Neurofurans: novel indices of oxidant stress derived from Docosahexaenoic Acid

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Running title: Oxidant stress products from DHA

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ABSTRACT.

Isoeicosanoids are free radical catalyzed isomers of the enzymatic products of arachidonic acid (AA). They are formed in situ in cell membranes, are cleaved, circulate and are excreted in urine. Isomers of prostaglandin F2α – the F2-isoprostanes (iPs) have emerged as sensitive indices of lipid peroxidation in vivo. Analogous compounds formed from docosahexaenoic acid (DHA) are termed neuroprostanes (nPs) and are more abundant than iPs in brain. Isofurans (iFs) are another class of isoeicosanoids characterized by a substituted tetrahydrofuran ring. They are preferentially formed, relative to iPs, under conditions of elevated oxygen tension. Here, we report the discovery of neurofurans (nFs), the analogous family of compounds formed from DHA. Formation of nFs is characterized by mass spectrometry and confirmed by oxidation of DHA in vitro and following CCl4 administration in liver in vivo. It is demonstrated that the levels of nFs are elevated in the brain cortex of a mouse model of Alzheimer disease and are depressed in mouse brain cortex by deletion of p47phox, an essential component of the phagocyte NADPH oxidase. Measurement of the nFs may ultimately prove useful in diagnosis, timing and selection of dose in the treatment and chemoprevention of neurodegenerative disease.

Introduction:

Isoprostanes (iPs) are prostaglandin (PG) isomers derived by free radical attack on esterified arachidonic acid (AA) in cell membranes (1). They are then cleaved, presumably by phospholipases, circulate in plasma and are excreted in urine, where they can be quantified by immunologic methods or by mass spectrometry. Isomers of PGF2α – F2-iPs – have been measured in urine as indices of in vivo lipid peroxidation (2). Compounds analogous to the F2-iPs may be formed from other fatty acid substrates. For example, neuroprostanes (nPs) are iPs derived from the ω-3 fatty acid, docosahexaenoic acid (DHA) (3). Given that DHA is more abundant than AA in brain, the nPs may prove to be a
more attractive biomarker of neurodegeneration than are the iPs (4). Consequently, there is considerable interest in the use of these compounds as indices of progression in neurodegeneration, such as in Alzheimer disease (AD) (5).

The isofurans (iFs) are a family of free radical-induced peroxidation products of AA (6). They are even more abundant than iPs in tissues as diverse as kidney and hippocampus in the rat and may offer an adjunctive approach to the assessment of oxidant stress in vivo (6, 7). They are formed preferentially under conditions of elevated oxygen tension (6).

Here we report the characterization of the analogous compounds derived from DHA – the neurofurans (nFs). Quantitative assessment of nFs in vivo reveals modulated formation under conditions of elevated and diminished oxidant stress. Given the abundance of DHA in the brain, analysis of nFs may have particular value in the quantitative assessment of lipid peroxidation in neurodegenerative disease.

**Experimental Procedures:**

**In Vivo Oxidation: Analysis of Liver from CCl₄-Treated Mice.**

Treatment of mice with CCl₄ was used to induce oxidant injury to the liver as previously described (8). Three month-old C57/BL6 male mice were fasted overnight and then administered intraperitoneal (i.p.) injections of CCl₄ (4g/kg body weight). CCl₄ was mixed with canola oil (1:1 by volume). Mice were anesthetized with CO₂ prior to sacrifice at 0, 1, 2.5, 7.5, and 20 hours after CCl₄ administration, their livers were removed and rapidly frozen in liquid nitrogen prior to storage at -80°C. Total lipids were extracted using a modified Folch procedure (9). Briefly, frozen samples (0.1–0.5g) were suspended in 5 mL ice-cold chloroform: methanol (2:1, v/v) with 0.005% (w/v) butylated hydroxytoluene to suppress oxidation. Samples were homogenized using a TissueLyzerTM (Qiagen, Valencia, CA). The lipid extracts were mixed vigorously with 2.0 mL NaCl (0.9%, w/v) and the phases separated by centrifugation. After the upper phase was decanted, samples were transferred to clean tubes and the residual organic solvent was removed under a stream of nitrogen. Total lipids were dissolved in 0.5 mL methanol and stored at −80°C.

Lipid extracts were then saponified by adding 0.5 mL of 2.7 N KOH in 0.5 mL methanol. The mixture was then sonicated and mixed vigorously until thoroughly suspended, then heated at 37 °C for 30 min. The pH was adjusted to 3.0 with 1.2 mL 1N HCl. Next, 1.0 ng of [2H₄] 8, 12-iso-iPF₂₀−VI was added as an internal standard. The samples were purified by solid phase extraction (SPE) using StrataX cartridges (Phenomenex, Torrance, CA). Purified lipid extracts were analyzed by LC/MS/MS.

**In Vitro Oxidation of DHA:**

DHA, obtained from Cayman Chemical Co. (Ann Arbor, Michigan) was dissolved in ethanol and added to 5 mL PBS, pH 7.4, to a final concentration of 20 mM. 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH; Sigma, St. Louis, MO) was used as an oxidant as described (6). Reactions were allowed to proceed at 37°C and aliquots were removed for analysis at 0, 2, 4, 8, 12, 24,
and 30 hours. Reactions were stopped by immersing the tube in an ice water bath. The aliquots were diluted by a factor of 100 with water. 1.5 ng of [2H4]8,12-iso-iPF2α-VI was added as internal standard to 10 μL aliquots of the diluted samples. They were then acidified to pH <3 with formic acid and extracted with ethyl acetate. The samples were then purified by SPE before LC/MS/MS analysis. AAPH concentrations of 0.01, 0.1, 1, 10, and 100 mM were used to examine dose dependent product formation. Three independent experiments were done for the time course and dose dependent formation experiments.

MS Analyses:

Unknown compounds were characterized by stable isotope dilution gas chromatography / electron capture / negative ionization (GC/EC/NI) /MS as the pentafluorobenzyl (PFB) ester, trimethylsilyl (TMS) ether derivative as described (6). [2H9]TMS ether derivatization was used to determine the number of the hydroxyl groups. Catalytic hydrogenation was used to reveal the number of double bonds. Compounds were also subjected to methoxime (MO) derivatization conditions to exclude the presence of ketone or aldehyde groups. Exposure to HCl excluded the possibility of epoxides. Fragmentation patterns of the unknown compounds were analyzed by electrospray (ES) MS/MS in the negative ion mode. [2H4]8, 12-iso-iPF2α-VI was used as an internal standard for quantitation.

Analysis of nFs in vivo:

(i) P47phox Knockout Mice:

Mice lacking with the P47phox component of NADPH oxidase (kindly provided by Steven M. Holland, M.D., NIAID) possess an impaired ability to generate superoxide (10). Ten knockout mice (5 male and 5 female) and ten wild type controls (5 male and 5 female) were anesthetized prior to sacrifice and removal of the brain at the age of 9 months. The brains were rapidly frozen in liquid nitrogen prior to placement at -80°C. Total lipids were extracted from the brains using a modified Folch procedure. Purified lipid extracts were analyzed by LC/MS/MS. [2H4]8, 12-iso-iPF2α-VI was added as an internal standard.

(ii) Tg 2576 transgenic mouse model of AD:

The transgenic mouse line Tg 2576 expressing the human amyloid precursor protein with the “Swedish” double mutation KM670/671NL driven by the hamster prior protein promoter (11) has been extensively characterized and is a widely used model of AD. These mice develop abundant extracellular amyloid deposits after 12-15 months (11, 12). Eight female Tg 2576 transgenic mice and 8 female littermate controls were anesthetized prior to sacrifice and removal of their brains at the age of 15 months. The brains were rapidly frozen in liquid nitrogen prior to placement at -80°C. Total lipids were extracted and purified as described above. Purified lipid extracts were analyzed by LC/MS/MS. [2H4]8, 12-iso-iPF2α-VI was added as internal standard.

Results:

Discovery of the nFs:
Purified lipid extracts from mouse liver were analyzed by LC/MS after treatment with CCl4. During the analysis, four ions detected near the region of the chromatogram in which the iPs eluted attracted attention. The ions were m/z 353 (F2-iPs), m/z 369 (iFs), m/z 377 (nPs) and a peak at m/z 393 (Fig1). Selected ion monitoring (SIM) analysis of m/z 393 revealed a region of incompletely resolved chromatographic peaks that eluted slightly earlier than the iPs, nPs and iFs in a reverse phase LC gradient (Fig2a). This ion is 16 Da higher than the quasi-molecular ion of nPs (m/z 377) and 24 Da higher than that of iFs (m/z 369). Following GC/EC/NI/MS analysis after PFB ester and TMS ether derivatization, there was a series of chromatographic peaks detected at m/z 609, which is again 16 Da higher than nPs (m/z 593) and 24 Da higher than iFs (m/z 585) (Fig2b).

Given these chromatographic, mass spectrometric, and functional similarities, it was surmised that the unknown compounds may share structural similarities with the known products of AA and DHA oxidation; for example, iF-like compounds derived from DHA. We sought to determine whether one could generate these compounds by oxidation of DHA. After free radical-initiated oxidation of DHA, total lipids were extracted with ethyl acetate and the samples were then purified and analyzed by LC/MS. A SIM mass chromatogram of m/z 393 yielded a pattern similar to that of the in vivo experiments (Fig3).

GC/EC/NI/MS analysis of the compounds as the [2H9]TMS ether derivatives resulted in a mass increase of 27 Da while retaining a similar chromatographic pattern, indicating the presence of three hydroxyl groups (Fig4). Retention of an almost identical chromatographic pattern with little residual at m/z 609 supports the contention that all of these compounds have three hydroxyl groups.

Catalytic hydrogenation resulted in a mass gain of 8 Da, indicating the presence of four double bonds (Fig. 5).

The presence of epoxide or carbonyl groups was excluded when treatment with HCl or methoxyamine HCl failed to alter the mass chromatogram (see supplement Fig 1).

Based on the mechanisms postulated for formation of the iFs (6), two plausible mechanisms for the formation of nFs are proposed: the cyclic peroxide cleavage pathway and the epoxide hydrolysis pathway (Fig.6,7). These two mechanisms together predict the formation of 16 distinct regioisomers, each comprised of 32 racemic diastereomers for a total of 512 compounds. The epoxide hydrolysis pathway is predicted to contribute to the formation of all 16 regioisomers, whereas the cyclic peroxide cleavage pathway is predicted to contribute to the formation of only eight of the sixteen regioisomers. The nomenclature *-epox or *-both were used in those pathways to be consistent with iFs. (6)

**Analysis of the Unknown Compounds by MS:**

The compounds purified from both in vivo and in vitro oxidation experiments were analyzed by infusion into an MS/MS while scanning product ions of m/z 393. Spectra averaged over a period
of approximately 9 minutes during the infusion are shown in Fig 8. The spectra obtained in vivo and in vitro are strikingly similar, differing mainly in the relative abundance of the various product ions. A prominent ion is present at m/z 393, representing the unfragmented precursor ion. A set of product ions were predicted from the postulated structures shown in Fig 9.

SRM analysis of some predicted transitions is shown in Fig 10. The transition of m/z 393 → 193 was the most abundant one, which could come from I-Both, I-Epox, II-Both and II Epox. Again, the unknown compounds revealed a similar pattern of SRM fragments whether derived in vivo or in vitro.

Overall, these data provide strong evidence that these unknown compounds are nFs.

**Formation of nFs in vitro:**

The formation of nFs and nPs by in vitro lipid oxidation reflected both the duration of exposure and the concentration of AAPH. The formation of nFs during oxidation of DHA using AAPH reached a maximum of roughly 15 ng/µg DHA by 6 hours (Fig 11). This effect was dose related and a maximal response was attained at roughly 10mM AAPH (Fig 12). However, unlike iFs, increasing oxygen tension did not detectably favor nF formation (6) (see supplement Fig 2).

**Formation of nFs in vivo:**

NFs could be readily detected in mouse liver. Formation of nFs increased dramatically, from a mean 141.3 ng/g tissue weight before CCl₄ injection to 412.2 ng/g at one hour and to 1330.6 ng/mg tissue weight at 2.5 hours after CCl₄ administration. While the nPs also increased dramatically, they were less abundant than the nFs (Fig 13). Unmetabolized nFs were below the detectable level in mouse plasma and urine in these experiments.

**nFs in the Tg2576 Transgenic Mouse Model of AD and p47phox Knockout Mice:**

Levels of iPs, nPs and nFs were significantly (p < 0.05) elevated in Tg2576 mouse brain cortex (7.1 vs 5.4 ng/g; 14.3 vs 10.5 ng/g and 173.2 vs 109.1 ng/g tissue weight, respectively compared to controls. A similar difference was not observed in the cerebellum, an area spared amyloid pathology (Fig. 14)

Deletion of p47phox, an essential component of the phagocyte NADPH oxidase (phox), renders murine microglial cells unable to produce superoxide (10). The levels of nFs were significantly (p < 0.05) reduced in the p47phox knockout mouse brain cortex from a mean of 156.2 ng/g to 99.3 ng/g tissue weight (Fig 15).

**Discussion:**

These studies report the discovery of a novel class of iF-like compounds, the nFs, formed in vivo and in vitro from the free radical-initiated peroxidation of DHA. Previous studies have demonstrated that iF formation is favored by high oxygen tension (6) and that the relative enrichment of brain with
DHA over AA is reflected in preferential formation of nPs over iPs (3). Our observations document the relatively greater formation of nFs than nPs in the brain cortex of Tg2576 mice, a widely used experimental model of AD, over their controls. It is likely that combined analysis of nPs and nFs would reflect lipid oxidation in DHA rich tissues such as brain more comprehensively than either analyte alone.

DHA is one of the main polyunsaturated fatty acids (PUFAs) in fatty fish (13) and fish oil supplements rich in DHA (14) are popular as food supplements, either alone or in combination with vitamins and minerals. Fish oils have pleiotropic effects of potential benefit in the treatment of dyslipidemias (15), hypertension (16) and inflammation (17). Epidemiological studies have related cardiovascular health to a diet rich in fish consumption (18-21). Although they impact on biomarkers of risk, limited evidence for a beneficial effect of fish oils or high fish consumption on clinical outcomes presently derives from randomized trials (22-26). A potential mechanism by which beneficial effects might be transduced is via a shift from metabolic products of AA to those of ω-3 fatty acids, such as eicosapentaenoic acid and DHA (14, 27, 28). Aside from the enzymatic products of these fatty acids, the biological importance of their relatively greater susceptibility to lipid peroxidation than ω-6 analogs is unknown. Thus, on the one hand, ω-3 PUFAs might act as a more efficient “sink” for free radical generation than does AA. On the other, their peroxidation products might directly impair cellular function. In either situation, peroxidation products such as nPs and nFs have the virtue of being chemically stable and as such, represent attractive quantitative analytes of oxidant stress in brain, enriched as it is in DHA (29). DHA is highly concentrated in the brain, particularly in the gray matter, where it comprises approximately 30% of the total fatty acids in aminophospholipids (29). However, DHA is not synthesized by neurons. Instead, it is synthesized and secreted by astrocytes and subsequently taken up by neurons (30). DHA is thought to be important for brain development (31); DHA deficiency is associated with abnormalities in brain function (32).

There is intense interest in the identification of biomarkers of oxidant stress that might reflect the periodic episodes of inflammation that characterize the clinical course of neurodegenerative disease (4). Such indices, particularly if available in noninvasively acquired biomaterials, such as urine, might prove particularly useful in guiding the timing and dosing of antioxidant or antiinflammatory therapies subject to clinical evaluation. Previously, we (33) and others (34) demonstrated that iPs are detectable in affected regions of the brains of patients who had died of AD and levels appear elevated in the cerebrospinal fluid of patients with the presumptive clinical diagnosis (35). However, the utility of urinary iPs in this regard has proven controversial. A series of studies indicated greater free radical damage in DHA- than AA-containing compartments in diseased regions of AD brain, and suggest diminished reducing capacity in DHA-containing compartments (34, 36, 37). The brains of AD patients are relatively deficient in
DHA in the gray matter of the frontal lobe and hippocampus (38). Thus, quantification of DHA oxidation products might be expected to be a more sensitive indicator of oxidative stress in brain than measurement of either iPs or iFs. Here, we report that the peroxidation products of DHA are more abundant in the brains a mouse model of AD than those in their WT controls.

In these studies, nFs were identified in vitro and in vivo using a variety of complementary chemical and MS approaches involving LC/ES/MS/MS and GC/EC/NI/MS. The likely mechanism of their formation is outlined in Figures 6 and 7. Augmentation and depression of oxidant stress in vivo were achieved by administration of CCl₄ and deletion of p47phox respectively and attendant alterations in nF generation were observed in liver. Consistent with the increase in iF formation under conditions of high oxygen tension, nF generation increased when ambient oxygen was increased from 0 to 21%. However, to our surprise no further alteration was detected at 100% oxygen. One possibility is that nFs might get overoxidized under these conditions due to "skipped dienes" (double bonds interspaced with a methylene group) which are absent in iFs. A failure to identify nFs in urine constrains their applicability as indices of oxidant stress. This may reflect their retention in tissues at the site of free radical attack on DHA, their failure to clear rapidly into urine and/or their rapid breakdown to unmeasured metabolites. Further study will be necessary to discriminate between these options.

In summary, we report the discovery that iF-like compounds, the nFs, are formed in vivo as products of the nonenzymatic free radical-catalyzed peroxidation of DHA. The fact that nFs are readily detectable in normal brain from animals at concentrations significantly higher than other classes of isoeicosanoids is consistent with their representing important products of the oxidative metabolism of DHA. It also implies ongoing oxidant stress in the central nervous system. Elucidation of the biological effects of these novel compounds and the factors which influence their formation and metabolism will likely provide valuable insights into the pathophysiology of oxidant stress in the nervous system. Measurement of the nFs may ultimately prove useful in diagnosis, timing and selection of dose in the treatment and chemoprevention of neurodegenerative disease.

References:

3. Roberts LJ 2nd, Montine TJ, Markesbery WR, Tapper AR, Hardy P, Chemtob S,


15. Phillipson BE, Rothrock DW, Connor WE, Harris WS, Illingworth DR.  
Reduction of plasma lipids, lipoproteins, and apoproteins by dietary fish oils in 
patients with hypertriglyceridemia.  

16. Knapp HR, FitzGerald GA.  
The antihypertensive effects of fish oil. A controlled study of polyunsaturated fatty 
acid supplements in essential hypertension.  

17. Mori TA, Beilin LJ.  
Omega-3 fatty acids and inflammation  

Sasaki J, Hishida H, Itakura H, Kita T, Kitabatake A, Nakaya N, Sakata T, Shimada K, 
Shirato K; Japan EPA lipid intervention study (JELIS) Investigators.  
effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic 
patients (JELIS): a randomised open-label, blinded endpoint analysis.  

19. Murphy KJ, Meyer BJ, Mori TA, Burke V, Mansour J, Patch CS, Tapsell LC, Noakes M, 
Clifton PA, Barden A, Puddey IB, Beilin LJ, Howe PR.  
Impact of foods enriched with n-3 long-chain polyunsaturated fatty acids on 
erthrocyte n-3 levels and cardiovascular risk factors.  

20. Erkkila AT, Matthan NR, Herrington DM, Lichtenstein AH.  
Higher plasma docosahexaenoic acid is associated with reduced progression of 
coronary atherosclerosis in women with CAD.  

Lau J.  
n-3 Fatty acids from fish or fish-oil supplements, but not alpha-linolenic acid, benefit 
cardiovascular disease outcomes in primary- and secondary-prevention studies: a 
systematic review.  

22. Brouwer IA, Zock PL, Camm AJ, Bocker D, Hauer RN, Wever EF, Dullemeyer C, 
Effect of fish oil on ventricular tachyarrhythmia and death in patients with 
implantable cardioverter defibrillators: the Study on Omega-3 Fatty Acids and 
Ventricular Arrhythmia (SOFA) randomized trial.  

Effects of n-3 long-chain polyunsaturated fatty acids on depressed mood: systematic 
review of published trials.  

Visual acuity and cognitive outcomes at 4 years of age in a double-blind, randomized 
trial of long-chain polyunsaturated fatty acid-supplemented infant formula  
Early Hum Dev. 2007 May; 83(5):279-84. Epub 2007 Jan 18.
Omega-3 fatty acid treatment in 174 patients with mild to moderate Alzheimer disease: OmegAD study: a randomized double-blind trial.

26. Makrides M, Duley L, Olsen SF
Marine oil, and other prostaglandin precursor, supplementation for pregnancy uncomplicated by pre-eclampsia or intrauterine growth restriction

27. Knapp HR, Reilly IA, Alessandrini P, FitzGerald GA.
In vivo indexes of platelet and vascular function during fish-oil administration in patients with atherosclerosis.

Enzymes and receptors of prostaglandin pathways with arachidonic acid- vs. eicosapentaenoic acid-derived substrates and products.
J Biol Chem. 2007 May 22;

29. Skinner ER, Watt C, Besson JA, Best PV.
Differences in the fatty acid composition of the grey and white matter of different regions of the brains of patients with Alzheimer's disease and control subjects.
Brain. 1993 Jun;116 ( Pt 3):717-25

30. Moore SA, Yoder E, Murphy S, Dutton GR, Spector AA.
Astrocytes, not neurons, produce docosaheaxaenoic acid (22:6 omega-3) and arachidonic acid (20:4 omega-6).

31. Hogyes E, Nyakas C, Kiliaan A, Farkas T, Penke B, Luiten PG.
Neuroprotective effect of developmenta docosahexaenoic acid supplement against excitotoxic brain damage in infant rats.

32. Xiao Y, Wang L, Xu RJ, Chen ZY.
DHA depletion in rat brain is associated with impairment on spatial learning and memory.

33. Pratico D, MY Lee V, Trojanowski JQ, Rokach J, FitzGerald GA.
Increased F2-isoprostanes in Alzheimer's disease: evidence for enhanced lipid peroxidation in vivo.

34. Reich EE, Markesbery WR, Roberts LJ 2nd, Swift LL, Morrow JD, Montine TJ.
Brain regional quantification of F-ring and D-/E-ring isoprostanes and neuroprostanes in Alzheimer's disease.

35. Montine TJ, Beal MF, Cudkowicz ME, O'Donnell H, Margolin RA, McFarland L, Bachrach AF, Zackert WE, Roberts LJ, Morrow JD.
Increased CSF F2-isoprostane concentration in probable AD.


Footnotes;

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The abbreviations used are: arachidonic acid (AA), 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH), Alzheimer disease (AD), docosahexaenoic acid (DHA), electron capture (EC), gas chromatography (GC), isoprostanes (iPs), isofurans (iFs), liquid chromatography (LC), mass spectrometry (MS), negative ionization (NI), neurofurans (nFs), neuroprostanes (nPs), prostaglandin (PG), selective ion monitoring (SIM), selective reaction monitoring (SRM) solid phase extraction (SPE), trimethylsilyl (TMS).

Fig 1  Negative ion mass spectrum from an extract of mouse liver after CCl₄ infusion  The spectrum shown is an average of scans across the region of the chromatogram in which iPs (m/z 353), iFs (m/z 369), and nPs (m/z 377) eluted. The ion at m/z 393 is 24 Da higher than iFs and 16 Da higher than nPs.

Fig 2 A Selected ion monitoring (SIM) analysis of an extract of mouse liver after CCl₄ infusion A SIM analysis of m/z 393 reveals a series of incompletely resolved chromatographic peaks that elute slightly earlier than the iPs (m/z 353), nPs (m/z 377) and iFs (m/z 369) in a reverse phase LC gradient.

B GC/MS analysis of an extract of mouse liver after CCl₄ infusion  There are chromatographic peaks that elute slightly later than the iPs (m/z 569), iFs (m/z 585) and nPs (m/z 593) after PFB ester, TMS ether derivatization and SIM analysis by gas chromatography/electron capture/negative ionization mass spectrometry (GC/EC/NI/MS).

Fig 3 LC/MS comparision of in vivo and in vitro products SIM analysis at m/z 393 revealed similar profiles when the products of in vitro oxidation of DHA were compared with an extract of mouse liver after CCl₄ infusion.
Left: In vitro  Right: In vivo

Fig 4 GC/MS determination of hydroxyl groups No significant peaks are detectable in the products of in vitro oxidation of DHA at m/z 636 after formation of the PFB ester, TMS derivative. However,
following formation of the $[^2H_9]$TMS ether derivative, intense peaks appear at m/z 636 while no peaks are evident at m/z 609, indicating the presence of three hydroxyl groups.

**Fig 5** GC/MS determination of double bonds  Prior to hydrogenation, no significant peaks are present at m/z 617. However, following hydrogenation, intense peaks appear at m/z 617 while no peaks appear at m/z 609, indicating the presence of four double bonds.

**Fig 6** The cyclic peroxide cleavage pathway of nF formation

**Fig 7** The epoxide hydrolysis pathway of nF formation

**Fig 8** LC/MS/MS comparison of in vitro and in vivo products. Product ion scans of m/z 393 from in vitro DHA oxidation and from an extract of mouse liver after CCl4 infusion are strikingly similar, differing mainly in the relative abundance of the various product ions.

**Fig 9** Product ion formation from the proposed nF structures.

**Fig 10** LC/MS/MS analysis of the products of in vitro DHA oxidation  SRM analysis of some predicted transitions of m/z 393 from the in vitro oxidation of DHA is shown. Results from an extract of mouse liver after CCl4 infusion were similar.

**Fig 11** The time course of nF formation during in vitro oxidation of DHA with 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH). nFs were more abundant than the nPs at all time points and reached a maximum level of approximately 15 ng/µg DHA at 6 hours (SRM analysis of m/z 393 → 193 and 377 → 101)

**Fig 12** The dose response relationship between nF and nP formation and 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) concentration during in vitro oxidation of DHA. (SRM analysis of m/z 393 → 193 and 377 → 101)

**Fig 13** The formation of nFs in liver following intraperitoneal injection of CCl4 (4 g/kg). Time after injection is indicated on the x axis. (SRM analysis of m/z 393 → 193 and 377 → 101)

**Fig 14** Relative amounts of iPs, nPs and nFs in the brain cortex of Tg 2576 transgenic mice and non-transgenic littermates (controls). (SRM analysis of m/z 393 → 193 and 377 → 101)

A. All three classes of peroxidation compounds were significantly elevated in AD brain cortex  *P<0.05  **P<0.01

B. No significant difference was noted in levels of iPs, nPs and nFs in the cerebellum of Tg 2576 vs control mice.

**Fig 15** nF levels in p47 phox knockout mice. (SRM analysis of m/z 393 → 193 and 377 → 101) nF levels were depressed significantly in the brain cortex of p47phox knockout mice compared to wild type (WT) controls. *p<0.05 n=10
Figure 1
Figure 2A

Relevant Abundance

Time (min)

0 15 30

m/z 353.3

m/z 377.3

m/z 369.3

m/z 393.3
Figure 2B
Figure 3

**In vitro**

**In vivo**
Figure 4

$d_0$-TMS derivatization

$d_9$-TMS derivatization

m/z 609

m/z 636
Figure 5

Before hydrogenation

Relevant Abundance

$m/z$ 609

Time (min)

After hydrogenation

Relevant Abundance

$m/z$ 609

Time (min)

$m/z$ 617

Time (min)
Fig 6

Cyclic Peroxide Cleavage Pathway
Figure 8

In vivo

Relevant Abundance

100

50

0

m/z

123 137 167 193 213 225 313 331 349 375 393

In vitro

Relevant Abundance

100

50

0

m/z

123 137 167 193 213 225 313 331 349 375 393
### a. Main MS/MS fragments of neurofuranes

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<th>m/z</th>
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<sup>a</sup> C<sub>6</sub>H<sub>9</sub>O<sub>2</sub> [(113+H)-H]<sup>-</sup>

<sup>b</sup> C<sub>8</sub>H<sub>13</sub>O [(125-H)-H]<sup>-</sup>

<sup>c</sup> C<sub>8</sub>H<sub>11</sub>O<sub>2</sub> (IV), C<sub>9</sub>H<sub>15</sub>O (V) [(139-H)-H]<sup>-</sup>

<sup>d</sup> C<sub>11</sub>H<sub>17</sub> [(149+H)-H]<sup>-</sup>

<sup>e</sup> C<sub>9</sub>H<sub>13</sub>O<sub>2</sub> [(153-H)-H]<sup>-</sup>

<sup>f</sup> C<sub>11</sub>H<sub>17</sub> [(149+H)-H]<sup>-</sup>

<sup>g</sup> C<sub>12</sub>H<sub>19</sub>O [(179-H)-H]<sup>-</sup>

<sup>h</sup> C<sub>12</sub>H<sub>17</sub>O<sub>2</sub> [(193+H)-H]<sup>-</sup>

<sup>i</sup> C<sub>11</sub>H<sub>19</sub>O<sub>4</sub> [(215-H)-H]<sup>-</sup>

<sup>j</sup> C<sub>13</sub>H<sub>21</sub>O<sub>4</sub> [(225+H)-H]<sup>-</sup>

<sup>k</sup> C<sub>11</sub>H<sub>17</sub>O<sub>4</sub> [(245-H)-H]<sup>-</sup>

<sup>l</sup> C<sub>21</sub>H<sub>30</sub>O<sub>2</sub> [394-(CO<sub>2</sub>+2H<sub>2</sub>O+H)]<sup>-</sup>

<sup>m</sup> C<sub>21</sub>H<sub>32</sub>O<sub>3</sub> [394-(CO<sub>2</sub>+H<sub>2</sub>O+H)]<sup>-</sup>

<sup>n</sup> C<sub>21</sub>H<sub>34</sub>O<sub>4</sub> [394-(CO<sub>2</sub>+H)]<sup>-</sup>

<sup>o</sup> C<sub>22</sub>H<sub>32</sub>O<sub>5</sub> [394-(H<sub>2</sub>O+H)<sup>-</sup>]

*Fig 9*
b. MS/MS fragmentation sites of neurofurans

Main fragments of Neurofuranes

I-Both

I-Epox

II-Both

II-Epox

III-Both

III-Epox

IV-Both

IV-Epox

V-Both

V-Epox

VII-Both

VII-Epox

VIII-Both

VIII-Epox
Figure 10

Graphs showing the abundance of mz 393.3 at different time points:
- mz 393.3>113.1
- mz 393.3>123.1
- mz 393.3>187.2
- mz 393.3>193.3

Time (min)
Figure 11

[Graph showing the comparison of Neurofurans and Neuroprostanes over time (hours)]

- Neurofurans: Initially increase rapidly, then stabilize at around 20 ng/ug DHA from 10 hours onwards.
- Neuroprostanes: Show a slower increase, peaking at around 5 ng/ug DHA at 10 hours and then stabilizing.

Time (hours): 0 to 30
ng/ug DHA: 0 to 20

Legend:
- Green line with error bars: Neurofurans
- Blue line with error bars: Neuroprostanes
Figure 12
Fig 14

a

![Bar chart showing ng/g brain for iPs, nPs, and nFs. Red bars represent controls with n=11, and blue bars represent Tg 2576 with n=7.](chart-a.png)

b

![Bar chart showing ng/g brain for iPs, nPs, and nFs. Red bars represent controls with n=11, and blue bars represent Tg 2576 with n=7.](chart-b.png)
Fig 15
Neurofurans: Novel indices of oxidant stress derived from docosahexaenoic acid
Wen-Liang Song, John A. Lawson, Dermot Reilly, Joshua Rokach, Chih-Tsung Chang,
Benoit Giasson and Garret A. FitzGerald

J. Biol. Chem. published online October 6, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M706124200

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