FORMATION OF HIGHLY REACTIVE A-RING AND J-RING ISOPROSTANE-
LIKE COMPOUNDS (A$_4$/J$_4$-NEUROPROSTANES) IN VIVO FROM
DOCOSAHEXAENOIC ACID

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Running Title: Formation of cyclopentenone neuroprostanes.

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SUMMARY

Free radical-initiated oxidant injury and lipid peroxidation have been implicated in a number of neural disorders. Docosahexaenoic acid is the most abundant unsaturated fatty acid in the central nervous system. We have previously shown that this 22-carbon fatty acid can yield, upon oxidation, isoprostane-like compounds termed neuroprostanes, with E/D-type prostan rings (E\textsubscript{4}/D\textsubscript{4}-neuroprostanes). Eicosanoids with E/D-type prostan rings are unstable and dehydrate to cyclopentenone-containing compounds possessing A-type and J-type prostan rings, respectively. We thus explored whether cyclopentenone neuroprostanes (A\textsubscript{4}/J\textsubscript{4}-neuroprostanes) are formed from the dehydration of E\textsubscript{4}/D\textsubscript{4}-neuroprostanes. Indeed, oxidation of docosahexaenoic acid in vitro increased levels of putative A\textsubscript{4}/J\textsubscript{4}-neuroprostanes 64-fold from 88 ± 43 ng/mg of docosahexaenoic acid to 5463 ± 2579 ng/mg of docosahexaenoic acid. Chemical approaches and liquid chromatography/electrospray ionization tandem mass spectrometry definitively identified them as A\textsubscript{4}/J\textsubscript{4}-neuroprostanes. We subsequently showed these compounds are formed in significant amounts from a biological source, rat brain synaptosomes. A\textsubscript{4}/J\textsubscript{4}-neuroprostanes increased 13-fold, from a basal level of 89 ± 72 ng/mg protein to 1187 ±217 ng/mg (n=4), upon oxidation. We also detected these compounds in very large amounts in fresh brain tissue from rats at levels of  97 ± 25 ng/g brain tissue (n=3) and from humans at levels of 98 ± 26 ng/g brain tissue (n=5), quantities that are nearly an order of magnitude higher than other classes of neuroprostanes. Owing to the fact that A\textsubscript{4}/J\textsubscript{4}-neuroprostanes contain highly reactive cyclopentenone ring structures, it would be predicted that they readily undergo Michael addition with glutathione and adduct covalently to proteins. Indeed, incubation of A\textsubscript{4}/J\textsubscript{4}-neuroprostanes in vitro with excess...
glutathione resulted in the formation of large amounts of adducts. Thus, these studies have identified novel, highly reactive A/J-ring isoprostane-like compounds that are derived from docosahexaenoic acid in vivo. The fact they are readily detectable in large quantities and are highly reactive provides a basis for exploring the role of these compounds in the pathogenesis of neurological disease associated with oxidant brain injury.

Abbreviations used: DHA, docosahexaenoic acid; IsoP, isoprostane; NP, neuroprostane; PG, prostaglandin; GSH, glutathione; AAPH, 2,2'-azobis(2-amidinopropane) hydrochloride; PFB, pentafluorobenzyl; 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; TMS, trimethylsilyl; MTBE, methyl t-butyl ether; BSTFA, N,O-Bis(trimethylsilyl)-trifluoracetamide.
Docosahexaenoic acid (C22:6ω3, DHA) is estimated to account for approximately 30% of the total fatty acids in brain tissue aminophospholipids (1,2). DHA is believed to be important for brain development (3), and its deficiency is associated with abnormalities in brain function (4). Oxidation of DHA has been an area of intense research interest since lipid peroxidation has been implicated in the pathogenesis of various central nervous system diseases, among them neurodegenerative disorders including Alzheimer’s disease (3, 5-8, 9).

We have previously reported that DHA is readily oxidized to generate 22-carbon isoprostane (IsoP)-like compounds, termed neuroprostanes (NPs) (3,10). The formation of NPs from DHA is similar to the formation of IsoPs from arachidonic acid, and proceeds via the generation of highly unstable endoperoxide intermediates (3,10). These intermediates can undergo reduction in vitro and in vivo to form NPs containing F-type prostane rings (F₄-NPs) or can isomerize to molecules with E-type and D-type prostane rings (E₄/D₄-NPs). Levels of these compounds are significantly increased in the temporal and parietal cortices obtained from patients with Alzheimer’s disease compared to control subjects (11). DHA is concentrated in neurons and we have thus suggested that NP formation may be a useful marker of selective neuronal oxidative injury.

It is well known that eicosanoids containing E-type and D-type prostane rings are unstable and readily dehydrate in aqueous solutions to cyclopentenone-containing compounds (12). For example PGE₂ and PGD₂ can dehydrate to PGA₂ and PGJ₂, respectively. PGJ₂ can undergo further metabolism to Δ₁²-PGJ₂ and 15-deoxy-Δ₁²,₁⁴-PGJ₂. Cyclopentenone PGs have attracted considerable interest because of their ability to modulate cell proliferation and differentiation. For example, various compounds of the
A- and J-series have been shown to inhibit proliferation with a G\(_1\) cell cycle arrest and to induce differentiation (13-15). The concentrations of compounds required for this activity are frequently in the micromolar range or above. On the other hand, at concentrations in the nanomolar range, A- and J-series PGs induce proliferation (16-18). One limitation of the studies reported to date with cyclopentenone PGs is that there is marked paucity of evidence that they are formed in vivo (19,20).

Previously, we reported that analogous to the formation of A-ring and J-ring PGs from the dehydration of cyclooxygenase-generated PGE\(_2\) and PGD\(_2\), A\(_2\)/J\(_2\)-IsoPs are generated in vitro and in vivo from IsoPs containing E-type and D-type prostane rings (E\(_2\)/D\(_2\)-IsoPs) (21). The biological relevance of this observation relates to the fact that A\(_2\)/J\(_2\)-IsoPs contain \(\alpha,\beta\)-unsaturated carbonyl moieties and are thus highly reactive and readily adduct, via Michael addition, glutathione (GSH) and protein thiols. Indeed, we have shown that in the presence of GSH and cellular GSH transferase, the A-ring IsoP 15-A\(_2\t\)-IsoP (8-iso-PGA\(_2\)) rapidly conjugates GSH (21). The adduction of relevant reducing substances and proteins by endogenously generated IsoPs may be responsible for some of the adverse effects of oxidant stress in vivo.

Analogous to the formation of A\(_2\)/J\(_2\)-IsoPs from the dehydration of E\(_2\)/D\(_2\)-IsoPs, it would be postulated that E\(_4\)/D\(_4\)-NPs can undergo dehydration resulting in the generation of cyclopentenone NPs, termed A\(_4\)/J\(_4\)-NPs. The mechanism of formation of A\(_4\)/J\(_4\)-NPs from DHA 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 to form eight bicyclic endoperoxide intermediate regioisomers (not shown). These
regioisomers then undergo rearrangement to generate eight E₄-NP and eight D₄-NP regioisomers. Each regioisomer is theoretically comprised of eight racemic diastereoisomers for a total of 256 E-ring and D-ring compounds. These molecules subsequently undergo dehydration to A-ring and J-ring compounds. Loss of one chiral center reduces the total number of potential A₄/J₄-NPs to 128. The Eicosanoid Nomenclature Committee has established and approved a nomenclature system for the IsoPs, 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 C₁ (22). By applying this system to NPs, A-ring and J-ring regioisomers can be designated as the 4-series A₄/J₄-NPs, the 7-series A₇/J₇-NPs, 11-series, etc. This is comparable to the series obtained with D₄/E₄-NPs (3). Herein, we present evidence that A₄/J₄-NPs are, in fact, formed in significant amounts in vitro and in vivo from the free radical catalyzed peroxidation of DHA.
EXPERIMENTAL PROCEDURES

Materials- Docosahexaenoic acid, dimethylformamide, and undecane were purchased from Aldrich (Milwaukee, WI). Pentafluorobenzyl (PFB) bromide, methoxyamine HCl, and diisopropylethylamine were from Sigma (St. Louis, MO). 2,2’-Azobis (2-amidinopropane) hydrochloride (AAPH) was from Kodak (Rochester, NY). \[^{2}H_{3}\text{-Methoxyamine HCl}\] was from Cambridge Isotope Laboratories, Inc. (Andover, MA). N,O-Bis(trimethylsilyl)-trifluoracetamide (BSTFA) was from Supelco (Bellefonte, PA). \[^{2}H_{9}\text{-N,O-Bis(trimethylsilyl)-acetamide}\] was from CDN Isotopes (Pointe-Claire, Quebec). C-18 and Silica Sep-Pak cartridges were from Waters Associates (Milford, MA). 60ALK6D TLC plates were from Whatman (Maidstone, UK). \[^{2}H_{4}\text{-PGA}_{2}\] and \[^{2}H_{4}\text{-PGE}_{2}\] were from Cayman Biochemicals (Ann Arbor, MI). B&J Inert SPE System Columns (Glass Sep-Paks) were from Burdick and Jackson (Muskegon, MI).

Oxidation of DHA- DHA was oxidized in vitro using an iron/ADP/ascorbate mixture, as previously described (23).

Purification and Analysis of Unesterified \(A_{4}/J_{4}\)-NPs- Samples obtained from oxidation of DHA or from other sources were extracted in ethyl acetate and subsequently converted to O-methyloxime derivatives by incubation with methoxyamine HCl in pyridine for 45 minutes at 55°C. \(A_{4}/J_{4}\) NPs were then extracted using C-18 Sep-Pak cartridges as described (3,21). The eluted samples were converted to O-methyloxime PFB ester derivatives, purified by thin layer chromatography (TLC) using 60ALK6D silica gel plates employing a solvent system of hexane/acetone (70:30, v/v) as previously
utilized for A<sub>2</sub>/J<sub>2</sub>-IsoPs (21). Compounds migrating in the region from 2 cm below to 1.5 cm above the methyloxime PFB ester of PGA<sub>2</sub> (R<sub>f</sub> = 0.27-0.54) were scraped, extracted with ethyl acetate, converted to trimethylsilyl (TMS) ether derivatives and quantified by stable isotope dilution techniques employing gas chromatography (GC)/negative ion chemical ionization (NICI)/mass spectrometry (MS). The standard used for quantification of A<sub>4</sub>/J<sub>4</sub>-NPs was [<sup>2</sup>H<sub>4</sub>]PGA<sub>2</sub>. The major ions generated in the NICI mass spectra of the PFB ester, O-methyloxime, TMS ether derivatives of A<sub>4</sub>/J<sub>4</sub>-NPs and the [<sup>2</sup>H<sub>4</sub>]PGA<sub>2</sub> are the carboxylate anions at m/z 458 and m/z 438, respectively. Quantification of A<sub>4</sub>/J<sub>4</sub>-NPs was performed based on integration of peak areas. GC/NICI/MS was carried out using either a Hewlett-Packard 5890 GC/MS (Palo Alto, CA) or a Thermo-Finnigan Voyager GC/MS (San Jose, CA).

**Analysis of A<sub>4</sub>/J<sub>4</sub>-NPs as Piperidyl-Enol-Trimethylsilyl Ether Derivatives** - Treatment of PGA<sub>2</sub> with BSTFA and piperidine has been shown to convert it to a piperidyl-enol-TMS ether derivative, which is specific for A-ring prostanooids (21). Thus, we analyzed for the formation of this derivative of A<sub>4</sub>/J<sub>4</sub>-NPs. PFB esters of putative cyclopentenone-NPs were treated with a 1:1 mixture of BSTFA/piperidine for 1 hr at 60°C and analyzed by GC/NICI/MS monitoring the carboxylate anions at m/z 586 for the NPs and m/z 566 for [<sup>2</sup>H<sub>4</sub>]PGA<sub>2</sub>. In some experiments, compounds were exposed to catalytic hydrogenation after conversion to piperidyl-enol-TMS ether derivatives as described (24).
Purification of A₄/J₄-NPs by High-Performance Liquid Chromatography (HPLC) and Analysis by Liquid Chromatography (LC)/Electrospray Ionization (ESI)/MS-

Compounds generated by in vitro oxidation of DHA were purified by normal phase HPLC using a 25 cm x 4.6 mm Econosil SI column with 5µm particles employing an isocratic solvent system of hexane/isopropanol/acetic acid (97:3:0.1, v/v/v) at a flow rate of 1 ml/min. A₄/J₄-NPs eluted in retention volumes 13-37 ml. Fractions were analyzed by GC/MS for A₄/J₄-NPs. Selected fractions that contained substantial amounts of A₄/J₄-NPs were acidified with 1N 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 a 5cm x 2.1mm Zorbax C-18 column (Agilent Technologies, Palo Alto, CA). The solvent system was a gradient consisting of 5 mM ammonium acetate/acetonitrile/acetic acid (90:10:0.1, v/v/v) to 5 mM ammonium acetate/acetonitrile/acetic acid (10:90:0.1, v/v/v) over the course of 10 minutes at a flow rate of 200µL/min. The auxiliary gas pressure was 10 l/min, and the sheath gas pressure was 60 lb/in². The voltage on the capillary was 20.0 V, the capillary temperature was 200°C, and the tube lens voltage was 75.0V. Parent ions were scanned from m/z 300-400. Collision induced dissociation (CID) of molecular ions of putative A₄/J₄-NPs in these fractions was performed from 20 eV to 30 eV scanning daughter ions from m/z 50 to 400. The spectrum shown was obtained at 25eV. The CID gas was argon with a pressure set at 2.5 mTorr. Spectra were displayed by averaging scans across chromatographic peaks. LC/MS was carried out using a Finnigan TSQ 7000 instrument (San Jose, CA).
Preparation and Oxidation of Rat Brain Synaptosomes - Brain tissue was obtained from male Sprague-Dawley rats, homogenized, and synaptosomes isolated by Ficoll gradient centrifugation (25). Lipid peroxidation was initiated by the addition of AAPH (final concentration of 5mM). Incubations were carried out at 37°C for 2 hrs. The reactions were terminated by placing the samples at -80°C and lipids extracted by the method of Folch (3). Esterified Aβ/A4-NPs in phospholipids were hydrolyzed using chemical saponification with potassium hydroxide (26), following treatment with methoxyamine HCl in chloroform/methanol 2:1 for 1 hour at room temperature. Purification was performed as for free Aβ/A4-NPs and levels of Aβ/A4-NPs were then quantified by GC/MS. Data are expressed relative to protein concentration as determined by the Pierce BCA assay (3).

Preparation of Aβ/A4-NPs from Rat and Human Brain Tissue - Tissue samples were obtained from adult male Sprague-Dawley rats and post-mortem human parietal lobe tissue (informed consent obtained). Samples were homogenized in chloroform/methanol 2:1 containing BHT (0.005%) and triphenylphosphine (10 mg/ml) to prevent autooxidation. 2 mls of 0.9% NaCl were added to the samples; they were then shaken, centrifuged, and the aqueous layer discarded. The samples were dried under nitrogen and resuspended in 1 ml chloroform and phospholipids containing Aβ/A4-NPs were separated from other lipids as follows. A glass silica Sep-Pak (500 mg) was prewashed with 5 ml hexane followed by 5 ml chloroform. Each sample was applied to the Sep-Pak and initially extracted with 12 ml of hexane/MTBE (200:3, v/v). Unoxidized cholesterol
esters and triglycerides elute in this fraction. Subsequently, the column was extracted with 12 ml methanol/MTBE (5:95, v/v) to remove oxidized non-polar lipids and cholesterol. A final extraction using 15 ml MTBE:methanol:ammonium acetate (0.01M) (5:8:2 v/v/v) elutes oxidized and unoxidized phospholipids. In preliminary studies, >98% of A₄/J₄-NPs eluted in the phospholipid fraction. Further purification of A₄/J₄-NPs was identical to that employed for the rat synaptosomes as noted above.

Conjugation of A₄/J₄-NPs with Glutathione (GSH) In Vitro—

A₄/J₄-NPs (approximately 100 ng) obtained from the in vitro oxidation of DHA were purified by Sep-Pak extraction and TLC and incubated in 0.1 M KPO₄ buffer (pH 6.5) in the presence of a 10-fold excess (approximately 1 microgram) of GSH and 1 mg bovine liver GSH transferase (Sigma) containing a mixture of GSH transferases at 37°C for 2 hours. The incubation mixture was then acidified to pH 3 and extracted with 2 volumes MeCl₂. Unconjugated A₄/J₄-NPs were measured in the organic fraction by GC/MS and conjugated NPs represented the difference between the amount of A₄/J₄-NPs added to the incubation versus that amount present in the organic fraction.

Subsequently, A₄/J₄-NP-GSH adducts from a separate incubation were definitively identified by LC/MS. Adducts were purified by extraction using a C18 Sep-Pak cartridge preconditioned with acetonitrile and 0.1 M ammonium acetate (pH 3.4) (21) and eluted with 10 ml 95% ethanol and analyzed by LC/MS. LC was carried out using a MAGIC 2002 LC system (Michrom BioResources, Auburn CA) operating in the isocratic mode with the mobile phase of H₂O: acetonitrile: acetic acid (77:22.9:0.1, v/v/v) and compounds separated on an Eclipse XDB-C18 column (2.1 mm x 50 mm, 5 μm particle
size; Agilent, Palo Alto CA) at a flow rate of 75 \( \text{L/min} \). Following on-line chromatography, samples were characterized employing a Finnigan TSQ-7000 (San Jose CA) triple quadripole mass spectrometer operating in the positive ion mode. An electrospray source was fitted with a 100 \( \text{m} \) internal diameter deactivated fused silica capillary column and used nitrogen for both sheath and auxiliary gas, operating at 60 psi and 10 \( \text{L/min} \), respectively. The ESI potential was maintained at 3.5 kV, the heated capillary at 20 V and 200\( ^\circ \text{C} \), and the tube lens 70 V. For tandem MS, parent compounds were collisionally activated at an energy of –15 eV and under 2.5 mT argon. Data acquisition and analysis were performed using an Alpha work station (Digital Equipment Corp, Maynard MA) running Finnigan ICIS software, version 8.3.2.
RESULTS

Formation of \(A_4/J_4\)-NP in vitro - A representative selected ion current chromatogram obtained from the oxidation of DHA in vitro with iron/ADP/ascorbate for 6 hours is shown in Figure 2. The two large chromatographic peaks shown in the m/z 438 channel represent the syn- and anti-O methyloxime isomers of the internal standard \([^2\text{H}_4]\text{PGA}_2\). In the upper m/z 458 ion current chromatogram are a series of chromatographic peaks eluting over approximately a two minute interval. These compounds possessed a molecular mass predicted for \(A_4/J_4\)-NPs. In addition, it would be predicted that the retention time of \(A_4/J_4\)-NPs on GC should be longer than that of the deuterated \(\text{PGA}_2\) internal standard because the former compounds contain two additional carbon atoms (3). As for \(\text{PGA}_2\) and \(\text{PGJ}_2\), as well as for the \(A_2/J_2\)-IsoPs, that have similar chromatographic properties on TLC and GC and identical molecular masses, it is not possible to differentiate between A-type and J-type prostane rings in the putative NP compounds detected in the m/z 458 ion current chromatogram shown in Figure 2. However, since both \(\text{PGE}_2\) and \(\text{PGD}_2\) readily dehydrate in aqueous solutions to form \(\text{PGA}_2\) and \(\text{PGJ}_2\), respectively, it is expected that the \(D_4\)-NPs and \(E_4\)-NPs would dehydrate to form \(A_4\)-NPs and \(J_4\)-NPs.

Additional experimental approaches were undertaken to provide further evidence that the compounds represented by the chromatographic peaks in the m/z 458 ion current chromatogram were \(A_4/J_4\)-NPs. First, the m/z 457 ion current chromatogram contained no chromatographic peaks, indicating that the peaks in the m/z 458 chromatogram are not natural isotope peaks of compounds generating an ion of less than m/z 458. Analysis of putative \(A_4/J_4\)-NPs as \([^2\text{H}_9]\)-TMS ether derivatives resulted in a shift of the m/z 458
chromatographic peaks up 9 Da to m/z 467, indicating the presence of 1 hydroxyl group (data not shown). When these compounds were analyzed as [\textsuperscript{2}H\textsubscript{3}]-O-methyloxime derivatives, the m/z 458 chromatographic peaks all shifted up to m/z 461, indicating the presence of 1 carbonyl group (not shown). Subsequently, analysis following treatment with BSTFA/piperidine resulted in the formation of piperidyl-enol-TMS ether derivatives (Figure 3A). The amount of A\textsubscript{4}/J\textsubscript{4}-NPs analyzed as this derivative was calculated to be 547 ± 232 ng/mg DHA (mean ± S.E., n=3), which is less than the amount formed when compounds are analyzed as O-methyloxime, TMS ether derivatives. This discrepancy can be explained by the fact that we have found that, whereas treatment of PGA\textsubscript{2} with BSTFA/piperidine efficiently converts it to a piperidyl-enol-TMS ether derivative, only small amounts of this derivative are formed with PGJ\textsubscript{2}. By analogy, therefore, only a portion of the mixture of A\textsubscript{4}/J\textsubscript{4}-NPs would be expected to form a piperidy-enol-TMS ether derivative. Finally, putative A\textsubscript{4}/J\textsubscript{4}-NPs were analyzed following catalytic hydrogenation as piperidyl-enol-TMS ether derivatives. Prior to hydrogenation, there were no chromatographic peaks present 10 Da above the m/z 586 in the m/z 596 chromatogram. However, following hydrogenation, intense chromatographic peaks appeared at m/z 596 (Figure 3B) with the loss of chromatographic peaks at m/z 586, indicating the presence of 5 double bonds. It should be noted that it was not possible to analyze putative A\textsubscript{4}/J\textsubscript{4}-NPs following hydrogenation as O-methyloxime TMS ether derivatives owing to the presence of interfering chromatographic peaks 10 Da above m/z 458. Collectively, these data indicated that the compounds represented by the chromatographic peaks in the m/z 458 ion current chromatogram shown in Figure 2 have the functional groups and the number of double bonds predicted for A\textsubscript{4}/J\textsubscript{4}-NPs.
To provide direct evidence that the compounds analyzed by selected ion monitoring MS were A₄/J₄-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 A₄/J₄-NPs as determined by GC/MS were then analyzed by LC/MS. Notably, A₄/J₄-NPs eluted over a very broad volume from 13-37 ml using this HPLC solvent system (Figure 4A). Subsequently, 1 ml fractions were analyzed by LC/MS. The predicted [parent molecule-H]⁻ ion, hereafter referred to as [M-H]⁻, for A₄/J₄-NPs is m/z 357. Figure 4B shows the selected ion monitoring chromatogram of the ion at m/z 357 obtained from one of the analyses of putative cyclopentenone NPs eluting from the HPLC at 18 ml. In this fraction, A₄/J₄-NPs elute from the LC column as a relatively broad set of peaks over about 1 minute. 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 357 resulted in the formation of a number of relevant daughter ions that would be predicted to be common to all of the A₄/J₄-NPs, including m/z 339 ([M-H]-H₂O)⁻, m/z 313 ([M-H]-CO₂)⁻, and m/z 295 ([M-H] - H₂O - CO₂)⁻. Other prominent daughter ions are present that may result from fragmentation of specific A₄/J₄-NP regioisomers. Because of the limited amount of material and the lack of chemically synthesized A- and J-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, these ions can be potentially explained as follows (3,27-31). They include, m/z 259 ([M-H]-C₅H₆O₂ (C₁-C₅))⁻ (10-, 13-,17- and 20- series regioisomer), m/z 247
([M-H]-CH2=CHCH2CH=CHCH2CH3)− (14-series regioisomer), m/z 241 ([M-H]-C5H6O2 (C1-C5)-H2O)− (10-, 13-, 17-, and 20- series regioisomer), m/z 215 ([M-H]-CO2-CH3CH2CH=CHCH2CHO)− (17-series regioisomer) and ([M-H]-CHOCH2CH=CHCH2COOH)− (7-series regioisomer), and m/z 175 ([M-H]-CHOCH2CH=CHCH2CH=CHCH2CH2COOH)− (10 series regioisomer). These data suggested that the mass spectrum shown in Figure 4C represented a mixture of A4/J4-NPs that would be predicted to be formed. Although not shown, analysis of other HPLC fractions by CID revealed similar mass spectra although the relative abundance of different daughter ions varied significantly suggesting that various regioisomers likely eluted from the HPLC in different retention volumes. It is of interest to note that many of the daughter ions that are shown in Figure 4C resulting from the fragmentation of A4/J4-NPs are those we have previously reported for D4/E4-NPs (3). This would be predicted to be the case owing to the fact that A4/J4-NPs are dehydration products of D4/E4-NPs. Taken together, the data shown above provide direct evidence for the formation of a series of A4/J4-NPs generated from the peroxidation of DHA.

Having provided significant evidence for the formation of A- and J-ring NPs 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 5A. For comparison, formation of the precursors of A4/J4-NPs, the D4/E4-NPs, in the same incubations is shown in Figure 5B. Levels of A4/J4-NPs increased dramatically in a time dependent manner from 88 ± 43 ng/mg DHA to 5463 ± 2579 ng/mg DHA at 24 hours, a 62-fold increase (n=4). By comparison, levels of D4/E4-NPs also increase with time from 93 ± 64 ng/mg DHA to 1980 ± 1849 ng/mg DHA at 12 hours, a 21-fold increase.
Interestingly, however, levels decline thereafter, possible due to the dehydration of D$_4$/E$_4$-NPs to A- and J-ring compounds. Also of note is the fact that the total amounts of A$_4$/J$_4$-NPs are significantly greater than D$_4$/E$_4$-NPs at all time points suggesting that the formation of A- and J-ring compounds may be favored over other classes of NPs.

We next examined whether A$_4$/J$_4$-NPs are formed from the peroxidation of DHA esterified in phospholipids since studies to this point had utilized only free DHA. For these experiments, we explored the generation of NPs in synaptosomes isolated from adult rat brain. Synaptosomes are composed of sealed off neuronal and glial processes and are a widely used model for the study of central nervous system gray matter metabolism (3,25). Synaptosomes were oxidized with AAPH, a water soluble free radical generator that induces an oxidative stress. As shown in Figure 6, incubation of synaptosomes for 2 hours with AAPH increased levels of A$_4$/J$_4$-NPs from 89 ±72 ng/mg protein to 1187 ± 217 ng/mg protein, a 13-fold rise. In comparison, we have previously reported that levels of both E/D-ring and F-ring NPs are generated in peroxidizing synaptosomes that are at least an order of magnitude lower than A$_4$/J$_4$-NPs, supporting the notion again that the formation of A/J-ring NPs is favored over other classes of compounds (3). Further, these findings suggest that quantification of A$_4$/J$_4$-NPs may be a more sensitive index of lipid peroxidation in certain biological models of central nervous system oxidative damage.

**Formation of A$_4$/J$_4$-NPs in vivo**—We undertook experiments to determine whether A$_4$/J$_4$-NPs are present esterified in brain lipids in vivo in both animals and humans. Putative A$_4$/J$_4$-NPs were analyzed as free compounds following chemical hydrolysis of
compounds esterified in brain lipid extracts from normal rats. A representative ion current chromatogram obtained from one of these analyses is shown in Figure 7. The chromatographic peaks in the lower m/z 438 ion current chromatogram represent the O-methyloxime isomers of the internal standard [\(^{2}\)H\(_{4}\)]PGA\(_{2}\). Of note, in this analysis, the first isomer is significantly smaller than the second and elutes just before the larger isomer. In the upper m/z 458 chromatogram are a series of peaks which have molecular masses and retention times expected for A\(_{4}\)/J\(_{4}\)-NPs. The pattern of peaks representing A- and J-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 458 ion current chromatogram in Figure 7 represent A\(_{4}\)/J\(_{4}\)-NPs formed in vivo. The compounds were analyzed as [\(^{2}\)H\(_{9}\)]trimethylsilyl ether derivatives, which resulted in a shift of the chromatographic peaks in the m/z 458 ion current chromatogram to m/z 467 (data not shown). This shift of 9 Da indicates the presence of 1 hydroxyl group. Analysis of the compounds as [\(^{2}\)H\(_{3}\)]O-methyloxime derivatives resulted in the shift of the m/z 458 chromatographic peaks up 3 Da to m/z 461, indicating the presence of one carbonyl group (data not shown). Subsequently, analysis following treatment with BSTFA/piperidine resulted in the formation of a new series of chromatographic peaks at m/z 586 with disappearance of peaks at m/z 458, supporting the contention that these compounds can form piperidyl-enol-TMS ether derivatives (data not shown). Taken together, these experiments provide evidence that the compounds represented in the m/z 458 chromatogram in Figure 7 have the expected number of functional groups that are predicted for A\(_{4}\)/J\(_{4}\)-NPs.
Levels of A₄/J₄-NPs esterified in the lipids of rat brain were determined to be 97±25 ng/g (n=3). These amounts are significantly higher by nearly an order of magnitude than those reported for either D/E-ring or F-ring NPs. We also quantified amounts of non-esterified A₄/J₄-NPs in rat brain tissue and they were <5% of esterified levels. Subsequently, levels of A₄/J₄-NPs were measured in parietal cortex from 5 humans obtained at autopsy. No individual had known neurological disease at the time of death. Figure 8 shows a representative ion current chromatogram from a human brain sample. Again, the two O-methyloxime isomers of the [²H₄]PGA₂ internal standard are present in the m/z 438 chromatogram. In the upper m/z 458 chromatogram are a series of peaks which have molecular masses and retention times expected for A₄/J₄-NPs. The chromatographic pattern of compounds is very similar to that obtained from the incubation of DHA in vitro. Levels of putative A₄/J₄-NPs were found to be 98 ± 26 ng/g of brain tissue (n=5). As with rat brain tissue, quantities of A/J-ring NPs exceed those of E/D-ring and F-ring NPs by at least 5-fold, supporting the contention that formation of cyclopentenone NPs in the human central nervous system is favored.

Adduction of A₄/J₄-NPs with GSH in vitro- One of our major interests in determining whether cyclopentenone NPs are formed in vivo is related to the fact that A₄/J₄-NPs should be highly reactive because they contain α,β-unsaturated-carbonyl moieties and would thus be susceptible to nucleophilic addition reactions (21). Thus, we sought to determine whether purified A₄/J₄-NPs obtained from the oxidation of DHA in vitro would conjugate GSH in the presence of GSH transferase. We had previously shown that cyclopentenone eicosanoids including the isoprostane 15-A₂­t-IsoP rapidly
adduct GSH in the presence of GSH transferase (21). Because of the limited availability of A$_4$/J$_4$-NPs, we examined whether significant conjugation of these compounds with GSH occurred within 2 hours, a time at which the majority of 15-A$_{2t}$-IsoP is largely conjugated in vitro. Formation of GSH conjugates of cyclopentenone NPs was assessed by determining the percent of NPs that did not extract into methylene chloride at pH 3. We found that after 2 hours 58 ± 13% of the A$_4$/J$_4$-NPs present in incubations was in the form of a polar conjugate that would not extract (n=3). In similar studies, approximately 70% of 15-A$_{2t}$-IsoP formed a conjugate with GSH (21). These data suggest that like cyclopentenone IsoPs, A$_4$/J$_4$-NPs are reactive molecules capable of undergoing nucleophilic addition.

Subsequently, putative A$_4$/J$_4$-NP-GSH adducts were definitively identified by LC/MS. Adducts were partially purified from an incubation mixture and subjected to LC as described. The expected molecular ion [M-H]$^-$ for A$_4$/J$_4$-NP-GSH adducts is m/z 666. Selected ion monitoring analysis of material eluting from the LC showed multiple m/z 666 chromatographic peaks from 5-6 minutes, presumably representing GSH adducts of different A$_4$/J$_4$-NP stereoisomers. A composite CID spectrum was obtained by summing scans over these peaks and is shown in Figure 9. As is apparent, the molecular ion [M+H]$^+$ is present at m/z 666. Relevant daughter ion include m/z 648 ([M+H]-H$_2$O)$^+$, m/z 341 ([M+H]-GSH-H$_2$O)$^+$ (the eicosanoid portion of the molecule) and m/z 308 ([M+H]-A$_4$/J$_4$-NP)$^+$, confirming that identity of A$_4$/J$_4$-NP adducts of GSH.
DISCUSSION

These studies report the identification of a novel class of cyclopentenone eicosanoids, A$_4$/J$_4$-NPs, formed in vivo from the free radical-initiated peroxidation of DHA. DHA is a major unsaturated fatty acid in neural tissues. Oxidative damage to neuronal structures such as DHA-rich cellular membranes has been implicated in a number of neurodegenerative diseases (3,5-8,9). Nonetheless, the identification, at a molecular level, of products that are formed from the oxidation of DHA has been largely lacking. This provided the impetus for the studies reported herein.

The mechanism of formation of A$_4$/J$_4$-NPs from DHA is outlined in Figure 1. We have previously reported that both F-ring and E/D-ring NPs are formed by the same mechanism with E$_4$/D$_4$-NPs resulting from isomerization of endoperoxide intermediates while F$_4$-NPs are generated by reduction of the endoperoxides (3,10). A$_4$/J$_4$-NPs are formed by dehydration of E$_4$/D$_4$-NPs. It should be noted from the present studies that in all biological systems examined to date in vitro and in vivo, cyclopentenone NPs are formed in significantly larger amounts than either D/E-ring or F-ring NPs. These findings strongly suggest that the formation of cyclopentenone NPs is a favored route of DHA oxidative metabolism. In addition, these findings support the notion that quantification of A$_4$/J$_4$-NPs may be a more sensitive indicator of DHA oxidation and neural tissue oxidative stress than other classes of NPs. Factors that may influence the formation of A/J-ring NPs over other NPs are not known although we have previously shown that agents such as GSH and other thiols reduce IsoP endoperoxide intermediates resulting in generation of F$_2$-IsoPs over E$_2$/D$_2$-IsoPs (26). It might thus be predicted that
the presence of reducing substances would analogously decrease cyclopentenone eicosanoid formation although this issue has not been explored. The present studies also suggest that the dehydration of \( \text{E}_4/\text{D}_4 \)-NPs to \( \text{A/J-ring} \) NPs occurs non-enzymatically since we detected significant amounts of the latter compounds when pure DHA was oxidized in vitro. On the other hand, it cannot be ruled out that the dehydration of \( \text{E}_4/\text{D}_4 \)-NPs in vivo occurs, to some extent, enzymatically.

Like other classes of NPs, the \( \text{A}_4/\text{J}_4 \)-NPs are formed in situ esterified in phospholipids and are then presumably released in the free form by a phospholipase(s) (3). We have previously demonstrated that \( \text{E}_4/\text{D}_4 \)-NP-containing phospholipids are substrates for bee venom phospholipase \( \text{A}_2 \). The type(s) of mammalian phospholipase that might be responsible for hydrolysis of NPs from phospholipids in vivo, however, is unknown, although it has previously been reported that the activity of phospholipase \( \text{A}_2 \) is enhanced toward oxidized phospholipids (32). Understanding the enzyme and regulatory factors controlling the formation and hydrolysis of NP-containing phospholipids is of considerable importance because they would be predicted to be very distorted molecules and thus profoundly affect biophysical properties of neuronal membranes. Therefore, in settings of oxidant stress, enhanced formation of NP-containing phospholipids could alter neuronal membrane fluidity, integrity, and function.

The discovery of the formation of cyclopentenone NPs in vivo opens up numerous new avenues for scientific inquiry. The reactive nature of these compounds conferred by the \( \alpha,\beta \)-unsaturated carbonyl moiety that characterizes them provides a basis for hypotheses regarding their potential role in the pathogenesis of oxidant injury. Cyclopentenone PGs have been shown to exert unique biological properties. They inhibit
cellular proliferation via their ability to modulate a variety of growth-related and stress-induced genes, induce apoptotic cell death at higher concentrations, and activate the peroxisome proliferator-activated receptor-γ (33-41). The reactive α,β-unsaturated carbonyl seems essential for many of the biological actions. In this regard, one might anticipate that some of these biological effects could also be mediated by cyclopentenone NPs, particularly in tissues such as the central nervous system where they are formed in significant amounts. As noted, cyclopentenone NPs rapidly undergo glutathione transferase-catalyzed conjugation with GSH, analogous to PGA₂ and 15-A₂t-IsoP (21). This adduction may also contribute to cellular and tissue injury in settings of oxidative stress through depletion of endogenous antioxidants. It has been shown that conjugation of cyclopentenone PGs with GSH eliminates the biological activities of these reactive compounds (42). Thus, much remains to be known about the underlying molecular mechanisms that might mediate the biological effects of cyclopentenone NPs.

In these studies, A₄/J₄-NPs were identified in vitro and in vivo using a variety of complementary chemical and MS approaches using LC/ESI/MS/MS. A relatively small body of literature exists regarding the characterization of eicosanoids using LC/MS compared to GC/MS. Nonetheless, our present findings are consistent with our previous studies and reports by other investigators (3,27-31). As noted, Figure 4C is a mass spectrum likely representing a number of A₄/J₄-NPs. Ions comprising the loss of combinations of H₂O and CO₂ are readily apparent. In addition, other prominent ions can be appreciated which apparently derived from specific A₄/J₄-NPs regioisomers. Of note are the abundant fragments at m/z 259 and m/z 241. These ions are consistent with fragmentation at C₅ resulting in the loss of 98 Da from the parent ion of several D₄/E₄-NP
regioisomers (10-,13-,17-, and 20-series compounds). The m/z 241 ion has an additional loss of water. Previous reports have noted a similar loss of 98 Da for various fatty acids, IsoPs and NPs analyzed by LC/MS (3,27-31). Another noteworthy fragment ion that can be appreciated in the mass spectrum shown in Figure 4C is m/z 175. This ion can be explained, at least in part, as an alkyl-directed product resulting from the loss of carboxy-containing neutral fragments from the parent molecule. Such fragmentation patterns have been previously reported for both oxygenated and unoxygenated fatty acids.

In summary, we report the discovery that A/J-ring NPs are formed in vivo as a products of the nonenzymatic free radical-catalyzed peroxidation of DHA, a major unsaturated fatty acid in neural tissue. The fact that A₄/J₄-NPs are readily detectable in normal brain from animals and humans at concentrations significantly higher than other classes of NPs suggests the compounds represent an important pathway of the oxidative metabolism of DHA. In addition, these findings support the notion that there is significant 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 will likely provide valuable insights into the pathophysiology of oxidant injury in the nervous system.

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FIGURE LEGENDS

**Figure 1.** A-C  Pathways for the formation of A\(_d/J_4\)–NPs by the nonenzymatic peroxidation of DHA.

**Figure 2.** Selected ion current chromatogram obtained form the analysis of A\(_d/J_4\)-NPs generated during iron/ADP/ascorbate-induced oxidation of DHA in vitro. The two large peaks in the m/z 438 ion current chromatogram represent the syn and anti-O-methylloxime isomers of the \[^2\text{H}_4\] PGA\(_2\) internal standard. The series of peaks in the m/z 458 chromatogram represent putative A\(_d/J_4\)-NPs. The amount of A\(_d/J_4\)-NPs represented is 2038 ng/mg DHA.

**Figure 3.** Analysis of the putative A\(_d/J_4\)-NPs generated in vitro as piperidyl-enol-TMS ether derivatives prior to after catalytic hydrogenation. A) Analysis of compounds prior to hydrogenation. The peaks in the m/z 586 chromatogram are consistent with the formation of piperidyl-enol-TMS ether derivatives of cyclopentenone neuroprostanes. The major peak in the m/z 566 chromatogram represents the \[^2\text{H}_4\] PGA\(_2\) internal standard. No compounds were detected 10 Da above m/z 586 at m/z 596 before hydrogenation. B) After catalytic hydrogenation, the m/z 586 peaks shifted up 10 Da indicating the presence of 5 double bonds while the internal standard represented by the major chromatographic peak shifted upwards 6 dalton to m/z 572 indicating the presence of 3 double bonds.
Figure 4. A) Straight phase HPLC analysis of $A_4/J_4$-NPs generated from the oxidation of DHA in vitro. Quantities of $A_4/J_4$-NPs in HPLC fractions were measured by GC/MS. Material eluting in fraction 18 that is shown in Figures 4B and 4C is denoted by the asterisk. Analysis was carried out by normal phase HPLC using a 25 cm x 4.6 mm Econosil SI column employing an isocratic solvent system of hexane/isopropanol/acetic acid (97:3:0.1, v/v/v at a low rate of 1 ml/min. B) Selected ion monitoring chromatogram of the [parent molecule-H] $^-$ (M) ion at m/z 357 form LC/ESI/MS analysis of putative $A_4/J_4$-NPs obtained from prior HPLC purification of oxidized DHA in vitro. Material analyzed by LC/MS eluted from the HPLC in a fraction volume at 18 ml. LC was carried out using a 5cm x 2.1mm Zorbax C-18 column (Agilent Technologies, Palo Alto, CA). The solvent system was a gradient consisting of 5 mM ammonium acetate/acetonitrile/acetic acid (90:10:0.1, v/v/v) to 5 mM ammonium acetate/acetonitrile/acetic acid (10:90:0.1, v/v/v) over the course of 10 minutes at a flow rate of 200µL/min. C) LC/ESI/MS analysis of $A_4/J_4$-NPs generated in vitro. Material represented in the m/z 357 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 peaks. See the text for structural characterization of particular ions denoted in the figure.
Figure 5. Time course of formation of (A) A₄/J₄-NPs and (B) E₄/D₄-NPs during oxidation of DHA in vitro by incubation with an iron/ADP/ascorbate mixture. Data are expressed as mean ± standard error. N=4 experiments.

Figure 6. Effect of incubation of rat brain synaptosomes with AAPH (5 mM) for 2 hours. Data are expressed as mean ± standard error. N=3 experiments.

Figure 7. Selected ion current chromatogram obtained from the analysis of A₄/J₄-NPs esterified in rat brain tissue. The series of peaks shown in the m/z 458 chromatogram represent putative A₄/J₄-NPs. The two peaks in the m/z 438 ion current chromatogram represent the syn and anti-O-methyloxime isomers of the [²H₄] PGA₂ internal standard. The first methyloxime isomer in this figure is very small and is just to the left of the larger isomer. The amount of A₄/J₄-NPs present was 94 ng/g of brain tissue.

Figure 8. Selected ion current chromatogram obtained from the analysis of A₄/J₄-NPs esterified in post-mortem human temporal lobe brain tissue. The series of peaks shown in the m/z 458 chromatogram represent the putative A₄/J₄-NPs, and the two peaks in the m/z 438 ion current chromatogram represent the syn and anti-O-methyloxime isomers of the [²H₄] PGA₂ internal standard. The calculated amount of A₄/J₄-NPs present was 74 ng/g of brain tissue.

Figure 9. LC/ESI/MS/MS analysis of the GSH conjugates of A₄/J₄-NPs. The parent ion is m/z 666. Material was subjected to collision induced dissociation at –15 eV and the
dauther ions scanned from 100 to 700. Spectra were obtained by averaging scans across
the eluting LC chromatographic peak. See text for structural characteristics of major
fragment ions.
Figure 1
Figure 1
Figure 1
FIGURE 2
FIGURE 3
Figure 4
Figure 4

B

$m/z = 357$
Figure 4
Figure 5

A

A4/J4 Neuroprostanes (ng/mg DHA)

Time (hrs)

Figure 5
Figure 5
FIGURE 7

Retention Time (min)

Relative Intensity (Arbitrary Units)

$m/z = 458$

$m/z = 438$

$[^2H_4]PGA_2$

Retention Time (min)
**FIGURE 8**
Figure 9
Formation of highly reactive A-ring and J-ring isoprostane like compounds (A4/J4-neuroprostanes) In Vivo from docosahexaenoic acid

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