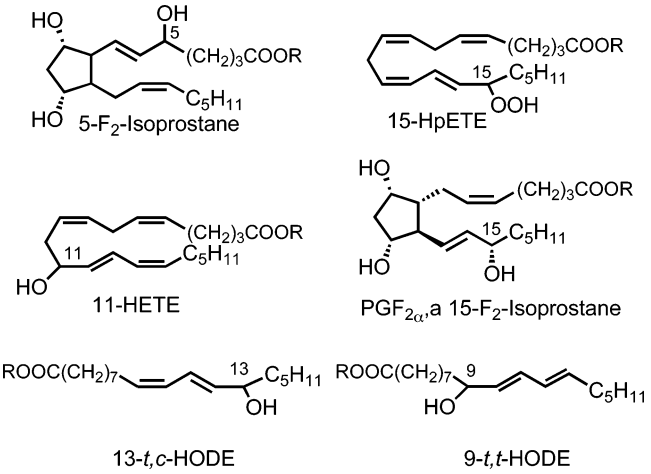
Scheme 1



The delocalized carbon radical shown in Scheme 1 is formed by abstraction of the weakly bonded hydrogen at C-13 of the chain.[7] Two other regioisomeric carbon radicals, not shown in the scheme, are formed by abstraction of the hydrogens bonded at C-7 and C-10, and regioisomeric IsoPs as well as hydroperoxy eicosatetraenoates (HpETEs) and hydroxy eicosatetraenoates (HETEs) are formed from these radicals. A nomenclature system for the IsoPs has been established and approved by the Eicosanoid Nomenclature Committee in which the different regioisomer classes are defined by the position of the side-chain hydroxyl relative to the carboxyl carbon which is designated as C-1. Each IsoP regioisomer is theoretically composed of eight racemic diastereomers. One of the 32 F_2-IsoP stereoisomers, $PGF_{2\alpha}$, is formed enzymatically by the action of prostaglandin synthase (COX) enzymes on arachidonate. The structure of an IsoP regioisomer, 5-F_2-IsoP, as well as those of 15-HpETE and 11-HETE, is illustrated below.



Quantification of F_2-IsoPs has been shown to be one of the most reliable markers to assess oxidant stress status and lipid peroxidation in vivo. In addition, F_2-IsoPs have been shown to exert potent vasoconstrictive activities, suggesting a pathogenic role for these compounds in diseases such as atherosclerosis.[8]

The oxidation product mixture from linoleate is simpler than the arachidonate product profile. The major primary products are the four hydroxyoctadecadienoates (HODEs), two of which have *trans,trans* conjugated dienes, the other two having *trans,-cis* diene geometry.[9] Two of the structures are illustrated above.

Eicosapentaenoic acid (C20:5, ω-3; EPA) is the most abundant polyunsaturated fatty acid (PUFA) in fish oil. The other important PUFA in fish oil is docosahexaenoic acid (C22: 6, ω-3; DHA) Studies in animals as well as human epidemiological studies, and more recently clinical intervention trials, suggest that fish consumption or dietary fish oil supplementation reduces the incidence of important diseases including atherosclerosis and sudden death, neurodegeneration, and various inflammatory disorders.[10] Although the mechanisms by which these beneficial effects occurs is unknown, a potentially important anti-atherogenic and antiinflammatory mechanism of ω-3 PUFAs is their interference with arachidonic acid peroxidation that generates pro-inflammatory F_2-IsoPs.

We report here on an examination of the effect of lipid composition on the distribution of oxidation products formed from arachidonate esters in a controlled free radical oxidation of well-defined lipid mixtures that include the ω-3 PUFAs. We also describe the effect of ω-3 PUFAs on the formation of IsoPs formed from arachidonate esters in vivo and we conclude that ω-3 lipid content has a significant effect on the distribution of oxidation products formed from ω-6 lipids. Peroxidation of lipid mixtures rich in highly unsaturated PUFA esters yield lower levels of the pro-inflammatory isoprostanes than are formed in oxidations of lipid mixtures having lower unsaturation levels.

Results

In vivo Studies. To determine the effect of unsaturated lipid levels on peroxidation product yields in vivo, we carried out preliminary exploratory experiments on rodents fed a diet including or lacking EPA. We supplemented rodents with diets containing 0% and 0.28% EPA for 8 weeks. Subsequently, animals were sacrificed and tissue lipids extracted and analyzed for F_2-IsoPs. In one set of studies, levels of F_2-IsoPs were quantified in livers from mice supplemented with 0.28%EPA. Liver tissue was examined because exposure of rodents to carbon tetrachloride initiates massive lipid peroxidation in that organ. Levels of fatty acid and F_2-IsoPs were measured at

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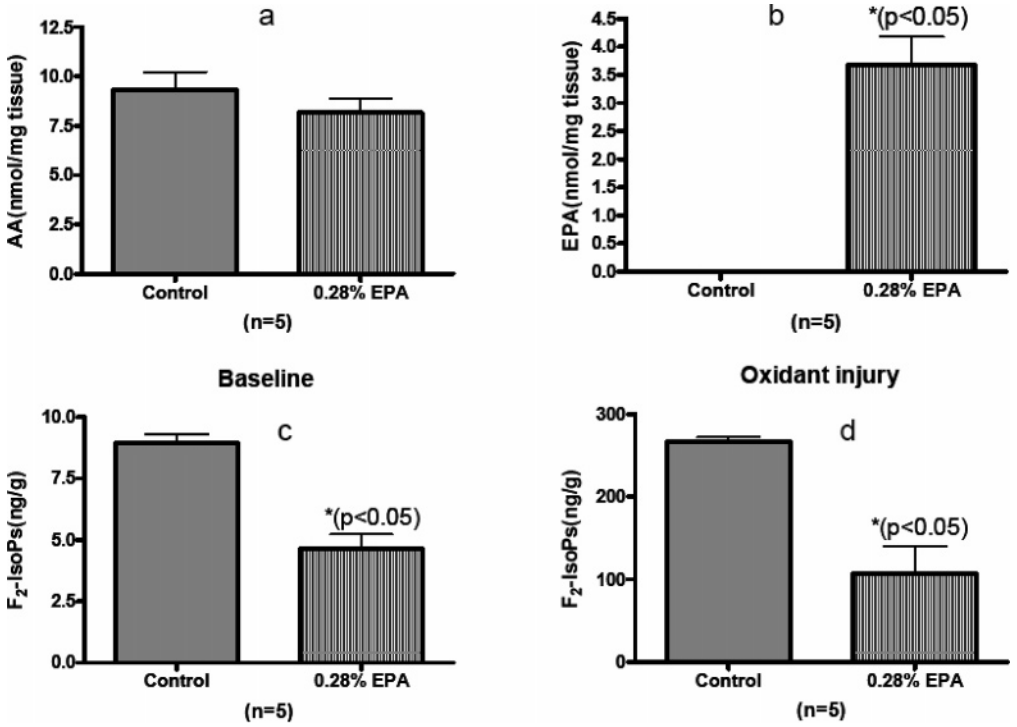

Figure 1. Arachidonic acid, EPA and F_2-IsoP levels in livers of mice on EPA-enriched diets. (a) Arachidonate levels in livers of control and EPA-fed mice. (b) EPA levels in liver after 8 weeks EPA dietary supplements. (c) Baseline levels of F_2-IsoP in livers of control and EPA-fed mice. (d) Levels of F_2-IsoP in livers of control and EPA-fed mice after CCl_4 treatment.

baseline for the EPA mice and control animals by well-established GC/MS procedures. F_2-IsoPs were also determined for EPA mice and controls after administration of carbon tetrachloride. The results of this study are presented in Figure 1. Levels of arachidonate in livers of EPA mice were marginally reduced (Figure 1a) compared to control animals, but there was a remarkable reduction of F_2-IsoPs in the EPA mice at baseline (Figure 1c) or after CCl_4-induced oxidative stress (Figure 1d). Significantly elevated EPA levels were observed in liver after 8 weeks EPA dietary supplementation (Figure 1b).

Chemical System Chosen for Study. Given the in vivo result that F_2-IsoP levels are significantly reduced in tissues having only marginally lower levels of the arachidonate precursor, we designed experiments to probe the effect of lipid unsaturation levels on arachidonate peroxidation product profiles. To minimize complications, we selected a protocol that controls for the rate of initiation and minimizes the number of steps between the peroxidation event and the analysis of stable end products. The compounds analyzed include the arachidonate-derived IsoPs, HETEs, and oxidation products derived from linoleic acid (HODEs). Oxidation products from EPA and DHA were not assayed in this protocol although methods development is ongoing for the analysis of oxidation products from those fatty acids.[11]

Mixtures of pentafluorobenzyl (PFB) esters of palmitic (16:0), oleic (18:1), linoleic (18:2), arachidonic (20:4), eicosapentaenoic (20:5), and docosahexaenoic (22:6) acids, and the free radical initiator 2,2′-azobis(2,4-dimethyl-4-methoxyvaleronitrile) (MeOAMVN) were reacted under air in thin-films at 37 °C. Samples were taken at 1, 2, and 3 h, and the samples were analyzed for consumption of starting material by gas chromatography and were then reduced with triphenylphosphine. Triphenylphosphine reduces hydroperoxides to alcohols (i.e. HpETEs → HETEs) and bicyclic endoperoxides to cyclopentane diols (G_2-IsoPs → F_2-IsoPs). The advantage of this protocol is that only one chemical step (PPh_3 reduction) and no prepurification steps exist between the oxidation and analysis. PFB esters of HETEs and F_2-IsoPs are assayed directly by HPLC/MS/MS with appropriate isotopically labeled internal standards as described in the following section.

The in vitro lipid mixtures studied were chosen roughly on the basis of plasma lipid profiles of fatty acids found in humans as a function of dietary lipid intake.[12] In this published study, 15 women took a safflower oil-rich diet providing 10.5 g/d of linoleate and a fish oil-rich diet, providing 2.0 g/d of EPA and 1.4 g/d of DHA in a three-treatment crossover trial. Plasma levels (in mmol/L) of unsaturated fatty acids from those on the safflower diet were approximately: 18:1 (ω-9) 1.8; 18:2 (ω-6) 4.1; 20:4 (ω-6) 0.75; 20:5 (ω-3) <0.1; 22:6 (ω-3) 0.1 mmol/L. Plasma levels resulting from the fish oil diet were: 18:1 (ω-9) 1.6; 18:2 (ω-6) 3.1; 20:4 (ω-6) 0.6; 20:5 (ω-3) 0.6; 22:6 (ω-3) 0.4 mmol/L.

The MeOAMVN-initiated oxidation of the four lipid mixtures shown in Table 1 was carried out. Of the lipids included in the mixtures, palmitate is not oxidizable, and oleate is a poor substrate for free radical oxidation. Linoleate,[13] arachidonate, EPA, and DHA are all highly oxidizable with the expected reactivity order being linoleate, arachidonate, EPA, and DHA

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Table 1. Mixtures of Fatty Acid Esters Oxidized

fatty acid PFB-ester	Mixture I (F.O.) (%)	Mixture II (S.O.) (%)	Mixture III (20:4) (%)	Mixture IV (18:2) (%)
palmitate; 16:0	33	43	33	33
oleate; 18:1, ω-9	14	16	59	59
linoleate; 18:2, ω-6	33	33		8
arachidonate; 20:4, ω-6	8	8	8	
EPA; 20:5, ω-3	8			
DHA; 22:6, ω-3	4			

due to the increasing number of C−H bonds allylic to two alkenes in these substrates.

Mixtures I, II, and III have equivalent amounts of arachidonate present, whereas mixture IV has linoleate present as the only oxidizable substrate for comparison with the other mixtures. Mixture I contains EPA and DHA in addition to linoleate and arachidonate, while Mixture II contains linoleate and arachidonate as the only readily oxidizable lipids. Palmitate and oleate[14] are used as nonoxidizable diluents in all of the systems studied. While there is not an exact correspondence, Mixture I approximates the PUFA lipid content of a plasma resulting from a fish oil (F.O.) diet, and mixture II is representative of safflower-oil (S.O.) diet plasma.

HPLC/MS/MS Analysis. Oxidation samples were analyzed by powerful liquid chromatography/mass spectrometry (LC/MS/MS) techniques.[15] Thus, quantitative information about diverse sets of peroxidation products was obtained by the use of selective reaction monitoring (SRM). The power of SRM is that in one LC/MS/MS assay, quantitative information of literally dozens of products can be obtained. In this protocol, HPLC separates isomers (A_1, A_2) of one product type from each other as well as from other product types (B_1, B_2). The separation is followed by an MS analysis in which a mass of interest (m_1, m_2) is selected in the first quadrupole of the MS, energy is added in the second quad, and a particular fragment (m_{fa}, m_{fb}) is detected in the third quadrupole of the instrument. The power of the SRM protocol in LC/MS/MS is that essentially three separations are carried out in one run. The first separation is by HPLC, the second is the selection of particular ions of interest in quad 1 of the MS, and the third is the selection of specific fragments unique for the products of interest that are detected. This assay provides a complete profile of oxidation products formed, as opposed to a typical IsoP GC/MS assay that reports on only one of many possible products. Furthermore, the LC/MS/MS protocol requires less derivatization and sample preparation than does the GC/MS assay which involves thin-layer chromatography and TMS derivatization.

Oxidation samples were chromatographed on a C-18 reverse-phase column with a methanol/0.1% aqueous acetic acid gradient which provides partial separations of HODEs, HETEs, along with the F_2-isoprostanes. As an example of the power of this technique, Figure 2 presents selected ion monitoring (SIM) and SRM LC/MS/MS chromatograms obtained in one run for HODEs, HETEs, and F_2-IsoPs in a sample derived from peroxidation of Mixture I. A host of products are formed, but in this chromatogram only ions and fragments from the compounds shown are printed. Figure 2A shows a composite of compounds detected that are HODEs, HETEs, and F_2-IsoPs. Figure 2B shows all of the HODEs that are formed, while 2C shows only the two 9-HODEs that differ by stereochemistry of the conjugated diene.

In Figure 2D SIM shows all compounds that have m/z of 319, i.e., all HETEs. It shows that the 5- and 15-HETEs are the major HETEs formed due to the fact that their precursor peroxyl radicals cannot cyclize to endoperoxides. In Figure 2E is shown only the SRM from 5-HETE; the other HETEs are not observed because they do not fragment to ions having $m/z = 115$. Chromatograms F and G of Figure 2 show analyses of the IsoPs. The SIM shown in Figure 2F shows all compounds that have an $m/z = 353$ (F_2-IsoPs), whereas 2G shows only the 5-series IsoPs by monitoring the fragmentation shown.

Arachidonate and Linoleate Oxidation Products: F_2-IsoPs, HETEs, and HODEs. In Table 2 are presented the results of arachidonate consumption and products formed (IsoPs and HETEs) in the oxidation of Mixtures I−III. Analyses in triplicate were implemented on triplicate samples. In general, arachidonate consumption is less for mixtures containing other oxidizable lipids as is the yield of F_2-IsoPs formed from arachidonate consumed. There appears to be no time dependence in yields of F_2-IsoPs formed, whereas yields of HETEs appear to decrease over time. HETEs are formed in higher yield in mixtures made up from more unsaturated lipids (Mixture I) than in those having a lower unsaturation index (Mixture III).

Four linoleate oxidation products were assayed in oxidations carried out on the systems that include linoleate: Mixtures I, II, and IV. HODEs are generally the only primary oxidation products formed from linoleate, and one instructive parameter that provides information about the medium of oxidation is the ratio of *trans,cis* conjugated diene HODEs (9- and 13-hydroxy) to the *trans,trans* diene HODEs (9- and 13-hydroxy).[9,16] This product ratio for the HODEs is presented in Table 3 for the three mixtures studied. HODE *trans,cis/trans,trans* product ratios are higher in mixtures made up from more unsaturated lipids (Mixture I) than in those having a lower unsaturation index (Mixture IV).

Discussion

One can conceive of multiple mechanisms by which dietary ω-3 PUFAs could affect peroxidation profiles from arachidonate- and linoleate-derived products. Among these mechanisms are: (1) *Lipid displacement*: a dietary increase in ω-3 PUFAs can displace ω-6 PUFAs in tissues and fluids, resulting in lower levels of peroxidation products from the ω-6 PUFAs. (2) *Product Profiles*: ω-3 PUFAs can alter the product profile of the ω-6 PUFAs consumed during peroxidation. (3) *Competitive peroxidation*: ω-3 PUFAs are more oxidizable than ω-6 lipids, and they can compete effectively for propagating peroxyls and alter the propagation/termination events, changing the consumption levels of ω-6 PUFAs. We consider each of these effects below.

Lipid Displacement. The effect of diet on composition of fluids and tissues is complex and poorly understood. In the course of studies on the effect of dietary ω-3 PUFAs on lipid peroxidation, we carried out an 8-week EPA feeding regimen on rodents. Liver tissue showed a small reduction in arachidonate levels in the EPA animals compared to controls, but the

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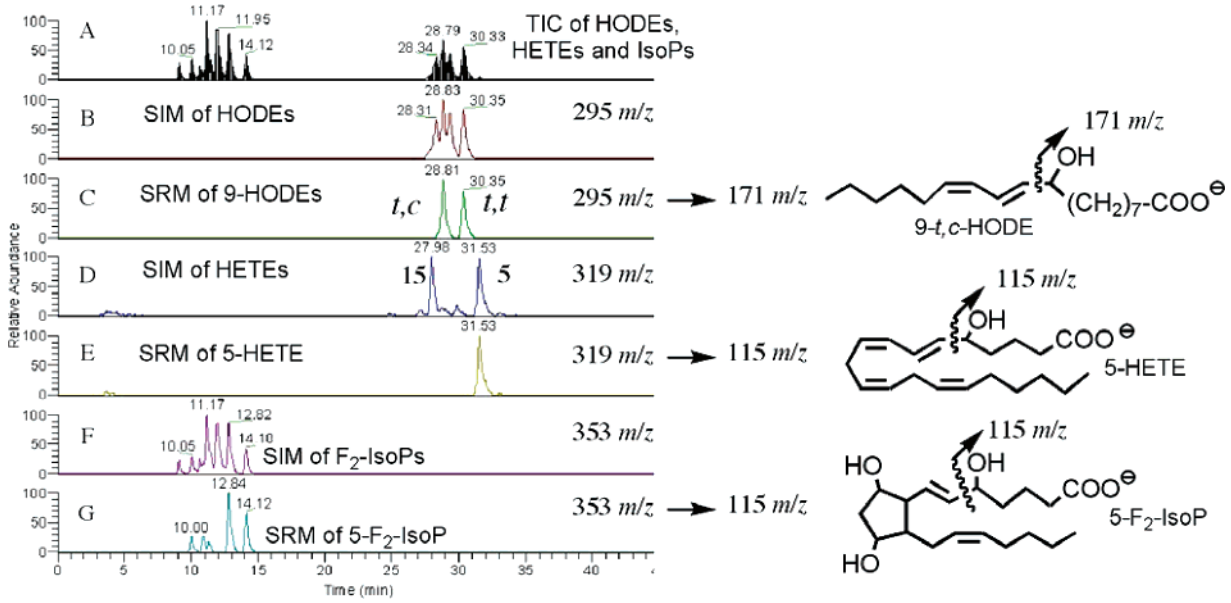


Figure 2. LC/MS analysis of fish oil peroxidation mixture.

Table 2. Product Yields from Free Radical Oxidation of Mixtures I−III[a]

	1 h	2 h	3 h
	Mixture I (F.O.)		
20:4 consumed[b]	2%	9%	15%
F_2-IsoPs	7.3 ± 2.8%	5.8 ± 1.6%	6.8 ± 1.8%
HETEs	5.0 ± 1.9%	5.3 ± 2.9%	5.5 ± 2.0%
mg F_2-IsoP[c]/g 20:4	1.5	5.2	10
	Mixture II (S.O.)		
20:4 consumed[b]	3%	10%	21%
F_2-IsoPs	10.7 ± 1.5%	15.3 ± 1.6%	12.8 ± 3.1%
HETEs	3.3 ± 1.7%	2.5 ± 1.3%	1.6 ± 0.7%
mg F_2-IsoP[c]/g 20:4	3.2	15	27
	Mixture III (20:4)		
20:4 consumed[b]	5%	21%	30%
F_2-IsoPs	18.1 ± 4.1%	18.6 ± 3.8%	16.1 ± 2.2%
HETEs	2.7 ± 1.8%	1.1 ± 0.2%	0.9 ± 0.3%
mg F_2-IsoP[c]/g 20:4	9	39	48

[a] Oxidations were carried out at 37 °C with MeOAMVN initiator. Samples were reduced with triphenylphosphine, isotopically labeled internal standards were added. Consumption of fatty acid esters was determined by GC; HETEs, HODEs, and IsoPs were analyzed by APCI LC/MS/MS in the SRM mode. Triplicate analyses of triplicate runs were carried out to give the standard error shown. [b] GC analysis of 20:4 PFB ester vs palmitate PFB ester as internal standard. [c] Based on 20:4 consumed and yield of IsoPs determined.

Table 3. HODE *trans, cis/trans, trans* Product Ratios for Oxidation Mixtures[a]

	1 h	2 h	3 h
Mixture I (F. O.)	1.4 ± 0.2	1.1 ± 0.1	1.1 ± 0.2
Mixture II (S. O.)	0.73 ± 0.04	0.61 ± 0.02	0.65 ± 0.04
Mixture IV (18:2)	0.40 ± 0.01	0.38 ± 0.05	0.39 ± 0.01

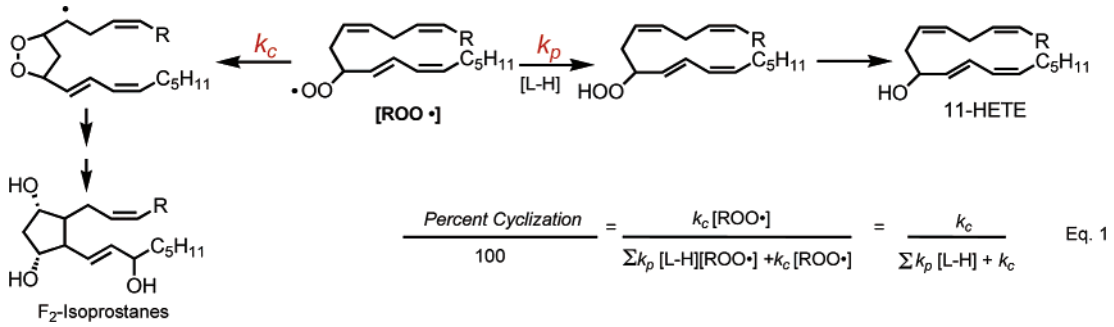
formation of arachidonate oxidation products, as indicated by assay of the F_2-IsoPs, was dramatically reduced in the EPA mice. The significant reduction of F_2-IsoPs in the EPA animals was observed at baseline and in animals exposed to a CCl_4-induced oxidative stress, Figure 1. Since these peroxidation products derived from arachidonate are thought to be pro-inflammatory, the observations may have consequences in pathologies associated with oxidative stress. These observations also stimulated us to investigate how lipid unsaturation levels could affect arachidonate peroxidation product profiles and to carry out the controlled oxidation studies reported here.

Arachidonate Product Profile. Oxidation of arachidonate in highly unsaturated lipid environments leads to lower yields of F_2-IsoPs and somewhat higher yields of HETEs based on arachidonate consumed as shown in Table 2. Cyclization is a unimolecular process, whereas the hydrogen atom (H-atom) transfer step is bimolecular, involving both the peroxyl radical and the H-atom donor, see Scheme 2. Equation 1 describes the relationship between lipid H-atom donors and product composition in terms of rate constants for cyclization and H-atom transfer.

The k_p for linoleate is 62 M^{-1} s^{-1} in organic solvents, and arachidonate, EPA, and DHA have propagation rate constants estimated to be 186, 248, and 310 M^{-1} s^{-1} based on the number of oxidizable C−H bonds in these lipids relative to those in linoleate. Local bilayer concentrations of lipids in biological membranes are approximated in the thin-film oxidations that we report here, with arachidonate concentrations in Mixtures I−III being about 0.25 M. On the basis of the PUFA concentrations relative to arachidonate, the Σk_p[L−H] term in the denominator of eq 1 is estimated to be 208 s^{-1} for Mixture I, 109 s^{-1} for Mixture II, and 47 s^{-1} for Mixture III. The yields of IsoPs for the mixtures (6%, 14%, 18%, respectively) decrease with increasing Σk_p[L−H], whereas the yields of HETEs increase with that parameter, consistent with the mechanism presented.

While Scheme 2 provides a framework for understanding the effect of lipid composition on product profiles, it nevertheless offers only a rough approximation of a very complex system. Indeed, a one-to-one correlation between F_2-IsoP yields and calculated *percent cyclization* is not expected since several products other than the F_2-IsoPs result from cyclization. For example, monocyclic peroxides, serial cyclic peroxides, and epoxides shown in Figure 3 have been identified as products that are formed from peroxidation of arachidonate, and each of these product types is formed after an initial peroxyl radical

Scheme 2



cyclization.[17] Furthermore, bicyclic endoperoxides such as 15-G_2-IsoP shown in Scheme 1 are unstable with respect to decomposition to levuglandins (isoketals), hydroxy-heptatrienoate (HHT), and the E_2, D_2, A_2, and J_2 IsoPs.[18] The mechanistic complexity described here suggests that a more rigorous analysis of the system based on eq 1 is unwarranted.

The yield of F_2-IsoPs formed from arachidonate peroxidation has been of interest since the compounds were first isolated and identified in vitro and in vivo. The currently accepted view of workers in the field is that the isoprostane pathway is only a minor one. An early in vitro study of arachidonate peroxidation in rat liver microsomes, for example, estimated a yield of F_2-IsoPs of less than 0.01% based upon arachidonate consumed.[19] Product yields of IsoPs depend on the conditions of oxidation and the stability of primary products formed. The conditions we have chosen for oxidation are reasonably mild, and aliquots are immediately reduced, minimizing the possibilities of decomposition to other product types during the reaction sequence. Our studies show that the isoprostanes may make up a substantial fraction of the peroxidation primary product profile. The arachidonate peroxidation product profile will, however, depend on the partitioning between the products shown in Figure 3, all of which are formed after cyclization. This partitioning in vitro or in vivo will depend on oxygen tension, H-atom donors present (including antioxidants), and the reducing nature of the medium.

Competitive Peroxidation. The data presented in Table 2 show that lipid composition also affects consumption of arachidonate in thin-film oxidations. We observe that the reaction of highly unsaturated mixtures, under conditions that are otherwise identical, generally results in lower arachidonate consumption than does oxidation of mixtures having a lower unsaturation index. Thus, percent arachidonate consumption at 1, 2, and 3 h of oxidation generally follows the order: Mixture III > Mixture II > Mixture I.

Arachidonate consumption should depend on the rate of initiation, the rate of termination, and the concentration of the oxidizable substrate according to eq 2

$$\frac{\mathrm{d}[20{:}4]}{\mathrm{d}t} = \sqrt{\frac{R_\mathrm{i}}{2k_\mathrm{t}}}\, k_\mathrm{p}[20{:}4] \qquad (2)$$

where R_i is the rate of radical generation, k_t is the rate constant for chain termination, and k_p is the rate constant for abstraction from arachidonate.

Arachidonate and initiator concentrations are comparable for all of the lipid mixtures; therefore, eq 2 does not provide a simple basis for understanding the arachidonate consumption results since [20:4] and presumably R_i are comparable for all of the mixtures. There is no guarantee, however, that highly oxygenated peroxyls derived from EPA and DHA will propagate or terminate with rate constants identical to those of the less oxygenated peroxyls derived from linoleate, and this could affect the overall consumption of arachidonate in the highly unsaturated lipid mixtures. In this regard, it is worth noting that Yazu et al. reported that EPA is less oxidizable than linoleate in a micellar environment, whereas it is more oxidizable than linoleate in an isotropic medium.[20] Neverthelesss, the relevant medium for study of peroxidation in vivo is not thin-film, micellar, or isotropic, but rather, it is the phospholipid bilayer. Oxidation experiments on appropriate bilayer model systems are currently in progress, and a detailed analysis of unsaturation index vs arachidonate consumption awaits the completion of those studies.

Linoleate Product Profile. Table 3 shows that product profiles of linoleate-derived HODEs also reflect the unsaturation index of the medium. More *trans,cis* products are formed in the highly unsaturated mixtures compared to the amounts formed in more saturated systems. This result is anticipated on the basis of earlier studies that describe the mechanism of HODE formation.[8,16] The *trans,cis* products are kinetic products that form by trapping the corresponding peroxyls with good H-atom donors such as the highly unsaturated PUFAs, see Scheme 3.

The *trans,cis/trans,trans* (*t,c/t,t*) product ratio and the known unimolecular fragmentation rate constants associated with the linoleate system have recently been used as a peroxyl radical clock to determine rate constants for H-atom transfer reactions. Thus, given a single H-atom donor of known concentration, one can determine k_p for that donor by determination of the *t,c/t,t* product ratio and application of eq 3 in Scheme 3.[21]

It is of interest to apply eq 3, which was derived for oxidations in isotropic media at 37 °C, to make predictions about the *t,c/t,t* product ratio in the thin-film oxidations reported here. We estimated the $\sum k_\mathrm{p}$[L−H] term to be 208 s^{-1} for Mixture I and 109 s^{-1} for Mixture II for purposes of discussion of the competition in Scheme 2, vide supra. Insertion of those values

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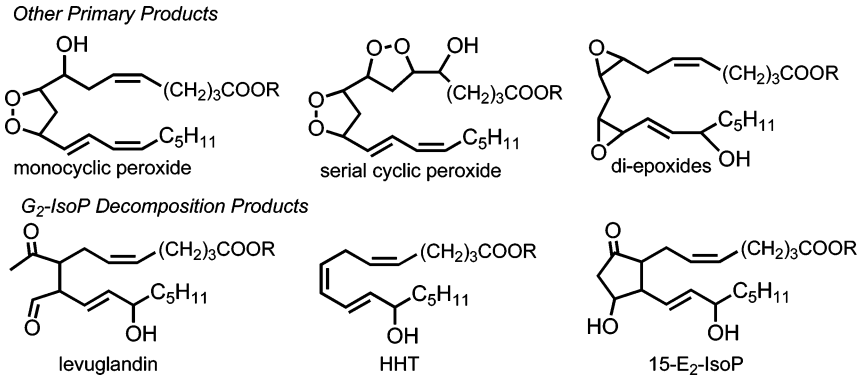
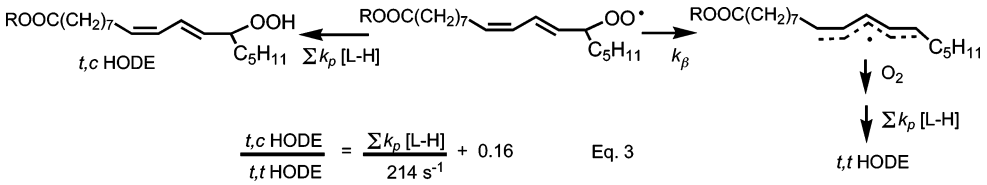
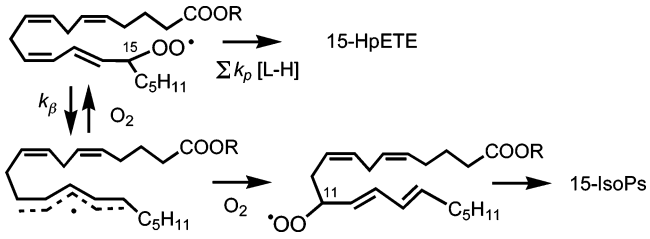


Figure 3. Products that form from peroxyl radical cyclization and/or endoperoxide decomposition.

Scheme 3



Scheme 4



in eq 3 predicts a *t,c/t,t* product ratio of 1.14 for Mixture I and 0.67 for Mixture II, in good agreement with the measured experimental values (*t,c/t,t* = 1.2 for Mixture I and 0.65 for Mixture II). This suggests, at a minimum, that the thin-film and solution oxidations are comparable for linoleate and that our concentration estimates for lipids in the thin-films is reasonable. The analysis also provides a kinetic basis for use of the *t,c/t,t* product ratio in studies of lipid peroxidation in vivo.[22]

One final mechanistic point relates the β-scission pathway shown in Scheme 3 with the IsoP yields determined for Mixtures I−III. Peroxyl radicals centered at C-5 and C-15 of the arachidonate chain cannot undergo *5-exo* cyclization and give IsoP products. In contrast, the peroxyl radicals substituted at C-8, -9, -11, or -12 of the 20-carbon arachidonate chain have structures that permit cyclization and IsoP formation. Note, however, that β-fragmentation of a 5- or 15-peroxyl gives a pentadienyl radical that can proceed on to IsoP products. The kinetic products of arachidonyl 5- and 15-peroxyls, formed preferentially in highly unsaturated lipid mixtures, are the 5- and 15-HpETEs, while the thermodynamic products are the IsoPs. This mechanism is described in Scheme 4 for the 15-peroxyl radical, and it introduces an additional factor to account for a reduction of IsoP product formation in highly unsaturated mixtures.

Summary

IsoPs are generally considered to be pro-inflammatory molecules that have been implicated in the pathophysiological consequences of oxidative stress. It is thus intriguing to propose that part of the mechanism by which fish oil prevents certain diseases associated with increased inflammation relates to its known ability to decrease IsoP generation.[23] Our studies show that lipid composition alone can affect the yield of IsoP formation. Thus, lipid mixtures containing highly unsaturated ω-3 fatty esters present in fish oil give lower yields of IsoPs than are formed in more saturated lipid mixtures, based upon arachidonate consumed. The results can be understood with reference to the peroxyl radical mechanism for formation of the IsoPs. Further understanding of the biological consequences of the formation of these novel compounds and factors influencing their formation and metabolism may provide valuable insights into the role of ω-3 fatty acids in human physiology and pathophysiology.

Experimental Section

Chemicals. All fatty acids were purchased from Nu-Chek Prep (Elysian, MN) and were of the highest purity (<99%). Pentafluorobenzyl bromide, diisopropyl ethylamine, triphenyl phosphine, and butylated hydroxyl toluene (BHT) were purchased from Aldrich Chemical Co. (Milwaukee, WI) and were used without further purification. Deuterated internal standards were purchased from Cayman Chemical Co. (Ann Arbor, MI) and were used without further purification. Pentafluorobenzyl (PFB) esters of the fatty acids and deuterated internal standards were synthesized by previously established methods. Purification of the fatty acid PFB-esters was performed using a Biotage SP-1 column chromatography system, and deuterated internal standards were purified by normal phase HPLC (0.5% 2-propanol/hexanes) using a Beckman Ultrasphere 5-μm silica column (2.0 mm × 25 cm). Concentration of 15(S)-HETE-d_8 was determined by UV−vis spectroscopy (ϵ = 27,000, λ_{max} = 236 nm) using an Agilent 8453 UV−vis spectrometer (Wilmington, DE) and $PGF_{2\alpha}$-d_4 by GC/MS/SIM peak integration versus previously determined concentration of $PGF_{2\alpha}$.

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Oxidation of Fatty Acid Mixtures, GC and LC/MS/MS Sample Preparation. Stock solutions (1.00 M) of each individual fatty acid PFB ester were prepared in benzene. An indicated amount of each fatty acid PFB ester was added, based on the ω-6, ω-9, safflower, and fish oil diets. PUFA mixtures were passed through a silica gel Sep-Pak column (5% ethyl acetate in hexanes), and 2.5 mL of each was collected to ensure the absence of any premature oxidation products. The sample was again concentrated by a flow of N_2. The thin-film was dissolved in benzene (250 μL), and 20−μL aliquots of the PUFA mixture and 10 μl of 1 mol % MeOAMVN solution were added to each individual oxidation vial. The solvent was once again removed by a flow of N_2, and the oxidations were allowed to proceed as thin-films at 37 °C. At 0-, 60-, 120-, and 180-min time frames the oxidations were stopped. Each sample was diluted with benzene (500 μL) and divided into two vials (250 μL in each vial). To one vial was added BHT (1 M, 25 μL), and the sample was analyzed by gas chromatography for fatty acid consumption. PPh_3 (1 M, 25 μL) was added to the second vial, an aliquot (150 μL) was removed, and the sample was concentrated by a flow of N_2. PPh_3 is known to reduce hydroperoxides and bicyclic endoperoxides, but its reaction with dialkyl peroxides and other cyclic peroxides is slow at room temperature.[5,6,24] The thin film was dissolved in a solution of methanol/H_2O (4:1, 350 μL) and a mixture (150 μL) of PFB-15(S) HETE-d_8 (0.0590 ng/μL) and PFB-$PGF_{2\alpha}$-d_4 (0.0525 ng/μL) was dissolved the thin-film as a solution of PFB esters in methanol/water (8:2) was added. The suspension was filtered through a Whatman 0.45 μm nylon filter. The solution was analyzed by LC/MS/MS for oxidation product formation.

Gas Chromatography. Gas chromatography flame ionization detection was performed using a Hewlett-Packard model 6890 series GC equipped with a 30-m length, 0.25-mm diameter, 0.2-μm film thickness AT Silar-90 column (AllTech, Columbia, MD) and an Agilent Technologies 7683 series autosampler. Initial temperature was set at 190 °C and held for 10 minutes. The temperature was then ramped at 10 °C/min to a final temperature of 240 °C and held for 10 minutes. Injector temperature was set at 220 °C, and helium carrier gas pressure was 12.2 psi. Concentrations of fatty acid PFB esters were referenced to that of palmitate PFB ester internal standard.

Mass Spectrometry. HPLC/MS/MS was conducted using a ThermoFinnigan TSQ Quantum Ultra equipped with a Finnigan Surveyor Autosampler Plus. Reversed phase HPLC was performed using a Supelco (4.6 mm × 25 cm) C-18 column with a gradient (80:20 to 90:10) of methanol/0.1% acetic acid in water at a flow rate of 1 mL/min. The MS was operated in the negative ion mode using atmospheric pressure chemical ionization (APCI) in the selective reaction monitoring (SRM) mode monitoring the fragmentation alpha to the alcohol for the HODEs, HETEs, and isoprostanes.[15] MS parameters were optimized for the 15(S)-HETE-d_8 and $PGF_{2\alpha}$-d_4 and were as follows: auxiliary gas pressure was set at 20 psi, sheath gas pressure was 19 psi, utilizing nitrogen for both. Discharge current was set at 20 eV, and the vaporizer temperature was set at 370 °C. Collision-induced dissociation (CID) for the HETEs and HODEs were optimized respectively at 14 and 20 eV for the isoprostanes under 1.5 mTorr of argon. Spectra were displayed by averaging the scans across the individual chromatography peaks. Data acquisition and analysis were performed using Xcalibur software version 1.4 (San Jose, CA).

Assays of F_2-IsoPs and Fatty Acid Content from Mouse Liver Tissue. Mice (strain C57BL/6J(B6)) were fed with a rodent AIN-93 diet supplemented with 0% and 0.28% EPA. After 8 weeks feeding, the mice were sacrificed. Liver tissues were removed, homogenized, and hydrolyzed to liberate free F_2-IsoPs. F_2-IsoPs in samples were then purified and analyzed as previously described. Fatty acid content in liver tissue was determined by GC.[25]

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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