Regiochemistry of Neuroprostanes Generated from the Peroxidation of Docosahexaenoic Acid in Vitro and in Vivo*

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Isoprostanes (IsoPs) are isomers of prostaglandins that are generated from the free radical-initiated peroxidation of arachidonic acid (C20.4 ω-6). IsoPs exert potent bioactivity and are regarded as the “gold standard” to assess oxidative stress in various human diseases. Analogously, autoxidation of docosahexaenoic acid (DHA, C22.6 ω-3) generates an array of IsoP-like compounds that are termed neuroprostanes (NPs). A major class of NPs identified in vitro and in vivo contains F-type prostane rings and are known as F4-NPs. A number of different F4-NP regioisomers are formed from the peroxidation of DHA. Among the eight possible regioisomeric groups, we hypothesize that 4- and 20-series NPs are generated in greater amounts than other classes because the precursors that lead to regioisomers other than those of the 4- and 20-series can be further oxidized to form novel dioxolane-IsoP-like compounds, analogous to those generated from arachidonate. Various mass spectrometric approaches, including electron capture atmospheric pressure chemical ionization mass spectrometry, were utilized to analyze NPs formed in vitro and in vivo based on their characteristic fragmentation in the gas phase. Experimental results were consistent with our hypothesis that 4- and 20-series NP regioisomers are preferentially generated. The discovery of regioselectivity in the formation of NPs will allow studies of the biological activities of NPs to focus on the more abundantly generated compounds to determine their role in modulating the pathophysiological consequences of DHA oxidation and oxidant stress.

Free radical-induced autoxidation of polyunsaturated fatty acids (PUFAs) has been implicated in numerous human disorders, including atherosclerosis, cancer, and various neurodegenerative diseases (1–3). Neuroprostanes (IsoPs) are prostaglandin (PG)-like compounds that are formed non-enzymatically in vivo from the peroxidation of arachidonic acid (C20.4 ω-6) by free radicals. A number of IsoPs have been identified that contain different functional groups on the prostane ring analogous to different classes of PGs. These include F4-IsoPs, D2/E2-IsoPs, A2/J2-IsoPs, and isothromboxanes (4, 5). A novel aspect of IsoP formation is that, unlike cyclooxygenase-derived PGs, IsoPs are generated in situ, esterified in phospholipids and other lipids, and subsequently cleaved from these lipid storage sites by various enzymes. In addition, IsoPs have different stereochemistry from PGs in that the former contain side chains that are predominantly in the cis configuration in relation to the prostane ring (6). In addition to novel aspects of their formation, quantification of IsoPs has emerged as one of the most accurate approaches to assess oxidant injury in vivo (4, 7). Furthermore, certain IsoPs exert potent biological activity (6).

Docosahexaenoic acid (C22.6 ω-3, DHA) is a fatty acid that is highly enriched in brain, particularly in gray matter, where it comprises ~25–35% of the total fatty acids in aminophospholipids, such as phosphatidylserine (8–10). Oxidation of DHA in the central nervous system has been implicated in various neurodegenerative disorders, including, importantly, Alzheimer disease. DHA is highly susceptible to autoxidation because it has six double bonds. We have previously shown that analogous to arachidonic acid, IsoP-like compounds can be generated from the free radical-catalyzed peroxidation of DHA both in vitro and in vivo. These compounds are termed neuroprostanes (NPs) (11–14). The first class of NPs discovered contained F-type IsoP rings and four double bonds and are designated F4-NPs. We have found that F4-NPs are markedly increased in the brain tissue of humans with Alzheimer disease supporting the contention that oxidation of DHA is increased in this disorder (12, 13).

Several studies have examined the regiochemistry and stereochemistry of IsoP formation (15–17). These were undertaken to provide a rationale for biological studies focusing on the most abundantly generated isomers. The free radical-initiated peroxidation of arachidonic acid and its esters generates complex mixtures of primary hydroperoxides and cyclic peroxides with dozens of regioisomers and diastereomers (15, 18). Four IsoP regioisomer classes are formed from the oxidation of arachidonate depending on the site of initial hydrogen atom abstraction and subsequent oxygen insertion (Scheme 1). 5-Series and 15-series IsoPs are produced from hydrogen atom abstraction at C-7 and C-13, respectively, whereas hydrogen atom abstraction at C-10 gives rise to 8-hydroperoxyeicosatetraenoic acid (8-HPETE) and 12-HPETE that subsequently generate 12-series and 8-series IsoPs, respectively. Among these four regioisomers, in vitro and in vivo studies have shown that 5- and 15-series IsoPs are formed in significantly greater amounts than 8- and 12-series IsoPs (19–21). The difference in the formation of various IsoP regioisomers is a consequence of

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§ The abbreviations used are: PUFA, polyunsaturated fatty acid; AP, atmospheric pressure; CI, chemical ionization; CID, collision-induced dissociation; DHA, docosahexaenoic acid; EC, electron capture; GC, gas chromatography; HPETE, hydroperoxyeicosatetraenoic acid; HpDHA, hydroperoxydocosahexaenoic acid; HPLC, high-performance liquid chromatography; Isp, isoprostane; LC, liquid chromatography; MS, mass spectrometry; NP, neuroprostane; PFB, pentafluorobenzyl; PG, prostaglandin; SIM, selective ion monitoring; SRM, selective reaction monitoring; TMS, trimethylsilyl.
that initial studies related to NP bioactivity should focus on 4-
regarding the relative formation of different NPs and suggest
various NP isomers. These findings have significant implications
results
other NP isomers. In addition, we report virtually identical re-
4- and 20-series NPs are generated in greater amounts than
MS. The results obtained are consistent with our hypothesis that
APCI-MS and by gas chromatography (GC)-electron ionization
mers generated from the autoxidation of DHA both
(SCHEME 2). Based on our previous studies of arachidonate per-
oxidation, we hypothesize that 4- and 20-series NPs will be pro-
duced in greater amounts than the other regioisomers because
the precursor radicals that lead to these two 4- and 20-series
NPs cannot further cyclize to form dioleolane-ISO-P-like compounds.
Herein, we provide experimental evidence to show that, indeed,
4- and 20-series NPs are two of the most abundant NP regio-
isomers generated from the autoxidation of DHA both in vitro and
in vivo. Several approaches were used. First, we purified the
various HpDHA isomers generated from the autoxidation of this
PUFA as described using semipreparative HPLC (24). We then
oxidized different HpDHA species and analyzed the NP products
using liquid chromatography atmospheric pressure chemical ion-
ization mass spectrometry (LC-APCI-MS). The MS fragmenta-
tion pattern for various NPs was extrapolated from our data for
ISOps and verified by collision-induced dissociation (CID).
Secondly, oxidation mixtures of DHA F4-NPs were studied by LC-
APCI-MS and by gas chromatography (GC)-electron ionization
MS. The results obtained are consistent with our hypothesis that
4- and 20-series NPs are generated in greater amounts than
other NP isomers. In addition, we report virtually identical results
in vivo when rodent brain and liver tissue are analyzed for
various NP isomers. These findings have significant implications
regarding the relative formation of different NPs and suggest
that initial studies related to NP bioactivity should focus on 4-
and 20-series compounds.

EXPERIMENTAL PROCEDURES

Materials—All lipid autoxidation reactions were carried out under
an atmosphere of oxygen unless otherwise noted. Air and argon were
passed through a bed of calcium sulfate desiccant. Benzene was dis-
tilled from sodium and stored over 4A molecular sieves. Oxygen (med-
ical grade) was obtained from A.L. Compressed Gases (Nashville, TN).
HPLC grade solvents were purchased from Burdick & Jackson (Musk-
ergon, MI) or EM Science (Gibbstown, NJ). All lipids were purchased
from Nu Chek Prep (Elysian, MN) or EM Science (Gibbstown, NJ). 17-F4c-NP
synthesized prior to use (25). N,N-Bis-(trimethylsilyl)trifluoracetamide
was purchased from Supelco Inc. (Bellefonte, PA). PGF
O
N
O
N
, [D-H]15-F2t-
IsoP (8-iso-PGF
O
N
O
N
), and 5- and 8-series F2-IsoP deuterated standards
were purchased from Cayman Chemicals (Ann Arbor, MI). 17-F2t-NP
was chemically synthesized, and
O exchange was performed accord-
ing to a procedure reported previously (12, 26). The unlabeled blank of
the standard is 2 parts/thousand. All other reagents were purchased
from Aldrich and used without further purification.

Lipid Identification and Separation Methods—Reactions involving
hydroperoxides were visualized by TLC using the standard is 2 parts/thousand. All other reagents were purchased
from Aldrich and used without further purification.
(254 nm) with a Mineralslag UVSL-25 hand lamp. Preparative TLC was carried out on silica gel 60ALK6D plates (Whatman).

Analytical HPLC was performed using a Waters Model 600E pump with a Waters 996 photodiode array detector. Millenium32 chromatography software (Waters Corp., Milford, MA) was utilized to control the array detector and to collect and process data. Pentafluorobenzyl (PFB) esters of IsoPs and NPs were analyzed by normal phase HPLC using 12% isopropanol in hexanes and a single analytical Beckman Ultrasphere 5-μm (4.6 mm × 25 cm) silica column or two narrow bore Beckman Ultrasphere 5-μm (2.0 mm × 25 cm) silica columns. A flow rate of 1 ml/min was used for analytical normal phase HPLC, whereas 0.2 ml/min was employed for narrow bore columns. Semipreparative HPLC was carried out using a Dynamax-60 Å 8-μm (83–121-C) silica column (21.4 mm × 25 cm) with a flow rate of 10 ml/min. For reverse phase HPLC analysis of PFB esters, a Phenomenex (Torrance, CA) Ultracarb ODS 5-μm 4.6 mm × 25 cm column was used at 1 ml/min flow rate with a gradient starting with 50% solvent A (95:5, water:MeOH) and 50% solvent B (5:95, water:MeOH), holding for 2 min and increasing to 100% B in 20 min and holding for 10 min.

Mass Spectrometry—On-line reverse phase liquid chromatography was carried out using the ThermoFinnigan Surveyor MS Pump 2.0 equipped with the columns mentioned above. Samples were analyzed using a ThermoFinnigan TSQ Quantum 1.0 SR 1 mass spectrometer. The APCI source was fitted with a deactivated fused silica capillary (100 μm, inner diameter). The mass spectrometer was operated in negative ion mode with a capillary temperature of 300 °C, vaporizer temperature of 460 °C, discharge current 20 and ~94 V tube lens voltage. Nitrogen was used as both the sheath gas and the auxiliary gas, at 21 and 3 p.s.i., respectively. For tandem MS experiments, collision energies were optimized between 20 and 30 eV under 1.5 millitorr of argon. Data acquisition and analysis were performed using Xcaliber software, version 1.3. Samples were introduced either by direct liquid infusion or HPLC. For normal phase HPLC, samples were introduced by a Hewlett-Packard 1090 HPLC system.

Gas chromatography MS was performed in chemical or electron ionization mode using a Hewlett-Packard HP5989A GC-MS instrument interfaced with an IBM Pentium computer system. GC was carried out using a 30-m, 0.25-mm diameter, 0.25-μm film thickness DB 1701 fused silica capillary column (Fison, Folsom, CA). The column temperature was programmed from 190 to 260 °C at 10 °C/min. Methane was used as the carrier gas at a flow rate of 1 ml/min. Ion source temperature was 250 °C, electron energy was 70 eV, and filament current was 0.25 mA.

Oxidation of DHA Methyl Ester to Primary Hydroperoxides—DHA methyl ester (500 mg, 1.46 mmol) was dissolved in 1.5 ml of acetonitrile to make a solution of 0.97 M. To the mixture were added 44 mg of N-methyl benzohydroxamic acid (2.92 mmol) and 0.05 equivalent of di-tert-butyl hyponitrite as a radical initiator (27). The reaction was stirred under oxygen at 37 °C for 24 h. The reaction was quenched by adding 2 mg of butylated hydroxytoluene, and N-methyl benzohydroxamic acid was removed by flash chromatography on silica gel. The hydroperoxides were analyzed and separated by analytical and semipreparative HPLC, and reasonable separation was obtained as described previously (24). The separated fractions of primary hydroperoxides were converted to more highly oxidized peroxides according to

Scheme 2

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our previous procedure (18). Alternatively, a mixture of all NP isomers derived from DHA was obtained by incubating DHA with di-tert-butyl hyponitrite. A similar method was employed to generate mixture of IsoPs from arachidonate as described previously (15).
were reduced by adding an excess amount of PPh₃ or by catalytic hydrogenation using palladium on carbon (28). The reduced lipid was subjected to basic hydrolysis using 1.0 M KOH for 60 min at 40 °C. After adjusting to pH 3, the mixture was loaded onto a C₁₈ Sep-Pak (Waters Corp.) cartridge that was preconditioned with 5 ml of methanol and then 5 ml of water. The column was washed with 5 ml of water and 5 ml of heptane. The eluent of 10 ml of ethyl acetate was collected. The solution was dried over Na₂SO₄. After evaporation of the solvent, the residue was dissolved in 200 μl of CH₃CN. To the resulting solution was added 200 μl of 10% (v/v) PFB bromide in acetonitrile and 100 μl of 10% (v/v) N,N-diisopropylethylamine in acetonitrile, and the mixture was kept at room temperature for 30 min. The reagent was dried under nitrogen, and the residue was subjected to TLC separation using a protocol reported recently (12). The PFB esters, after TLC separation, were either directly analyzed by LC-APCI-MS or were further derivatized to trimethylsilyl (TMS) derivatives for GC-MS analysis. For the latter, after evaporation of the ethyl acetate, 20 μl of N,O-Bis-(trimethylsilyl)trifluoroacetamide and 10 μl of dimethylformamide were added to the residue, and the mixture was incubated at 40 °C for 20 min. The reagents were dried under nitrogen, and the derivatives were dissolved in 10 μl of dry undecane for analysis by GC-MS.

**Purification and Analysis of F₄-NPs from Rat Brain—** The purification and derivatization scheme for the analysis of F₄-NPs was reported recently and utilized herein (12). Briefly samples of brain tissue (75–300 mg) were homogenized in 5 ml of ice-cold chloroform:methanol (2:1, v/v) containing butylated hydroxytoluene (0.005%). Esterified F₄-NPs in phospholipids were isolated and hydrolyzed using chemical saponification by adding 2 ml of 15% aqueous potassium hydroxide. The samples were acidified to pH 3 with 1 M HCl and diluted to 25 ml with pH 3 H₂O. 1 ng of a chemically synthesized 𝓓₀ 17-F₄c-NP standard was then added to the samples and purified by Sep-Pak extraction. Purified NPs were converted to PFB esters TMS ether derivatives and analyzed by GC-MS.

**FIG. 2.** Collision-induced dissociation results of selected NPs analyzed by LC-APCI-MS (parent ion m/z 377 with an offset of 30 eV). a, 10-series NP obtained from oxidation of purified 14-HpDHA; inset indicates the LC chromatogram that was averaged to generate the CID spectrum. b, 17-series NP obtained from oxidation of purified 13-HpDHA.
Animal Model of Oxidant Stress and Purification of NPs From Rat Livers—The extraction of lipids from livers of rats has been reported previously (29). In brief, after intragastric administration of CCl₄ (2 mg/kg) in corn oil to Sprague-Dawley rats for 2 h, the animals were anesthetized with pentobarbital (60 mg/kg) intraperitoneally and sacrificed. Livers were subsequently removed and 1–4 g of tissue immediately extracted to obtain a crude phospholipid mixture. After basic hydrolysis, PFB esterification and TMS derivatization were performed and NPs analyzed as described above.

RESULTS

Application of LC-APCI-MS to Study the Regioselectivity of Isoprostanes Formed from the Autoxidation of Arachidonic Acid—As noted, several reports have examined issues related to the stereochemistry of IsoP formation in vitro and in vivo (17, 20). Among the four IsoP regioisomers that are generated, 5- and 15-series molecules are formed in significantly greater amounts than 8- and 12-series IsoPs (Scheme 1). This selectivity in regioisomer formation is because of the generation of dioxolane-IsoPs from the precursors that lead to 8- and 12-series IsoPs, whereas the precursors of 5- and 15-series IsoPs cannot undergo further oxidation to form dioxolane-IsoPs. Additionally, for each IsoP regioisomer, it is hypothesized that eight diastereomers are generated. We have previously identified these eight diastereomers of the 15-series IsoPs utilizing GC-MS (15).

EC-APCI-MS has been utilized to structurally characterize various synthetic and biological molecules after electrophilic derivatization (30, 31). This technique is revolutionary because it combines the exquisite sensitivity and selectivity of EC with HPLC separation methods. We have utilized EC-APCI-MS to examine the formation of different arachidonate-derived IsoPs as a prelude to studying DHA-derived NP regioisomer generation. For these studies, a mixture of in vitro arachidonate oxidation products was initially reduced with triphenylphosphine and F₂-IsoP regioisomers were analyzed as PFB esters employing selective reaction monitoring (SRM). The results are illustrated in Fig. 1. Corroborating our previous report using GC-MS, 5- (Fig. 1a) and 15-series (Fig. 1d) IsoPs are formed in significantly greater abundance than 8- (Fig. 1c) and 12-series (Fig. 1b) compounds (20, 22). Because deuterated internal standards for 15- (Fig. 1e), 5- (Fig. 1f), and 8- (Fig. 1g)-series IsoPs have nearly identical responses in our SRM analysis, the relative intensity of individual regioisomers reflects the actual abundance.

EC-APCI-MS facilitates the characteristic fragmentation of the regioisomeric F₂-IsoPs in the gas phase of mass spectrometry. The four IsoP regioisomers can be divided into two groups when analyzed by MS depending on the position of the hydroxyl group on the side chain. 5- and 8-series IsoPs possess side chain hydroxyl groups before the cyclopentyl ring structure, whereas 12- and 15-series IsoPs contain hydroxyl groups after the ring. Fragmentation patterns of the IsoPs in the gas phase of MS largely depend on the location of the hydroxyl group (19, 20, 32). Thus, IsoPs with the hydroxyl before the ring (5- and 8-series) show predominantly /H₉₂₅₁ cleavage next to the hydroxyl groups (m/z 115 and m/z 127). On the other hand, 12- and 15-series IsoPs undergo multiple bond cleavages to generate the characteristic fragments (m/z 151 and m/z 193). These results support the contention that EC-APCI-MS can be used to study oxidation products derived not only from arachidonate but also from other PUFAs such as DHA.

Regioselectivity of Neuroprostanes Formed from the Autoxidation of DHA Analyzed by EC-APCI-MS—As noted, eight regioisomeric groups of NPs would be predicted to be formed from the autoxidation of DHA (Scheme 2). Understanding the stereochemistry of NP formation will provide the rationale to study the biological activity of the compounds that are formed in the greatest abundance. Oxidation of DHA would be pre-
dicted to lead to the generation of NPs and other highly oxidized products, as shown in Scheme 3A, in which the peroxidation of 10-HpDHA is used as an example. Besides the formation of the 14-series bicyclic endoperoxide 3b that serves as a precursor for 14-series NPs, other oxidation products, such as 3c, 3d, and 3e, would also be predicted to be generated in this process (33). As indicated in this scheme, 3b can be further oxidized to form dioxolane-IsoP-like compounds, 3c and 3d (22). The identification of compounds of 3c, 3d, 3e, 3f, and 3g, and other highly oxidized products from DHA under free radical conditions has recently been confirmed (data not shown). On the other hand, dioxolane-IsoP-like products cannot be formed from 8-HpDHA as shown in Scheme 3B. Therefore, the 4-series NPs 3i would be predicted to be generated in greater amounts than other regioisomers that can be further oxidized. Although the autoxidation pattern of DHA is much more complex than that of arachidonic acid, similar hypotheses based on our understanding of radical chemistry can be applied to predict the formation of NPs and other oxidation products of DHA. For the same reasons that 5- and 15-series IsoPs predominate over 8- and 12-series IsoPs when arachidonate is oxidized, we predict that not only 4-series, but also 20-series, NPs are the major regioisomers among the eight possible groups because the precursor peroxyl radical of both NPs cannot cyclize to form dioxolane-IsoP-like compounds.

EC-APCI-MS was utilized to examine the regiochemistry of NP formation. The predicted fragmentation pattern of each NP regioisomer is summarized in Scheme 4. The fragmentation patterns for F4-NPs can be extrapolated from those for IsoPs. To verify the predicted fragmentation of each regioisomeric F4-NP, we adopted the strategy of generating specific NP regioisomers from parent hydroperoxides. Fortunately, most of these 10 precursor hydroperoxides of DHA methyl esters (HpDHA) can be well separated by normal phase HPLC as we have described previously (24). Thus, a mixture of DHA hydroperoxides was generated by a free radical reaction using a good hydrogen atom donor N-methyl benzohydroxamic acid. After the separation of these DHA hydroperoxides by semipreparative HPLC, they were further autoxidized to generate specific classes of NPs. After derivatization of the autoxidation mixtures, the PFB esters were studied by EC-APCI-MS (15). CID of these PFB esters confirmed the predicted fragmentation for each NP.

**Fig. 4.** Total ion chromatogram and CID results of NPs from the *in vitro* oxidation of DHA analyzed by EC-APCI-MS using normal phase HPLC as described under “Experimental Procedures.” a, total ion chromatogram of m/z 377 with an offset of 30 eV; b, MS spectrum of m/z 377 averaged from 6 to 26 min. The numbers in bold indicate the characteristic fragments of each regioisomer.
regioisomer. Examples of a 10-series NP (from 14-HpDHA) and a 17-series NP (from 13-HpDHA) are illustrated in Fig. 2. As shown in Fig. 2a, the parent ion m/z 377 of 10-series NP gives rise to a specific daughter ion m/z 153 via α cleavage to the hydroxyl group on the side chain, whereas the 17-series NP generated m/z 219 through multiple bond dissociations as shown in Fig. 2b. It is apparent that there are more fragments generated from 17-series NPs than from 10-series NPs because fragmentation of the former involves multiple bond fragmentation. In addition, we have previously synthesized a 17-series NP, 17-F4c-NP, and shown that it gives an identical fragmentation pattern as that shown in Fig. 2b. The predicted fragmentation of other regioisomers as indicated in Scheme 4 has also been verified by this technique.

SRM experiments were then carried out to study the oxidation

**FIG. 5.** SRM of NPs resulting from the *in vitro* oxidation of DHA analyzed by EC-APCI-MS using normal phase HPLC with analytical silica column. a, 13-series NP SRM: m/z 377 to 193; b, 11-series NP SRM: m/z 377 to 137; c, 14-series NP SRM: m/z 377 to 173; d, 17-series NP SRM: m/z 377 to 179; e, 20-series NP SRM: m/z 377 to 259; f, 4-series NP SRM: m/z 377 to 101; g, 7-series NP SRM: m/z 377 to 113; h, 10-series NP SRM: m/z 377 to 153. The numbers on the right indicate the absolute intensity of the base peak.

**FIG. 6.** Mass spectrum of NP methyl esters after catalytic hydrogenation and TMS derivatization analyzed by gas chromatography-electron ionization-MS. Numbers in bold indicate the α cleavage of the isomeric NPs.
mixture from separated HpDHA isomers by LC-APCI-MS using reverse phase HPLC. The results illustrated in Fig. 3 were obtained from a fraction that has as its major component 13-HpDHA and as its minor component of 16-HpDHA (Fig. 3a) because these two hydroperoxides cannot be fully separated by our HPLC method. The identities of the hydroperoxides can be unambiguously made based on α cleavage of hydroxyl-DHA under the EC-APCI-MS conditions. These two starting hydroperoxides give rise to two NP regioisomers, 17-series (from 13-HpDHA) and 20-series (from 16-HpDHA). Although the 16-HpDHA is the minor component in this mixture, 20-series NPs are formed in greater amounts than 17-series NPs as indicated in Fig. 3b. These results clearly indicate that the NPs in the 20-series are major regioisomers because their precursor peroxyl radical cannot further cyclize to form dioxolane-like molecules.

After characterization of the MS fragmentation for each regioisomeric NP, experiments were carried out to identify these fragments in an oxidation mixture of DHA containing all NP regioisomers after it was reduced and derivatized to PFB esters. Total ion chromatography and CID analysis results are shown in Fig. 4. All of the predicted fragments corresponding to each regiosomer can be identified, and as is evident in Fig. 4b, 4- and 20-series are among the major peaks. Although it is extremely difficult to find exclusive characteristic fragments for all of the eight regioisomers, a mixture of all regioisomers resulting from the oxidation of DHA was analyzed by SRM. The results show that the formation of 4- and 20-series NPs is favored over other isomers as indicated in Fig. 5. The different peaks within each panel indicate the different diastereomers for each regiosomer. For example in Fig. 5c, eight peaks may represent the eight possible diastereomers of 20-series F4-NPs. These findings are consistent with our hypothesis that 4- and 20-series NPs are major regioisomers among the eight possible groups because they cannot undergo further oxidation. It is noteworthy, however, that the predominance of 4- and 20-series NPs over other NP regioisomers is significantly less than is observed for the formation of 5- and 15-series IsoP regioisomers compared with other IsoP isomers derived from arachidonate.

Historically GC-MS has been widely employed to study lipid oxidation products of arachidonate and other fatty acids after proper derivatization. Electron ionization generates a characteristic cleavage next to a hydroxyl group derivatized to a TMS ether. We employed this method to study the regiochemistry of the NP oxidation products derived from DHA. An autoxidation mixture of DHA methyl ester was hydrogenated and analyzed by electronic ionization MS after TMS derivatization. The results are shown in Fig. 6. When the GC chromatography region of NPs is averaged, the α cleavage of all eight regioisomers can be identified, and as predicted, 4- and 20-series predominate.

Neuroprostanes Are Formed from the Oxidation of DHA in Vivo—Having obtained information on the regioselectivity of NP formation in vitro, we next sought to study the regiochemistry of NP formation in vivo. DHA is enriched in brain and liver. Thus, lipid extracts from the liver of a rat treated with CCl4 to induce an oxidant stress were analyzed by LC-APCI-MS, and the results are shown in Fig. 7. The 20-series (Fig. 7a) and 4-series (Fig. 7b) of NPs are the major regioisomers generated, similar to our in vitro findings. Fig. 7c and d, shows the non-selective SRM of all regioisomeric NPs from rat liver and the results from an in vitro incubation, respectively. Strikingly similar patterns of NPs are observed, and these results indicate that 4- and 20-series NPs are major regioisomers from the autoxidation of DHA both in vitro and in vivo.

GC-MS employing EC chemical ionization in the negative mode has also been widely used to analyze IsoPs and NPs in vivo because this technique has extremely high sensitivity and selectivity. Thus, lipids extracted from rat brain were assayed using this technique for NP formation. Results were compared with DHA oxidized in vitro. The results are shown in Fig. 8. Selective ion monitoring (SIM) was carried out to scan m/z 593 (Fig. 8, a and b), which corresponds to the TMS derivatives of NPs, and m/z 597, which represents 13O-labeled internal standard (Fig. 8c). Theoretically there are 64 racemic F4-NP stereoisomers that can be generated from the oxidation of DHA, and the peaks in Fig. 8, a and b, reflect the complexity of this mixture. Again, virtually identical patterns of peaks are apparent in the lipid extract from rat brain and DHA oxidized in vitro, supporting the contention that similar amounts of different NP regioisomers are formed in vitro and in vivo.

DISCUSSION

IsoPs are formed from the free radical-induced autoxidation of arachidonic acid and its esters. Measurement of these compounds is regarded as the gold standard for the assessment of
oxidative stress in vivo. Analogous to IsoPs, NPs are generated from the oxidation of DHA both in vitro and in vivo. DHA is enriched in brain tissue, and it has been suggested that quantification of NPs may be useful in the assessment of oxidative damage to the central nervous system. Liver tissue also contains considerable amounts of DHA. Unlike arachidonate, limited research has been performed to understand the oxidation of DHA and the biological relevance of the resulting peroxidation products. Study of the NPs is a major focus of our efforts because elevated levels of these compounds have been detected in certain human disorders such as Alzheimer disease, suggesting that these compounds may play an important role in the pathophysiology of this disease (12).

Although it is predicted that the oxidation of DHA is much more complex than that of arachidonic acid, similar free radical-based mechanisms of peroxidation can be applied to understand the oxidation of this PUFA (Scheme 5) (34). DHA has six double bonds and five bis-allylic positions (namely at carbons 6, 9, 12, 15, and 18) where the first step of autoxidation and hydrogen atom abstraction occurs. When the hydrogen atom at C-9 is abstracted, a delocalized pentadienyl radical \( \text{5b} \) is formed. Molecular oxygen adds to either end of the pentadienyl radicals (C-7 or C-11) and forms peroxyl radicals such as \( \text{5c} \) at C-11. 11-HpDHA is generated when \( \text{5c} \) abstracts another hydrogen atom. Thus a total of 10 primary hydroperoxides can be formed from the oxidation of DHA. Peroxyl radical \( \text{5c} \) can undergo cyclization to form the cyclic intermediate \( \text{5d} \). This carbon-centered radical can cyclize again to form \( \text{5e} \), which adds molecular oxygen to form \( \text{5f} \). The bicyclic endoperoxide \( \text{5g} \) is generated from hydrogen atom abstraction. Compound \( \text{5g} \) is the parent intermediate to other NPs such as the 7-series NPs that contain F-type, D/E-type, or A/J-type prostane rings.

![Graphs showing GC-CI-MS of NPs from in vitro DHA oxidation (SIM m/z 593) (a), rat brain (SIM m/z 593) (b), and internal standard (SIM m/z 597, O\(^{18}\) 17-F\(_{4e}\)-NP) (c).]
There are also other potential pathways for the intermediate radicals that lead to the formation of various oxygenated DHA species. If molecular oxygen adds to $5d$ before it cyclizes, radical $5i$ will be generated. This pathway will lead to the formation of monocyclic peroxides and serial cyclic peroxides. It is noteworthy that the precursor radical $5f$ can undergo alternative cyclization to form the dioxolane-IsoP-like compound $5h$. However, the same type of precursor radicals for the formation of 4-series ($5j$) and 20-series ($5k$) NPs cannot cyclize. Therefore 4- and 20-series of NPs can be formed in greater amount than the other regioisomers. Our experimental results are consistent with this hypothesis.

In addition to the formation of dioxolane-IsoP-like compounds, there are other factors that may contribute to the regioselectivity of NP formation. The further oxidizability of parent NPs to generate other oxygenated species besides dioxolane-IsoPs is an additional factor that may alter isomer distribution. There are different numbers of bis-allylic positions in the NPs that may be peroxidized. 4-, 11-, 13-, and 20-series F$_4$-NP have two bis-allylic positions, whereas 7-, 14-, 10-, and 17-series of F$_4$-NP have only one such position (Scheme 2).

Reaction kinetics related to ease of oxidizability of NP precursors can also influence the formation of different oxidation products. Reaction rates are determined both by the rate constants and concentration of the reactants. The fact that a number of different DHA oxidation products can be detected indicates that these different reactions are competitive. For example, the formation of primary hydroperoxides (such as 11-HpDHA from $5e$) competes with further cyclization. The relative rate of these two reactions depends on the concentration of abstractable hydrogen atoms in the system. There are more primary hydroperoxides formed from the oxidation of DHA than from that of arachidonic acid because there are 67% more hydrogen atoms in bis-allylic positions in DHA. The formation of $5i$ and $5e$ depends largely on the oxygen concentration and rate constant of the 5-exo cyclization of carbon-centered radical $5d$ because the rate constant of molecular oxygen adding to this carbon radical is at a diffusion-controlled rate. The abstractable hydrogen atom concentration also plays an important role in the distribution of $5g$ and $5h$, and a higher concentration of hydrogen atom favors the formation of $5g$. In other words, the formation of dioxolane-IsoP-like compounds would be predicted to contribute less to the NP regiosomic distribution than to IsoPs. In favor of this hypothesis, our experimental results show a decreased regioselectivity of NPs compared with IsoPs. For IsoPs, 5- and 15-series compounds are formed in 10 times greater abundance than those of 12- and 8-series (Fig. 1), whereas 4- and 20-series NPs are only formed in 1–3-fold greater abundance than the other regioisomers (Fig. 5). However, when individual HpDHA are oxidized, the regioselective formation of 4- and 20-series NPs is much higher than those resulting from the oxidation of DHA (Fig. 3) presumably because there are many more extractable hydrogen atoms in DHA than in the individually separated DHA hydroperoxides. Therefore, the SRM results from DHA oxidation more likely mimic what happens in vivo than does oxidation of separated regioisomers.

The regioselective formation of different NPs will likely aid in studies related to the biology of these compounds. Currently, no information exists regarding the biological properties of the NPs. Based upon our current findings, it would seem prudent to examine the biological properties of the 4- and 20-series NPs because these compounds are formed in greater quantities than other NP isomers. Further work in this area, however, will
have to await the chemical synthesis of these molecules.

In summary, the present studies have examined the formation of various NP regioisomers. Several mass spectrometric approaches, including EC-APCI-MS, were utilized to analyze NPs formed in vitro and in vivo based on their characteristic fragmentation in the gas phase of MS. Experimental results are consistent with our hypothesis that 4- and 20-series NP regioisomers are preferentially generated. The discovery of regioselectivity in the formation of NPs will allow studies of the biological activities of these molecules. Although some NPs can be analyzed by high-resolution MS, the majority of NPs have to await the chemical synthesis of these molecules.

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