Neurofurans, Novel Indices of Oxidant Stress Derived from Docosahexaenoic Acid

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Isoeicosanoids are free radical-catalyzed isomers of the enzymatic products of arachidonic acid. They are formed in situ in cell membranes, are cleaved, circulate, and are excreted in urine. Isoomers of prostaglandin F$_2$α, the F$_2$-isoprostanes, have emerged as sensitive indices of lipid peroxidation in vivo. Analogous compounds formed from docosahexaenoic acid (DHA) are termed neuroprostanes and are more abundant than isoprostanes (iPs) in brain. Isofurans 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 CCl$_4$ 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.

Isoprostanes (iPs)$^2$ are prostaglandin 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 PGF$_2$α, F$_2$-iPs, have been measured in urine as indices of in vivo lipid peroxidation (2). Compounds analogous to the F$_2$-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 CCl$_4$-treated Mice**

Treatment of mice with CCl$_4$ 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 injections of CCl$_4$ (4 g/kg of body weight). CCl$_4$ was mixed with canola oil (1:1 by volume). The mice were anesthetized with CO$_2$ prior to sacrifice at 0, 1, 2.5, 7.5, and 20 h after CCl$_4$ administration, and 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 (0.1–0.5 g) were suspended in 5 ml of ice-cold chloroform: methanol (2:1, v/v) with 0.005% (w/v) butylated hydroxytoluene to suppress oxidation. The samples were homogenized using a TissueLyzer™ (Qiagen). The lipid extracts were mixed vigorously with 2.0 ml NaCl (0.9%, w/v), and the phases were separated by centrifugation. After the upper phase was decanted, the 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 of 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 of methanol.
Oxidant Stress Products from DHA

The mixture was then sonicated, mixed vigorously until thoroughly suspended, and then heated at 37 °C for 30 min. The pH was adjusted to 3.0 with 1.2 ml of 1 n HCl. Next, 1.0 ng of $[^{2}H_{4}]8,12$-iso-iPF$_{2\alpha}$-VI was added as an internal standard. The samples were purified by solid phase extraction using StrataX cartridges (Phenomenex, Torrance, CA). The purified lipid extracts were analyzed by LC/MS/MS.

In Vitro Oxidation of DHA

DHA, obtained from Cayman Chemical Co. (Ann Arbor, MI) was dissolved in ethanol and added to 5 ml of phosphate-buffered saline, pH 7.4, to a final concentration of 20 mM. $2,2^{\prime}$-Azobis(2-amidinopropane) hydrochloride (AAPH; Sigma) was used as an oxidant as described (6). The reactions were allowed to proceed at 37 °C, and the aliquots were removed for analysis at 0, 2, 4, 8, 12, 24, and 30 h. The 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 $[^{2}H_{4}]8,12$-iso-iPF$_{2\alpha}$-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 solid phase extraction 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 pentfluorobenzyl ester, trimethylsilyl (TMS) ether derivative as described (6). $[^{2}H_{4}]$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 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 MS/MS in the negative ion mode. $[^{2}H_{4}]8,12$-iso-iPF$_{2\alpha}$-VI was used as an internal standard for quantitation.

Analysis of nFs in Vivo

*p47$^{phox}$ Knock-out Mice—Mice lacking the p47$^{phox}$ component of NADPH oxidase (kindly provided by Steven M. Holland, M.D., NIAID, National Institutes of Health) possess an impaired ability to generate superoxide (10). Ten knock-out mice (five male and five female) and ten wild type controls (five male and five 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. $[^{2}H_{4}]8,12$-iso-iPF$_{2\alpha}$-VI was added as an internal standard.

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. $[^{2}H_{4}]8,12$-iso-iPF$_{2\alpha}$-VI was added as an internal standard.

RESULTS

Discovery of the nFs—Purified lipid extracts from mouse liver were analyzed by LC/MS after treatment with CCl$_{4}$. During the analysis, four ions were detected near the region of the chromatogram in which the iPs eluted attracted attention. The ions were m/z 353 (F$_{2}$-iPs), m/z 369 (iFs), m/z 377 (nPs), and a peak at m/z 393 (Fig. 1). 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 (Fig. 2a). 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 pentafluorobenzyl 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) (Fig. 2b).

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
FIGURE 2. a, 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 pentafluorobenzyl ester, TMS ether derivatization, and SIM analysis by GC/EC/NI/MS.
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 (Fig. 3).

GC/EC/NI/MS analysis of the compounds as the $[2H_9]$TMS ether derivatives resulted in a mass increase of 27 Da while retaining a similar chromatographic pattern, indicating the presence of three hydroxyl groups (Fig. 4). 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 supplemental Fig. S1).

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 (Figs. 6 and 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 was 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 

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 h (Fig 11). This effect was dose-related, and a maximal response was attained at roughly 10 mM AAPH (Fig 12). However, unlike iFs, increasing oxygen tension did not detectably favor nF formation (6) (see supplemental Fig. S2).

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 1 h and to 1330.6 ng/mg tissue weight at 2.5 h after CCl₄ administration. Although 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 Tg 2576 Transgenic Mouse Model of AD and p47phox Knock-out Mice—Levels of iPs, nPs, and nFs were significantly (p < 0.05) elevated in Tg 2576 mouse brain cortex (7.1 versus 5.4 ng/g; 14.3 versus 10.5 ng/g, and 173.2 versus 109.1 ng/g tissue weight, respectively, compared with 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 knock-out mouse brain cortex from a mean of 156.2 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 forma-
tion of nFs than nPs in the brain cortex of Tg 2576 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 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 \( \omega-3 \) 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 \( \omega-6 \) analogs is unknown. Thus, on the one hand, \( \omega-3 \) polyunsaturated fatty acids 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 \( \sim 30\% \) 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 anti-inflammatory 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-containing compartments than in 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

**FIGURE 7.** The epoxide hydrolysis pathway of nF formation.

**FIGURE 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.
Main MS/MS fragments of neurofurans

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a. C₆H₉O₂ [(113+H)-H]⁻
b. C₈H₁₃O [(125-H)-H]⁻
c. C₈H₁₁O₂ (IV), C₉H₁₅O (V) [(139-H)-H]⁻
d. C₁₁H₁₇ [(149+H)-H]⁻
e. C₉H₁₃O₂ [(153-H)-H]⁻
f. C₁₀H₁₇O [(169-H)-H]⁻
g. C₁₁H₁₉O [(179-H)-H]⁻
h. C₁₂H₁₇O₂ [(193+H)-H]⁻
i. C₁₁H₁₉O [(215-H)-H]⁻
j. C₁₂H₂₁O [(225+H)-H]⁻
k. C₁₁H₂₃O [(245-H)-H]⁻
l. C₁₂H₂₃O₃ [(394-(CO₂+H₂O+H)]⁻
m. C₁₂H₂₃O₄ [(394-(CO₂+H₂O+H)]⁻
n. C₁₂H₂₃O₅ [(394-(H₂O+H)]⁻
o. C₁₂H₂₃O₆ [(394-(H₂O+H)]⁻

FIGURE 9. Product ion formation from the proposed nF structures.
Here, we report that the peroxidation products of DHA are more abundant in the brains of a mouse model of AD than those in their wild type controls.

In these studies, nFs were identified in vitro and in vivo using a variety of complementary chemical and MS approaches involving LC/electrospray/MS/MS and GC/EC/NI/MS. The likely mechanism of their formation is outlined in Figs. 6 and 7. Augmentation and depression of oxidant stress in vivo were achieved by administration of CCl₄ and deletion of p47^{phox}, respectively, and attendant alterations in nF generation were observed in liver. Consistent with the

FIGURE 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. The results from an extract of mouse liver after CCl₄ infusion were similar.

FIGURE 11. The time course of nF formation during in vitro oxidation of DHA with AAPH. nFs were more abundant than the nPs at all time points and reached a maximum level of ~15 ng/μg DHA at 6 h (SRM analysis of m/z 393 → 193 and 377 → 101).

FIGURE 12. The dose response relationship between nF and nP formation and AAPH concentration during in vitro oxidation of DHA (SRM analysis of m/z 393 → 193 and 377 → 101).

FIGURE 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).
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 that 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 the diagnosis, timing, and selection of dose in the treatment and chemoprevention of neurodegenerative disease.

Acknowledgments—We acknowledge the contributions of Julien Ferrari, Eileen Callaghan, and Emanuela Ricciotti to this manuscript.

REFERENCES

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doi: 10.1074/jbc.M706124200 originally published online October 6, 2007

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

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