Formation of Novel D-Ring and E-Ring Isoprostane-like Compounds (D_4/E_4-Neuroprostanes) in Vivo from Docosahexaenoic Acid†

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Received August 26, 1999; Revised Manuscript Received December 2, 1999

ABSTRACT: Free radical-mediated oxidant injury and lipid peroxidation have been implicated in a number of neural disorders. We have reported that bioactive prostaglandin D_2/E_2-like compounds, termed D_2/E_2-isoprostanes, are produced in vivo by the free radical-catalyzed peroxidation of arachidonic acid. Docosahexaenoic acid, in contrast to arachidonic acid, is the most abundant unsaturated fatty acid in brain. We therefore questioned whether D/E-isoprostane-like compounds (D_4/E_4-neuroprostanes) are formed from the oxidation of docosahexaenoic acid. Levels of putative D_4/E_4-neuroprostanes increased 380-fold after oxidation of docosahexaenoic acid in vitro from 15.2 ± 6.3 to 5773 ± 1024 ng/mg of docosahexaenoic acid. Subsequently, chemical approaches and liquid chromatography electrospray ionization tandem mass spectrometry definitively identified these compounds as D_4/E_4-neuroprostanes. We then explored the formation of D_4/E_4-neuroprostanes from a biological source, rat brain synaptosomes. Basal levels of D_4/E_4-neuroprostanes were 3.8 ± 0.6 ng/mg of protein and increased 54-fold after oxidation ($n = 4$). We also detected these compounds in fresh brain tissue from rats at levels of 12.1 ± 2.4 ng/g of brain tissue ($n = 3$) and in human brain tissue at levels of 9.2 ± 4.1 ng/g of brain tissue ($n = 4$). Thus, these studies have identified novel D/E-ring isoprostane-like compounds that are derived from docosahexaenoic acid and that are formed in brain in vivo. The fact that they are readily detectable suggests that ongoing oxidative stress is present in the central nervous system of humans and animals. Further, identification of these compounds provides a rationale for examining their role in neurological disorders associated with oxidant stress.

Oxidant stress and peroxidation of lipids have been implicated in the pathogenesis of a variety of human diseases, including atherosclerosis, cancer, and neurodegenerative disorders such as stroke, Alzheimer's disease, and Huntington's disease (*1*−*7*). Quantification of isoprostanes has been shown to be a highly accurate marker of oxidative stress in vivo (*8*, *9*). Isoprostanes (IsoPs)[1] are prostaglandin (PG)-like compounds generated from the free radical-catalyzed peroxidation of arachidonic acid. Formation of these compounds proceeds through bicyclic endoperoxide PGH_2-like intermediates, which are reduced to PGF_2-like compounds (F_2-IsoPs) (*10*) or undergo rearrangement to PGD_2 and PGE_2-like compounds (D_2/E_2-IsoPs) (*11*) and thromboxane-like compounds (isothromboxanes) (*12*). In contrast to cyclooxygenase-derived PGs, IsoPs are formed in situ esterified to phospholipids and are released preformed, presumably by the action of a phospholipase A_2 (PLA_2) (*13*). In addition to the use of the IsoPs as markers of oxidative stress in vivo, several of these compounds have been shown to be capable of exerting potent biological activity and thus may mediate some of the pathophysiological sequelae of oxidant stress (*8*, *9*).

Docosahexaenoic acid (C22:6ω3, DHA) is an unsaturated fatty acid that is highly concentrated in the brain, particularly in the gray matter, where it comprises approximately 30% of the total fatty acids in aminophospholipids (*14*, *15*). Although DHA is concentrated in gray matter, it is not synthesized by neurons. Instead, DHA is synthesized and secreted by astrocytes and, subsequently, taken up by neurons (*16*). The function of this unsaturated fatty acid in the nervous system has not been fully elucidated; however, DHA is thought to be important for brain development, and DHA deficiency is associated with abnormalities in brain function (*17*).

† Supported by NIH Grants DK48831, CA77839, GM15431, GM42056, CA68485, DK26657, AG00774, and AG16835 and a grant from the Alzheimer's Disease and Related Disorders Association. J.D.M. is the recipient of a Burroughs Wellcome Fund Clinical Scientist Award in Translational Research.

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[1] Abbreviations: IsoP, isoprostane; PG, prostaglandin; PLA_2, phospholipase A_2; DHA, docosahexaenoic acid; NP, neuroprostane; AAPH, 2,2′-azobis(2-amidinopropane) hydrochloride; GC, gas chromatography; NICI, negative ion chemical ionization; MS, mass spectrometry; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; LC, liquid chromatography; ESI, electrospray ionization; CID, collision-induced dissociation.

10.1021/bi992000l CCC: $19.00
Published on Web 02/10/2000

Theoretically, IsoP-like compounds can be formed by the free radical-catalyzed peroxidation of any unsaturated fatty acid containing at least three interrupted carbon−carbon double bonds, including DHA. Because DHA is highly concentrated in nervous system tissue, quantification of IsoP-like compounds formed by the free radical-catalyzed peroxidation of this fatty acid, which we term neuroprostanes (NPs) (*18*), may provide a unique marker of oxidative injury to this tissue. Analogous to the IsoPs, the NPs could potentially exert biological activity. Moreover, the formation of the NPs esterified in phospholipids could lead to significant alterations in the biophysical properties of neuronal membranes, which might result in impaired neuronal function (*18*).

Recently, we and others reported the formation of F_4-neuroprostanes (F_4-NPs) that are generated from the free radical-catalyzed peroxidation of DHA both in vitro and in vivo (*18*−*20*). The mechanism of formation of the F_4-NPs is analogous to the mechanism of formation of F_2-IsoPs from arachidonic acid in which endoperoxide intermediates are reduced to F-ring compounds. Because the formation of the NPs proceeds through endoperoxide intermediates, we investigated the possibility that, in addition to F-ring NPs, compounds with a prostane D-ring and E-ring (D_4/E_4-NPs) may also be generated in vitro and in vivo by rearrangement of endoperoxide intermediates. The mechanism by which the D_4/E_4-NPs would be predicted to be formed is shown in Figure 1A−C. Initially, five DHA radicals are generated, and following the addition of molecular oxygen, eight peroxyl radicals result. Subsequently, the peroxyl radicals undergo endocyclization followed by addition of molecular oxygen which forms eight bicyclic endoperoxide intermediate regioisomers (not shown). These regioisomers then undergo rearrangement to generate eight D_4-NP and eight E_4-NP regioisomers. Each regioisomer is theoretically comprised of eight racemic diastereomers for a total of 256 D-ring and E-ring compounds. A nomeclature system for the IsoPs has been established and approved by the Eicosanoid Nomenclature Committee in which the different regioisomer classes are designated by the carbon number of the side chain where the hydroxyl is located with the carbonyl carbon designated as C-1 (*21*). Thus, in accordance with this system, the D- and E-ring NP regioisomers are similarly designated as 4-series D_4/E_4-NPs, 7-series D_4/E_4-NPs, etc. Herein, we present evidence which shows that D_4/E_4-NPs are, in fact, formed in significant amounts in vitro and in vivo from the free radical-catalyzed peroxidation of DHA.

MATERIALS AND METHODS

Materials. Docosahexaenoic acid, dimethylformamide, and undecane were purchased from Aldrich (Milwaukee, WI). Pentafluorobenzyl bromide, methoxyamine HCl, diisopropylethylamine, and *Apis mellifera* venom PLA_2 were from Sigma (St. Louis, MO). 2,2′-Azobis(2-amidinopropane) hydrochloride (AAPH) was from Kodak (Rochester, NY). [$^2H_3$]Methoxyamine HCl was from Cambridge Isotope Laboratories, Inc. (Andover, MA). *N*,*O*-Bis(trimethylsilyl)-trifluoroacetamide was from Supelco (Bellefonte, PA). [$^2H_9$]-*N*,*O*-Bis(trimethylsilyl)trifluoroacetamide was from Regis Chemical (Morton Grove, IL). [$^2H_9$]-*N*,*O*-Bis(trimethylsilyl)-acetamide was from CDN Isotopes (Pointe-Claire, PQ). C-18 and Silica Sep-Pak cartridges were from Waters Associates (Milford, MA). 60ALK6D TLC plates were from Whatman (Maidstone, U.K.). [$^2H_4$]-$PGF_{2\alpha}$ and [$^2H_4$]-PGE_2 were from Cayman Biochemicals (Ann Arbor, MI).

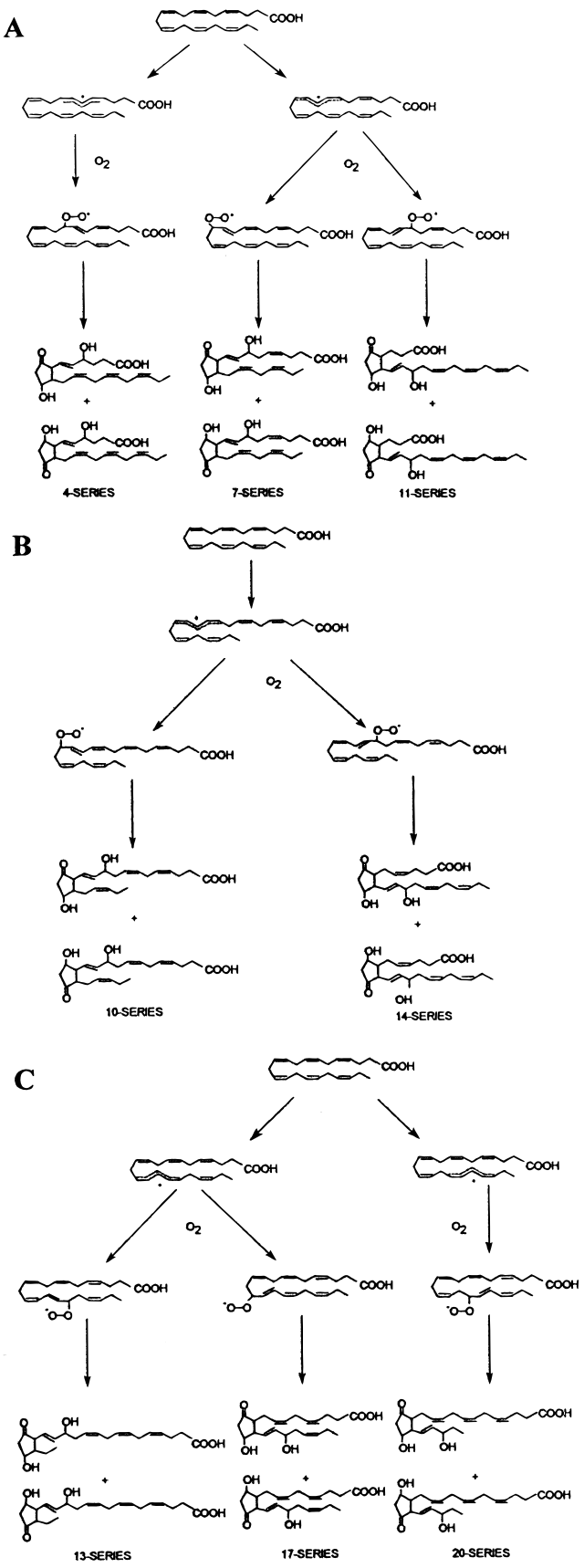

FIGURE 1: (A−C) Pathways for the formation of D_4/E_4-NPs by the nonenzymatic peroxidation of DHA.

Oxidation of DHA. DHA was oxidized in vitro using an iron/ADP/ascorbate mixture as described previously (*22*).

Purification and Analysis of D_4/E_4-NPs. Esterified D_4/E_4-NPs in phospholipids were quantified as free D_4/E_4-NPs following hydrolysis using either chemical saponification with potassium hydroxide (*23*) or enzymatic hydolysis employing *A. mellifera* venom PLA_2 (*24*) as previously described for the D_2/E_2-IsoPs. Both methods yielded quantitatively similar results. Free D_4/E_4-NPs were extracted using C-18 and silica Sep-Pak cartridges, purified by thin-layer chromatography (TLC), converted to *O*-methyloxime pentaflurobenzyl ester trimethylsilyl ether derivatives, and quantified by stable isotope dilution techniques employing gas chromatography (GC)/negative ion chemical ionization (NICI)/mass spectrometry (MS) using $[^2H_4]PGE_2$ as an internal standard (*23*). The technique that was employed is similar to that used to purify and analyze D_2/E_2-IsoPs except for the region scraped during TLC. The D_4/E_4-NP compounds were chromatographed as *O*-methyloxime pentaflourobenzyl ester derivatives on silica TLC plates (60ALK6D silica gel, Whatman), and a region that extended from 0.5 cm above the middle of a PGE_2 methyl ester standard to 4 cm above the leading edge of a PGD_2 methyl ester standard was scraped ($R_f = 0.34-0.83$). This area was determined to contain D_4/E_4-NPs by analyzing sequential small cuts of the TLC plate for compounds. D_4/E_4-NPs were detected by MS based on the $M - {}^{\bullet}CH_2C_6F_5$ ions (*m*/*z* 548 for D_4/E_4-NPs and *m*/*z* 528 for the $[^2H_4]PGE_2$ internal standard). Quantification of the total amounts of the D_4/E_4-NPs was achieved by integrating peak areas of material in the *m*/*z* 548 channel in comparison to the *m*/*z* 528 channel. GC/NICI/MS was carried out using a Hewlett-Packard 5890 instrument (Palo Alto, CA).

Purification and Analysis of F_4-NPs. F_4-NPs were purified, analyzed, and quantified as described previously (*18*).

Purification of D_4/E_4-NPs by High-Performance Liquid Chromatography (HPLC) and Analysis by Liquid Chromatography (LC)/Electrospray Ionization (ESI)/MS/MS. Compounds generated by the in vitro oxidation of DHA (approximately 10 μg total) were purified by reversed phase HPLC using a 25 cm × 4.6 mm Econosil C-18 column (Alltech, Deerfield, IL) with 5 μm particles using an isocratic solvent system of H_2O, acetonitrile, and acetic acid (68:32:0.1, v/v/v) at a flow rate of 1 mL/min. D_4/E_4-NPs eluted at a retention volume of 25−55 mL. Selected fractions containing large amounts of D_4/E_4-NPs were acidified with 1 N HCl, extracted with 2 volumes of ethyl acetate, dried, and redissolved in a small volume of ethanol. Fractions were then analyzed by LC/ESI/MS/MS in the negative ion mode using either a 15 cm × 2.1 mm Econosil C-18 column (Waters) or a 15 cm × 1 mm Zorbax C-18 column (MicroTech Scientific, Sunnyvale, CA). The solvent system employed in each case was a gradient consisting of 20 mM ammonium acetate/acetonitrile/acetic acid (90:10:0.1, v/v/v) to 20 mM ammonium acetate/acetonitrile/acetic acid (10:90:0.1, v/v/v) over the course of 10 min at a flow rate of either 0.2 mL/min (Waters column) or 50 μL/min (MicroTech column). The auxiliary gas pressure was 10 lb/in.^{2}, and the sheath gas pressure was 70 lb/in.^{2}. The voltage on the capillary was 20.0 V; the capillary temperature was 200 °C, and the tube lens voltage was 75 V. Parent ions were scanned from *m*/*z* 350 to 450. Collision-induced dissociation (CID) of molecular ions of putative D_4/E_4-NPs in these fractions was performed from −20 to −30 eV scanning daughter ions from *m*/*z* 50 to 400. Spectra that are shown were obtained at −25 eV. The CID gas was argon with a pressure set at 2.0 mTorr. Spectra were displayed by averaging scans across chromatographic peaks. LC/MS was carried out using a Finnigan TSQ 7000 instrument (San Jose, CA). Positive ion mass spectra were also examined, but they did not yield structurally useful information.

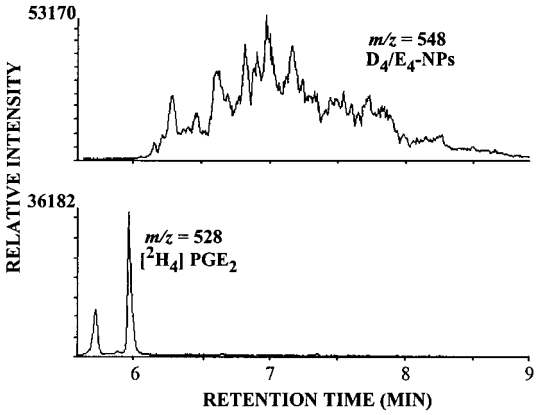

FIGURE 2: Selected ion monitoring chromatogram obtained from the analysis of D_4/E_4-NPs generated during iron/ADP/ascorbate-induced oxidation of DHA in vitro. The series of peaks in the *m*/*z* 548 chromatogram represents putative D_4/E_4-NPs, and the two peaks in the *m*/*z* 528 chromatogram represent the *syn*- and *anti*-*O*-methyloxime isomers of the $[^2H_4]PGE_2$ internal standard.

Preparation and Oxidation of Rat Brain Synaptosomes. Synaptosomes were isolated by Ficoll gradient centrifugation from male Sprague-Dawley rats (*25*). Lipid peroxidation was initiated by the addition of AAPH (final concentrations of 1−10 mM). Incubations were carried out at 37 °C for 24 h. The reactions were terminated by placing the samples at −80 °C. Levels of free F_4-NPs and D_4/E_4-NPs were quantitated following chemical saponification as described previously (*23*). Data are expressed relative to protein concentration as determined by the Pierce BCA assay.

Preparation of D_4/E_4-NPs from Rat and Human Brain Tissue. Lipids from the brains of adult male Sprague-Dawley rats or human frontal lobe tissue obtained post-mortem were extracted and hydrolyzed by reaction with *A. mellifera* venom PLA_2 and subsequently analyzed for free D_4/E_4-NPs (*23*).

RESULTS

Formation of D_4/E_4-NPs in Vitro. A representative selected ion current chromatogram obtained from the oxidation of DHA in vitro with an iron/ADP/ascorbate mixture is shown in Figure 2. The two chromatographic peaks in the lower *m*/*z* 528 ion current chromatogram represent the *syn*- and *anti*-*O*-methyloxime isomers of the internal standard $[^2H_4]$-PGE_2. In the upper *m*/*z* 548 ion current chromatogram are a series of chromatographic peaks eluting over an approximate 2 min interval. These compounds possessed a molecular mass predicted for the D_4/E_4-NPs. In addition, it would be predicted that the retention time of D_4/E_4-NPs on GC should be longer than that of the deuterated PGE_2 internal standard because the former compounds contain two additional carbon atoms. As for PGD_2 and PGE_2 as well as for D_2/E_2-IsoPs that have similar chromatographic properties on TLC and

GC and identical molecular masses, it is not possible to differentiate between the D-type and E-type prostane rings in the putative NP compounds detected in the *m/z* 548 ion current chromatogram shown in Figure 2. However, since the endoperoxide PGH_2 derived from the cyclooxygenase enzyme rearranges in aqueous solution to form both PGD_2 and PGE_2, it is expected that the NP endoperoxide intermediates also would rearrange to form both D-ring and E-ring NPs.

Additional experimental approaches were then undertaken to provide further evidence that the compounds represented by the chromatographic peaks in the *m/z* 548 ion current chromatogram are D_4/E_4-NPs. The *m/z* 547 ion current chromatogram contained no chromatographic peaks, indicating that the peaks in the *m/z* 548 chromatogram are not natural isotope peaks of compounds generating an ion of less than 548. Analysis of the putative D_4/E_4-NPs as a [$^2H_9$]-trimethylsilyl ether derivative resulted in a shift of the *m/z* 548 chromatographic peaks up 18 Da to *m/z* 566, indicating the presence of two hydroxyl groups (not shown). When these compounds were analyzed as a [$^2H_3$]-*O*-methyloxime derivative, the *m/z* 548 chromatographic peaks all shifted up to *m/z* 551, indicating the presence of one carbonyl group (not shown). Analysis of the putative D_4/E_4-NPs following catalytic hydrogenation is shown in Figure 3. Prior to hydrogenation, there were no chromatographic peaks present 8 Da above *m/z* 548 in the *m/z* 556 ion current chromatogram (Figure 3A,B). However, following hydrogenation, intense chromatographic peaks appeared at *m/z* 556 (Figure 3C) with the loss of the chromatographic peaks at *m/z* 548, indicating the presence of four double bonds. Collectively, these data indicate that the compounds represented by the chromatographic peaks in the *m/z* 548 ion current chromatogram have the functional groups and the number of double bonds predicted for the D_4/E_4-NPs.

To provide direct evidence that the compounds analyzed by selected ion monitoring MS were D_4/E_4-NPs, LC/ESI/MS/MS in the negative ion mode was employed. The material was purified before LC/MS analysis by HPLC, and eluted fractions containing significant amounts of putative D_4/E_4-NPs as determined by GC/MS were then analyzed by LC/MS. Notably, D_4/E_4-NPs eluted over a very broad volume from 27 to 51 mL using this HPLC solvent system (Figure 4A). We initially analyzed putative D_4/E_4-NPs eluting in a retention volume of 27−44 mL. The predicted [parent molecule − H]$^-$ ion, hereafter referred to as "M", for D_4/E_4-NPs is at *m/z* 375. Figure 4B shows the selected ion monitoring chromatogram of the ion at *m/z* 375 obtained from this analysis. D_4/E_4-NPs elute from the LC column as a broad chromatographic peak over approximately a 90 s interval. A composite collision-induced dissociation (CID) spectrum obtained by summing scans over the broad chromatographic peak in Figure 4B is shown in Figure 4C. CID of the ion at *m/z* 375 resulted in the formation of a number of relevant daughter ions that would be predicted to be common to all of the D_4/E_4-NP regioisomers, including *m/z* 357 [M − H_2O]$^-$, *m/z* 339 [M − $2H_2O$]$^-$, *m/z* 313 [M − H_2O − CO_2]$^-$, and *m/z* 295 [M − $2H_2O$ − CO_2]$^-$. Other prominent daughter ions are present that may result from fragmentation of specific D_4/E_4-NP regioisomers. Because of the limited amount of material and the lack of chemically synthesized D- and E-ring NPs, it is impossible to know with certainty the chemical structures of these smaller fragments. Nonetheless, on the basis of our previous work and studies by Murphy, Kerwin, and others characterizing fragmentation patterns of prostaglandins, isoprostanes, and other oxygenated fatty acids by LC/MS (*26*−*30*), these ions can be potentially explained as follows. They include *m/z* 255 [M − CH_3CH_2-CHO − H_2O − CO_2]$^-$ (20-series regioisomer) and [M − $CHOCH_2CH_2COOH$ − H_2O]$^-$ (4-series regioisomer), *m/z* 241 [M − $C_5H_6O_2$(C_1−C_5) − $2H_2O$]$^-$ (10-, 13-, 17-, and 20-series regioisomer), *m/z* 215 [M − $CHOCH_2CH$═$CHCH_2$-CH_3 − CO_2 − H_2O]$^-$ (17-series regioisomer) and [M − $CHOCH_2CH$═$CHCH_2CH_2COOH$ − H_2O]$^-$ (7-series regioisomer), *m/z* 193 CH_3CH═$CHCH_2CH$═$CHCH_2CH$═$CHCH_2$-CH_2COO^- (13-series regioisomer), *m/z* 175 [M − $CHOCH_2$-CH═$CHCH_2CH$═$CHCH_2CH_2COOH$ − H_2O]$^-$ (10-series regioisomer) and [M − $CHOCH_2CH$═$CHCH_2CH$═$CHCH_2$-CH_3 − H_2O − CO_2]$^-$ (14-series regioisomer), and *m/z* 147 [M − CH_2═$CHCHOHCH_2CH$═$CHCH_2CH$═$CHCH_2CH_3$ − CO_2 − H_2O]$^-$ (14-series regioisomer). These data suggested that the mass spectrum shown in Figure 4C represented a mixture of a number of the D_4/E_4-NP regioisomers that would be predicted to be formed.

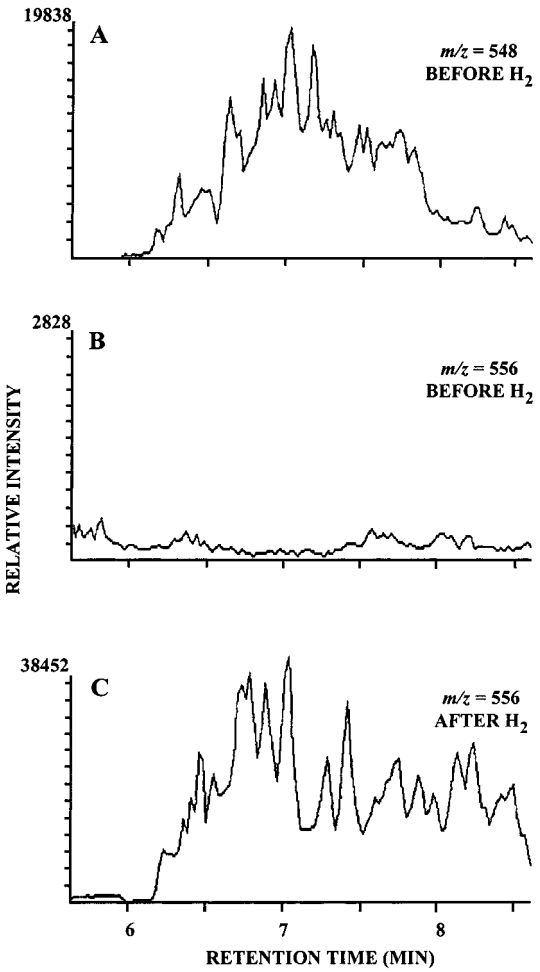

FIGURE 3: Analysis of putative D_4/E_4-NPs before and after catalytic hydrogenation. In the absence of hydrogenation, intense peaks are present in the *m/z* 548 ion current chromatogram (A) representing D_4/E_4-NPs. There are no peaks present 8 Da higher at *m/z* 556 (B). Following catalytic hydrogenation, intense peaks appear in the *m/z* 556 ion current chormatogram (C), indicating that the compounds represented by the peaks in the *m/z* 548 ion current chromatogram have four unsaturated double bonds.

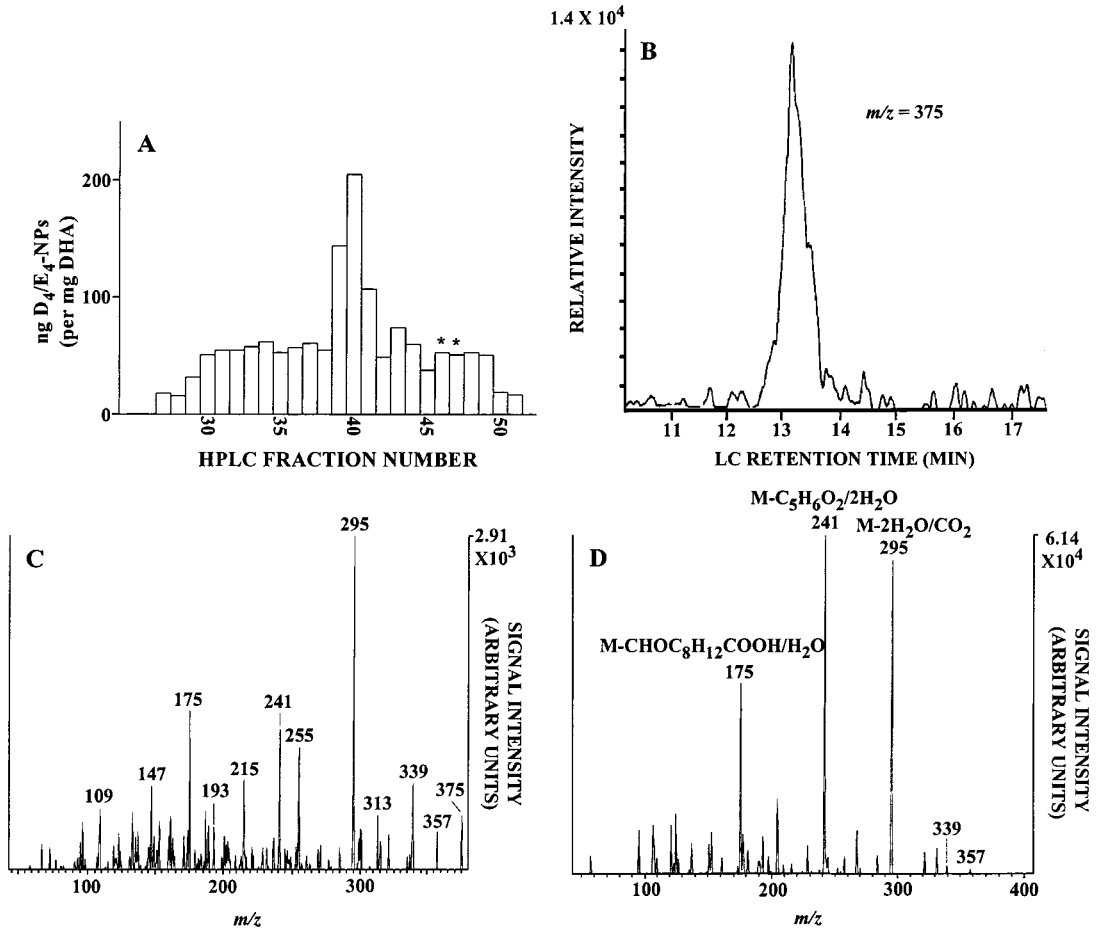

FIGURE 4: (A) Reversed phase HPLC analysis of D_4/E_4-NPs generated from the oxidation of DHA in vitro. Quantities of D_4/E_4-NPs in HPLC fractions were measured by GC/MS. Material eluting in fractions 46 and 47 (Figure 4D) is denoted by the asterisks. (B) Selected ion monitoring chromatogram of the [parent molecule − H]$^-$ (M) ion at *m/z* 375 from LC/ESI/MS analysis of putative D_4/E_4-NPs obtained from prior HPLC purification of oxidized DHA in vitro. Material analyzed by LC/MS eluted from the HPLC in fraction volumes of 27−44 mL. (C) LC/ESI/MS/MS analysis of D_4/E_4-NPs generated in vitro. Material represented in the *m/z* 375 chromatogram in panel B was subjected to CID at −25 eV, and daughter ions were scanned from *m/z* 50 to 400. Spectra were obtained by averaging scans across the chromatographic peak. See the text for structural characterization of particular ions denoted in the figure. LC was performed using a 15 cm × 2.1 mm C-18 column (Waters). (D) LC/ESI/MS/MS analysis of a 10-series D_4/E_4-NP generated from the oxidation of DHA in vitro. Material that was analyzed eluted from the HPLC system prior to LC/MS analysis in a fraction volume of 46−47 mL. The *m/z* 375 ion was subjected to CID at −25 eV, and daughter ions were scanned from *m/z* 50 to 400. Spectra were obtained by averaging scans across the peak that was observed. LC was performed using a 15 cm × 1 mm Zorbax C-18 column (Microtech Scientific).

Because the mass spectrum shown in Figure 4C likely represents a mixture of D_4/E_4-NPs, we subsequently undertook studies to determine whether we could separate individual regioisomers and characterize them by LC/MS. For these experiments, crude D_4/E_4-NPs were initially purified by HPLC and fractions were analyzed individually by GC/MS. Following localization of the D_4/E_4-NPs in the HPLC fractions, select fractions were combined and analyzed by LC/MS/MS. Figure 4D shows one of the CID mass spectra for *m/z* 375 obtained for material eluting from the HPLC purification at 46−47 mL. These fractions were analyzed because they contained substantially fewer D_4/E_4-NP regioisomers than other HPLC fractions. Interestingly, this spectrum represents a compound consistent with a 10-series D_4/E_4-NP. Daughter ions present in the spectrum that are common to all D_4/E_4-NP regioisomers include *m/z* 357 [M − H_2O]$^-$, *m/z* 339 [M − $2H_2O$]$^-$, *m/z* 331 [M − CO_2]$^-$, and *m/z* 295 [M − $2H_2O$ − CO_2]$^-$. In addition, two prominent daughter ions are also apparent at *m/z* 241 [M − $C_5H_6O_2$ − $2H_2O$]$^-$ and *m/z* 175 [M − $CHOCH_2CH{=}CHCH_2CH{=}CHCH_2CH_2COOH$ − H_2O]$^-$. In addition to this 10-series regioisomer, LC/MS analysis of other HPLC fractions gave spectra suggestive of other individual regioisomers (data not shown). Taken together, these data provide direct evidence for the formation of a series of D_4/E_4-NPs generated from the peroxidation of DHA.

Having provided significant evidence for the formation of D- and E-ring neuroprostanes in vitro, we next examined the time course of their formation. For these studies, DHA was oxidized using an iron/ADP/ascorbate mixture. The results are shown in Figure 5. As is evident, levels of D_4/E_4-NPs increased dramatically in a time-dependent manner to a maximum of 5773 ± 1024 ng/mg of DHA from baseline levels of 15.2 ± 6.3 ng/mg of DHA, a 380-fold increase (n = 3). Previously, we reported that using identical conditions, oxidation of DHA yields similar amounts of F_4-NPs (*18*).

We then examined whether D_4/E_4-NPs are formed from the peroxidation of DHA esterified in phospholipids since studies to this point had utilized free DHA only. For these experiments, we studied the generation of NPs in synaptosomes isolated from adult rat brain. Synaptosomes are composed of sealed-off neuronal and glial processes and are

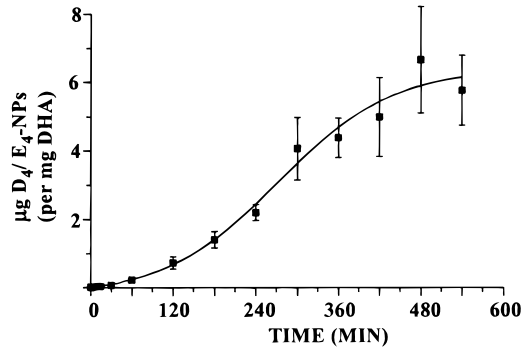

FIGURE 5: Time course of formation of D_4/E_4-NPs during oxidation of DHA in vitro by an iron/ADP/ascorbate mixture. Data are expressed as means ± the standard error of the mean.

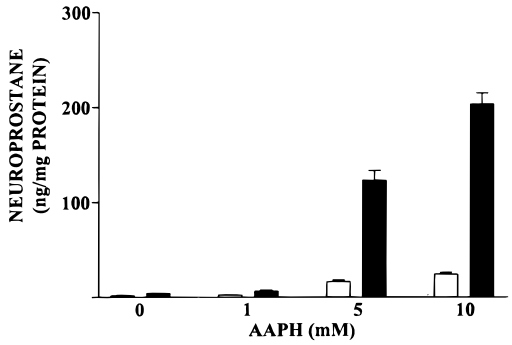

FIGURE 6: Effect of increasing concentrations of AAPH on formation of D_4/E_4-NPs and F_4-NPs in rat brain synaptosomes. Data for D_4/E_4-NPs are represented by the black bars and data for F_4-NPs by the white bars. Data are expressed as means ± the standard error of the mean.

a widely used model for the study of central nervous system gray matter metabolism (*25*). We compared the formation of D_4/E_4-NPs to F_4-NPs after reaction of synaptosomes with AAPH, a water soluble free radical generator that induces an oxidative stress. Concentrations of AAPH varied from 1 to 10 mM. Levels of nonesterified D_4/E_4-NPs were essentially undetectable in rat brain synaptosomes at baseline. Esterified D_4/E_4-NPs and F_4-NPs were quantitated as free compounds following base hydrolysis. The results are shown in Figure 6. There was a significant increase in the extent of formation of both D_4/E_4-NPs and F_4-NPs with increasing concentrations of AAPH. Baseline levels of D_4/E_4-NPs were 3.8 ± 0.6 ng/mg of protein and increased 54-fold after oxidation with 10 mM AAPH. Increases in the amounts of F_4-NPs were similar; however, absolute amounts of D_4/E_4-NPs were 8-fold greater than F_4-NP amounts after reaction with 5 ($p < 0.0001$, Student's *t* test) and 10 mM AAPH ($p < 0.0001$, Student's *t* test). This observation is similar to that in rat liver microsomes oxidized with an iron/ADP/ascorbate mixture where levels of D_2/E_2-IsoPs exceed those of F_2-IsoPs by 5-fold. Moreover, this suggests that quantification of D- and E-ring NPs may be a more sensitive index of lipid peroxidation in certain biological models of central nervous system oxidative injury.

Formation of D_4/E_4-NPs in Vivo. We undertook experiments to determine whether the D_4/E_4-NPs are present esterified in brain lipids in vivo in both animals and humans. Putative D_4/E_4-NPs were analyzed as free compounds following enzymatic hydrolysis of compounds esterified in brain lipid extracts from normal rats. A representative ion

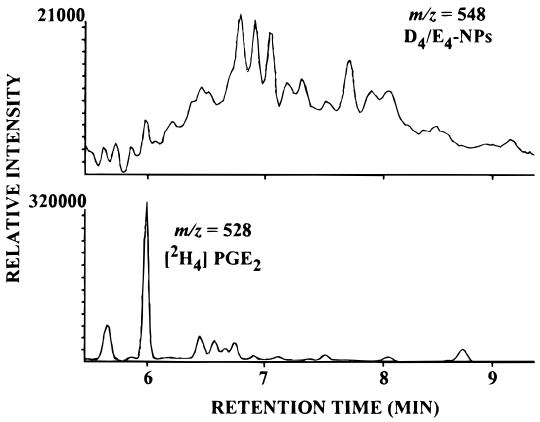

FIGURE 7: Selected ion current chromatogram obtained from the analysis of D_4/E_4-NPs esterified in rat brain tissue. The series of peaks in the *m*/*z* 548 chromatogram represents putative D_4/E_4-NPs, and the two peaks in the *m*/*z* 528 chromatogram represent the *syn*- and *anti*-*O*-methyloxime isomers of the $[^2H_4]PGE_2$ internal standard. The total amount of D_4/E_4-NPs present was approximately 15 ng/g of brain tissue.

current chromatogram obtained from one of these analyses is shown in Figure 7. The two chromatographic peaks in the lower *m*/*z* 528 ion current chromatogram represent the *syn*- and *anti*-*O*-methyloxime isomers of the internal standard $[^2H_4]PGE_2$. In the upper *m*/*z* 548 ion current chromatogram are a series of chromatographic peaks which have molecular masses and retention times expected for the D_4/E_4-NPs. The pattern of peaks representing D- and E-ring NPs was very similar to that obtained from the oxidation of DHA in vitro, except for some slight variation in relative peak heights. Analogous to studies performed in vitro, experiments were carried out to obtain further evidence that the chromatographic peaks in the *m*/*z* 548 ion current chromatogram of Figure 7 represent D_4/E_4-NPs formed in vivo. The compounds were analyzed as $[^2H_9]$trimethylsilyl ether derivatives, which resulted in a shift of the chromatographic peaks present in the *m*/*z* 548 ion current chromatogram to *m*/*z* 566 (data not shown). This shift of 18 Da indicates the presence of two hydroxyl groups. Analysis of the compounds as $[^2H_3]$-*O*-methyloxime derivatives resulted in the shift of the *m*/*z* 548 chromatographic peaks up 3 Da to *m*/*z* 551, indicating the presence of one carbonyl group (data not shown). The compounds were also analyzed by catalytic hydrogenation. Prior to hydrogenation, no peaks were present 8 Da above *m*/*z* 548 in the *m*/*z* 556 ion current chromatogram. However, following catalytic hydrogenation, the chromatographic peaks present in the *m*/*z* 548 ion current chromatogram disappeared and intense peaks appeared at *m*/*z* 556, indicating the presence of four double bonds (data not shown). Taken together, these experiments provide evidence that the compounds represented in the *m*/*z* 548 chromatogram in Figure 7 have the expected number of functional groups and double bonds that are predicted for the D_4/E_4-NPs.

Levels of D_4/E_4-NPs esterified in the lipids of rat brain were determined to be 12.1 ± 2.4 ng/g of brain tissue ($n = 3$) and are similar to or somewhat higher than those previously reported for F_4-NPs from normal whole rat brain (7.0 ± 1.4 ng/g of brain tissue) (*18*). We also quantified nonesterified amounts of D_4/E_4-NPs in rat brain tissue. They came to 0.8 ± 0.3 ng/g of brain tissue.

Subsequently, levels of D_4/E_4-NPs were quantified in frontal lobe gray matter from four humans who underwent autopsies. No individual had known neurological disease at the time of death. GC/selected ion monitoring MS analysis of the tissue for D_4/E_4-NPs yielded a series of *m*/*z* 548 chromatographic peaks virtually identical to those observed in Figure 7 (data not shown). Levels of D_4/E_4-NPs were found to be 9.2 ± 4.1 ng/g of brain tissue ($n = 4$). Additionally, F_4-NPs were quantified and levels were found to be 26.2 ± 7.0 ng/g of brain tissue ($n = 4$).

DISCUSSION

These studies report the identification of a novel class of D_2/E_2-IsoP-like compounds, D_4/E_4-NPs, formed in vivo by the free radical-induced peroxidation of DHA. DHA is a major unsaturated fatty acid in neural tissues (*14*, *15*). Oxidative damage to neuronal structures such as DHA-rich cellular membranes has been implicated in a number of neurodegenerative diseases (*5*). Nonetheless, the identification, at a molecular level, of products that are formed from the oxidation of DHA has been lacking. This provided the impetus for the studies reported herein.

The mechanism of formation of D_4/E_4-NPs from DHA is outlined in Figure 1. It is identical to that of F_4-NPs except that NP endoperoxide intermediates undergo rearrangement to form D- and E-ring compounds rather than reduction to F-ring NPs. It should be noted that depending on the biological system that we utilized in the studies presented here, levels of D_4/E_4-NPs are comparable to or in some cases exceed those of F_4-NPs, such as in synaptosomal preparations. These findings suggest that, in certain situations, quantification of D_4/E_4-NPs may be a more sensitive indicator of DHA oxidation and neural tissue oxidative stress than F_4-NPs. Factors that may influence the formation of D/E-ring NPs versus F-ring NPs are unknown, although we have shown previously that glutathione and other thiols are capable of reducing IsoP endoperoxides both in vitro and in vivo (*23*). The extent to which GSH modulates the formation of D_4/E_4-NPs versus F-ring NPs remains, however, to be determined.

Like D_2/E_2-IsoPs, D_4/E_4-NPs are formed in situ esterified in phospholipids and are then presumably released in the free form by a phospholipase(s) (*8*, *9*). We have demonstrated in this report that D_4/E_4-NP-containing phospholipids are substrates for bee venom PLA_2 in vitro. The type(s) of mammalian phospholipase that might be responsible for hydrolysis of D_4/E_4-NPs from phospholipids in vivo, however, is unknown, although it has previously been reported that the activity of PLA_2 is enhanced toward oxidized phospholipids (*31*). Understanding the enzyme and regulatory factors controlling the formation and hydrolysis of NP-containing phospholipids is of considerable importance because, analogous to IsoP-containing phospholipids, NP-containing lipids would be predicted to be very distorted molecules and thus profoundly affect biophysical properties of neuronal membranes (*18*). Thus, in settings of oxidant stress, enhanced formation of NP-containing phospholipids could alter neuronal membrane fluidity, integrity, and function.

In these studies, D_4/E_4-NPs were identified in vitro and in vivo using a variety of complementary chemical and MS approaches, including LC/ESI/MS/MS. A relatively small body of literature regarding the characterization of eicosanoids using LC/MS exists, although our findings are consistent with studies reported by other investigators (*26*−*30*). As noted, Figure 4C is a mass spectrum likely representing a number of D_4/E_4-NPs. Ions comprising the loss of combinations of H_2O or CO_2 are readily apparent. In addition, other prominent ions can be appreciated which apparently are derived from specific D_4/E_4-NP regioisomers. Of note is the abundant fragment at *m*/*z* 241. This ion is consistent with the loss of two molecules of water and fragmentation at C_5 resulting in the additional loss of 98 Da from the parent ion of several D_4/E_4-NP regioisomers (10-, 13-, 17-, and 20-series compounds). Previous reports have noted a similar loss of 98 Da for various fatty acids and IsoPs analyzed by LC/MS (*26*, *29*). Several other noteworthy fragment ions that can be appreciated in the mass spectrum shown in Figure 4C include *m*/*z* 255, 215, and 175. These ions can be explained, at least in part, as alkyl-directed product ions resulting from the loss of carboxyl-containing neutral fragments from the parent molecule. Such fragmentation patterns have been previously reported for both oxygenated and unoxygenated fatty acids (*26*, *27*).

An additional potentially important ramification of the discovery of D_4/E_4-NPs relates to the fact that these molecules, once hydrolyzed, could possess biological activity and thus, in this form, could mediate pathophysiological processes related to neural oxidative stress. Several observations made by us and others support this contention. We and others have previously shown that one E-ring IsoP produced in vivo, 15-E_{2t}-IsoP (8-iso-PGE_2), possesses potent biological activity ranging from effects on vascular smooth muscle to cellular activation (*11*, *32*). Of interest has been the observation that this IsoP may exert biological effects by interaction with a unique receptor (*8*, *9*). Thus, the possibility that D_4/E_4-NPs might also possess important biological actions that may be relevant to the pathophysiology of oxidative injury to the brain exists. This possibility is further supported by the finding that a compound structurally related to an F-ring NP, $PGF_{4\alpha}$, which is a 4-series F-prostaglandin corresponding to the structure expected from cyclooxygenase action on DHA, is approximately equipotent with cyclooxygenase-derived $PGF_{2\alpha}$ in contracting gerbil colonic smooth muscle strips (*15*), although these studies are very limited. Experiments on the biological activities of D_4/E_4-NPs, however, will have to await their chemical synthesis. These studies will be extremely important with respect to establishing the relevance of D_4/E_4-NP levels measured in neural tissue.

In summary, we report the discovery that D/E-ring-Isop-like compounds, termed D_4/E_4-NPs, are formed in vivo as products of the nonenzymatic free radical-catalyzed peroxidation of DHA, a major unsaturated fatty acid in neural tissue. The fact that D_4/E_4-NPs are readily detectable in normal brain tissue from animals and humans suggests ongoing oxidant stress in the central nervous system. 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 pathophysiology of oxidant injury in the nervous system.

ACKNOWLEDGMENT

The helpful advice of Dr. Richard Caprioli is acknowledged.

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BI992000L