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Neuronal Oxidative Injury and Biomarkers of Lipid Peroxidation

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Oxidative stress and lipid peroxidation

Oxidative stress is implicated as one of the major underlying mechanisms in variety of human diseases including cardiovascular and neurodegenerative diseases and even normal aging processes. Excess formation of free radicals may overwhelm the capacity of endogenous cellular antioxidant defense mechanisms, cause alterations in normal cell and organ physiology and even activate and/or accelerate disease processes. Increased generation of free radicals derived primarily from molecular oxygen can attack a variety of critical biological materials, including DNA, essential cellular proteins and lipids. The brain is especially susceptible to oxidative damage because of its great consumption of oxygen, glucose and energy, as well as relatively low levels of antioxidants (1,2). In addition, the high content of unsaturated lipids in the brain leads to pronounced lipid peroxidation, the central feature of oxidant injury in the brain.

Lipid peroxidation is the mechanism by which lipids are attacked by chemical species that have sufficient reactivity to abstract a hydrogen atom from a methylene carbon in their chain. Lipid peroxidation *in vivo*, through a free radical pathway, requires a polyunsaturated fatty acid (PUFA) and a reactant oxidant inducer that together form a free-radical intermediate. The free radical intermediate subsequently reacts with oxygen to generate a peroxy radical, which with unpaired electrons may additionally abstract a hydrogen atom from another PUFA. Greater the number of double bonds in the molecule and higher instability of hydrogen atom adjacent to the double bond explains why unsaturated lipids are particularly susceptible to peroxidation (3,4). Therefore, lipids are readily attacked by free radicals, resulting in the formation of a number of peroxidation products. F₂-isoprostanes (F₂-IsoPs) are one group of these compounds, which are derived by the free radical peroxidation of arachidonic acid (AA, C20:4, ω -6).

The measurement of the F₂-IsoPs, prostaglandin (PG)-like compounds is one of the most accurate approaches to measure oxidative injury *in vivo* (5). Initially, their formation involves the generation of four positional peroxy radical isomers of arachidonate, which

undergo endocyclization to PGG₂-like compounds. These intermediates are reduced to form four F₂-IsoP regioisomers, each of which can consist of eight racemic diastereomers (6). In contrast to cyclooxygenase (COX)-derived prostaglandins (PGs), non-enzymatic generation of F₂-IsoPs favors the formation of compounds in which the stereochemistry of the side chains has a *cis* orientation in relation to the prostane ring. A second important difference between F₂-IsoPs and PGs is that F₂-IsoPs are formed primarily *in situ*, esterified to phospholipids and subsequently released by a phospholipases (7,8), whereas PGs are generated only from free AA (6).

F₂-IsoPs analogs may be formed by peroxidation of other polyunsaturated fatty acid substrates, such as eicosapentaenoic acid (EPA, C20:5, ω -3) that lead to the production of F₃-IsoPs, and docosahexaenoic acid (DHA, C22:6, ω -3), which generates F₄-IsoPs. The latter compounds are also termed neuroprostanes (F₄-NeuroPs), due to the high levels of their precursor in brain (9). In contrast to AA, which is evenly distributed in all cell types in all tissues, DHA is highly concentrated in neuronal membranes (10). DHA is obtained mainly through dietary means as the human body can only minimally synthesize this fatty acid. DHA deficiency has been linked to slow mental development (11), while DHA supplementation has been linked to a variety of health benefits including decrease rates of neurodegenerative diseases (12). DHA can undergo oxidation both *in vitro* and *in vivo*, and increased units of unsaturation suggest its higher susceptibility to lipid peroxidation than AA. Thus, while the measurement of F₂-IsoPs provides an index of global oxidative damage in the brain, integrating data from both glial and neuronal cells, determination of F₄-NeuroPs permits the specific quantification of oxidative damage to neuronal membranes *in vivo*. In fact, to our knowledge, F₄-NeuroPs are the only quantitative *in vivo* marker of oxidative damage that is selective for neurons. This is particularly important because of the implication of oxidative damage and lipid peroxidation being causative factors in numerous neurodegenerative diseases (13,14).

Several methods have been developed to quantify the F₂-IsoPs from biological materials (15). Our laboratory uses a gas chromatography/ mass spectrometry (GC/MS) to quantify the F₂-IsoPs, methodology which was originally established at our University (by the pioneering work of Dr. Roberts and Dr. Morrow, Vanderbilt University Medical School) (6,7). More specifically, after isolation and derivatization of the F₂-IsoPs, we take advantage of stable isotope dilution, negative ion chemical ionization (NICI) GC/MS with select ion monitoring (SIM) for quantification. This methodology allows the lower limit of detection of the F₂-IsoPs to be in the low picogram range. These properties, along with the assay's high sensitivity and specificity, allow the F₂-IsoPs to be an excellent biomarkers of and the most robust and sensitive measure of oxidative stress *in vivo*. In addition, we will outline the difference in methodology for quantification of F₄-NeuroPs. Accordingly, we highlight these methods in the present review and address its advantages and shortcomings. Description on the methodologies for quantification of these biomarkers of oxidative injury is followed by concrete examples on measurements of F₂-IsoPs and F₄-NeuroPs in studies conducted by our laboratory.

2. Materials

1. Tissue samples, fresh or frozen. It is important to process tissue samples immediately after isolation or assure their immediate storage at -80°C for later quantification.
2. Blade homogenizer (e.g., Kinematica Polytron® PT 10–35; Brinkmann Instruments, Westbury, NY) and sonicator (e.g., Ultrasonic Bath, Fisher Scientific) are used for tissue processing.
3. Folch solution: 2:1 (v/v) chloroform/methanol, ice-cold, containing 0.005% (w/v) butylated hydroxytoluene (BHT; Sigma-Aldrich, cat. no. B1378). Free radical scavenging agent such as BHT is added to the organic solvent during extraction of phospholipids (18) to prevent oxidation and formation of F_2 -IsoPs.
4. Solution of NaCl (0.9%, w/v). Stored at room temperature.
5. Organic solvents including ethyl acetate, heptane, chloroform, ethanol, acetonitrile and methanol, with and without 0.005% (v/v) BHT. Solutions are prepared as volume/volume ration.
6. Fifteen percent potassium hydroxide solution (KOH, w/v) is used to release esterified isoprostanes.
7. One molar hydrochloric acid (1M HCl) and pH 3 water, adjusted by adding 1 N HCl is used to acidified the sample before solid-phase extraction (SPE) (see Note 3).
8. Deuterated standard, deuterium-labeled isoprostane, $[\text{}^2\text{H}_4]\text{15-F}_{2\text{t}}\text{-IsoP}$ (8-iso-PGF $_{2\alpha}$) (Cayman Chemical, Ann Arbor, MI, cat. no. 316351) (see Note 4).
9. Anhydrous Na_2SO_4 is used to dry ethyl acetate/heptane eluate from the C18 Sep-Pak (see Note 5).
10. Sep-Pak Plus C18 cartridge ((Waters Associates, Milford, MA, cat. no. WAT03657), Silica Sep-Pak cartridge (Waters, Milford, MA, cat. no. WAT036580) and 10-ml plastic syringe (Laboratory Supply; SMJ512878) are used for normal-phase solid-phase extractions (SPE).
11. Pentafluorobenzyl bromide (PFBB; Sigma-Aldrich, cat. no. 10105–2) and *N,N*-diisopropylethylamine (DIPE; Sigma-Aldrich, cat. no.D3887) are prepared as 10% solutions (v/v) in anhydrous acetonitrile.
12. Thin-layer chromatography (TLC) plates, 5×20 cm glass plates covered with a $250\text{ }\mu\text{m}$ layer of silica gel particles $60\text{ }\mu\text{A}$ in diameter (Partisil LK6D; Whatman, Maidstone, England, cat. no. WC486562IV) and TLC developing chamber (e.g., VWR)
13. TLC standard, prostaglandin $\text{F}_{2\alpha}$ (PGF $_{2\alpha}$) methyl ester is diluted in methanol (Cayman Chemical, Ann Arbor, MI, cat. no. 16011).

14. Ten percent phosphomolybdic acid in ethanol (Sigma Chemical Co., St. Louis, MO, cat. no. P4869) is used to visualize sample migration on TLC plates wormed with hot plate (e.g., Corning, cat. no. 6795–200).
15. Dimethylformamide (DMF, Sigma-Aldrich, cat. no. 6407) and undecane are stored over calcium hydride to prevent water accumulation (see Note 8).
16. Bis(trimethylsilyl)trifluoroacetamide (BSTFA, Supelco, cat. no. 33084) is stored at room temperature.
17. 15-ml polypropylene culture tube with cap, 20-ml scintillation vial, 5-ml glass Reacti-Vial with Teflon-lined cap (e.g., Supelco, cat. No.33299) and 1.5-ml microcentrifuge tube are used for sample processing/chemical reactions.
18. Nitrogen gas and methane is used for sample evaporations and mass spectrometry.
19. Temperature-controlled water bath, centrifuge, 95°C oven and hair dryer.
20. 15-m, 0.25-mm diameter, 0.25- μ m film thickness, DB1701 fused silica capillary GC column (Fisons, Folsum, CA) and Gas chromatography (GC)/mass spectroscopy (MS) system (e.g., Hewlett Packard 5982A interfaced with an IBM Pentium computer).

3. Methods

Measurement of F₂-IsoPs has revolutionized our ability to quantify oxidative injury *in vivo*. F₂-IsoPs are stable, robust molecules and are detectable not only in neuronal tissues but also in other tissues and biological fluids, such as plasma, urine, cerebrospinal fluid and bronchoalveolar lavage fluid. As F₂-IsoPs can be readily generated *ex vivo* in biological materials containing arachidonoyl-containing lipids, it is important to process the samples immediately after isolation or assure their immediate storage at –80 °C for later quantification. Formation of F₂-IsoPs does not occur if a free radical scavenging agent like BHT is added to the organic solvent during extraction of phospholipids (16) or if the samples are rapidly frozen in liquid nitrogen prior to placement at –80 °C.

3.1. Lipid extraction and hydrolysis of F₂-IsoPs-containing phospholipids in tissue samples

Formation of F₂-IsoPs occurs *in situ* in the phospholipid bilayer and then subsequently released in free form. This creates two forms of F₂-IsoPs, one that remains esterified in the membrane and a second that is hydrolyzed and released in free form. To quantify total F₂-IsoPs formation, both free and esterified F₂-isoPs are analyzed. It is necessary to extract the phospholipids from the tissue and release the F₂-isoPs from the phospholipids via base hydrolysis (Figure 1).

1. Fresh or frozen samples (0.05 –0.25 g) are added in ice-cold 5 ml of Folch solution (chloroform:methanol, 2:1, vol/vol) containing 0.005% BHT in a polypropylene culture tube with cap. The tissue is then homogenized with a blade homogenizer for approximately 30 sec. The second aliquot of ice-cold

Folch solution, added to a separate culture tube, is used to wash the blade homogenizer and to ensure that all sample tissue is recovered as tissue can adhere to or become lodged inside the blade of the homogenizer. The two aliquots are then combined, covered with a nitrogen blanket, and mixed every 10 min over 30 min at 25 °C to allow maximal extraction of lipids from homogenized tissue.

Note 1: Presence of BHT during extraction and hydrolysis is important in order to inhibit ex vivo formation of F₂-IsoPs; since polystyrene is not resistant to chloroform and its subsequent interference with the analytical procedures, it is recommended that lipid extraction be carried out in polypropylene tubes; Keep on ice.

2. The lipid extracts are mixed vigorously with 2.0 ml NaCl (0.9%, wt/vol), and the phases separated by centrifugation at $800 \times g$ for 10 min at 25 °C. After centrifugation, the upper aqueous layer is discarded and the lower organic layer is carefully separated from the intermediate semisolid proteinaceous layer. The organic layer is then evaporated to dryness under a stream of nitrogen.

Note 2: The organic layer and the proteinaceous layer can be readily separated by carefully pouring off the organic layer into a new culture tube. If the proteinaceous layer is small, because of the type and size of the tissue sample, it is often easier to remove the aqueous and proteinaceous layers simultaneously via suction. However, care must be taken not to compromise the organic phase if this approach is used.

3. Total lipids are dissolved in 0.5 ml methanol containing BHT (0.005%), stored at -80 °C or if further processed, 0.5 ml of aqueous KOH (15%) added to the residue, and thus lipid extracts are saponified to release esterified isoprostanes. The mixture is sonicated and mixed vigorously until thoroughly suspended and heated at 37 °C for 30 min to affect hydrolysis and release of the F₂-IsoPs. The mixture is then acidified to pH 3 with 1 M HCl (cca 1.2 ml) and diluted to a final volume of 10 ml with pH 3 water in preparation for purification of F₂-IsoPs with solid-phase extraction (SPE).

Note 3: It is important to dilute the methanol in this solution to 5% or less to ensure proper column extraction of free F₂-IsoPs in the subsequent purification procedure.

3.2. Sample purification for mass spectrometric analysis

1. Following acidification of the sample to pH 3 with 1 M HCl, 200 to 1000 pg of deuterated standard is added. The mixture is vortexed and F₂-IsoPs isolated using reversed-phase and normal-phase solid-phase extractions (SPE).

Note 4: The internal standard is a deuterium-labeled isoprostane, [²H₄]15-F_{2t}-IsoP (8-iso-PGF_{2a}). The amount of internal standard added depends on the levels of F₂-IsoPs in the sample as well as the sensitivity of the mass spectrometer. For low-level samples such as cerebrospinal fluid (CSF), less internal standard needs

to be added. Samples that consist of a particularly large amount of tissue will require more internal standard. This is because complex tissues such as brain, despite our best purification efforts, will still contain some unwanted compounds that may potentially have the same m/z (mass-to-charge) ratio as the internal standard when analyzed by GC/MS. Increasing the amount of internal standard to 1000 pg in these samples minimizes the variability in the internal standard ion channel due to contamination in the tissue sample.

2. A 10-ml plastic syringe is used to elute the sample and subsequent solvents through the Sep-Pak cartridge. For reverse phase, Sep Pak Plus C18 columns (each cartridge contains 500 mg of C18) are preconditioned with 5 ml methanol (flow rate, ~1.0 ml/min) and 7.0 ml H₂O (adjusted to pH 3.0 with 1.0N HCl). Once the sample has been added, the column is washed sequentially with 10 ml of water (pH 3) and 10 ml of heptane, which removes non-polar contaminants including un-oxidized AA. The F₂-IsoPs are eluted with 10 ml of ethyl acetate/heptane (50:50, vol/vol) into a 20-ml scintillation vial.
3. The ethyl acetate/heptane eluate from the C18 Sep-Pak is then dried over anhydrous Na₂SO₄ and applied to a silica Sep-Pak cartridge (each cartridge contains 500 mg of silica), which has been preconditioned with 5 ml of ethyl acetate. Once the sample has been added, the column is washed with 5 ml of ethyl acetate and the F₂-IsoPs are eluted with 5 ml of ethyl acetate/methanol (50:50, vol/vol) into a 5-ml glass react-a-vial (with Teflon-lined cap). For normal-phase SPE, Sep Pak Plus Silica columns are used with a flow rate of ~0.5 ml/min throughout.

Note 5: Drying of ethyl acetate/heptane eluate should be completed promptly, as Na₂SO₄ has been shown to adsorb lipids to some degree. Care must be taken not to transfer any Na₂SO₄ to the silica Sep-Pak cartridge.

3.3. Conversion of F₂-IsoPs to corresponding pentafluorobenzyl (PFB) esters

1. Isoprostanes isolated in ethyl acetate/methanol eluate by SPE are dried at 37 °C under a nitrogen stream, and derivatized to pentafluorobenzyl esters. Samples are vigorously mixed with 40 µl pentafluorobenzyl bromide: anhydrous acetonitrile (10:90, vol/vol) plus 20 µl diisopropylethylamine: anhydrous acetonitrile (10:90, vol/vol). Following reaction at 37 °C for 20 min, the esters are dried under a nitrogen stream and dissolved in 50 µl chloroform:methanol (2:3, vol/vol).

Note 6: Do not work outside of a well-ventilated hood because PFBB is a potent lachrymator.

3.4. Tin layer chromatography

1. Thin-layer chromatography (TLC) is accomplished with 5 cm × 20 cm glass plates covered with a 250 µm layer of silica gel particles 60 Å in diameter. Just before use, the plates are washed with ethyl acetate:ethanol (90:10, vol/vol), activated at 95 °C for 20 min, and cooled in a dessicator. A TLC chamber is lined

with filter paper and conditioned 30 min with 100 ml chloroform: ethanol (93:7, vol/vol).

2. Dissolved samples in chloroform: methanol (50 μ l) are applied to the upper half of pre-adsorbent in four pre-scored lanes, and dried 5–10 s with a hair dryer. Sample plates are added to both ends of the chamber. In contrast, TLC standard (5 μ g of the methyl ester of PGF_{2 α} /5 μ l CH₃OH) is applied to a separate plate that is positioned towards the center of the TLC chamber. After the chamber is rapidly closed, solvent is allowed to migrate 13 cm, and the plates removed.
3. Samples are scraped from silica plates in the region of the TLC standard and visualized by spraying with a 10% solution of phosphomolybdic acid in ethanol followed by heating. The areas 1 cm below and 1 cm above PGF_{2 α} (R_f ~0.15) are scraped and extracted from the silica with 1 ml of ethyl acetate.
4. Following centrifugation at $13,000 \times g$ for 3 min at 4 °C, isoprostane pentafluorobenzyl esters in the ethyl acetate are transferred into a virgin microcentrifuge tube and stored at –80 °C or samples further processed for the GC-MS analysis.

Note 7: Ethyl acetate should be carefully removed without disrupting the silica pellet in the bottom of the tube (silica may affect the instrument's sensitivity). Avoid applying samples to the first 1 cm of the plate and do not spray the sample plate.

3.5. Formation of trimethylsilyl ether derivatives and quantification of F₂-IsoPs

1. Once dried under a nitrogen stream, samples are dissolved in 8 μ l dimethylformamide (DMF) and mixed with 20 μ l bis (trimethylsilyl) trifluoroacetamide (BSTFA) to convert the residue to the trimethylsilyl ether derivatives.
2. After heating for 5.0 min at 37 °C, silylated samples are dried at 37 °C under a nitrogen stream, redissolved in 20 μ l of undecane, which has been dried over calcium hydride, and transferred into autosampler vial for GC-MS analysis.

Note 8: *DMF should be stored over calcium hydride to prevent water accumulation. Similarly to the amount of internal standard that is added to a sample, consideration should be given to the amount of undecane that is used to dissolve the derivatized sample. The amount added will depend on the levels of F₂-IsoPs. Samples that are rich in F₂-IsoPs will require greater amounts of undecane to keep them from overloading the column during GC. Likewise, low-level samples will require less undecane in order for the GC/MS signal to be of sufficient intensity for optimal quantification.*

3. For quantification of F₂-IsoPs, we routinely use a Hewlett Packard 5982A GC/MS system interfaced with an IBM Pentium computer. GC is performed using a 15-m, 0.25-mm diameter, 0.25- μ m film thickness, DB1701 fused silica capillary column. The column temperature is programmed from 190 to 290° at 20°/min. Methane is used as the carrier gas for NICI at a flow rate of 1 ml/min.

Ion source temperature is 250 °C, electron energy is 70 eV, and the filament current is 0.25 mA.

The major ions generated in the NICI mass spectra of the pentafluorobenzyl ester, tris-trimethylsilyl ether derivatives of F₂-IsoP are *m/z* 569 and corresponding ion for the [²H₄]15-F₂-IsoP internal standard, *m/z* 573 (Figure 2). For quantification purposes we compare the height of the pick containing derivatized F₂-IsoPs (*m/z* 569) with the height of the deuterated internal standard peak (*m/z* 573). Quantification of the F₂-IsoPs levels may be also achieved by comparing the areas of the appropriate peaks in the *m/z* 569 SIM chromatogram of the F₂-IsoPs to that of the peak of the internal standard in the *m/z* 573 SIM chromatogram (Figure 2).

The coefficient of variance for the assay is routinely less than 8%.

3.6. Simultaneous quantification of F₂-IsoPs and F₄-NeuroPs

1. When dealing with brain tissue, we often analyze our biological samples for lipid peroxidation products derived from both AA and DHA simultaneously. Similarity between two assays and the use of a common internal standard allows us to use a single sample to measure both families of compounds by splitting the sample before TLC step. In this case, sample is dissolved in twice the amount of chloroform: methanol (100 µl) after conversion to PFB esters. Then 50 µl are spotted on two separate lanes and the TLC plates are scraped accordingly, 1 cm above and below the methyl ster PGF_{2α} standard for the F₂-IsoPs and 1 cm below and 4 cm above the standard for the F₄-NeuroPs. This effectively splits a biological sample into two, allowing for individual analysis of both F₂-IsoPs and F₄-NeuroPs.
2. For quantification of F₄-NeuroPs we evaluate ions generated in the NICI mass spectra as *m/z* 594 and corresponding ion for the [²H₄]15-F₂-IsoP internal standard, *m/z* 573 (Figure 2). However, for quantification purposes we compare the area of the pick containing derivatized F₄-NeuroPs (*m/z* 593) with the area of the internal standard peak (*m/z* 573).

4. Timing and troubleshooting

In general, 12 samples can be assayed for F₂-IsoPs and/or F₄-NeuroPs in approximately 12 hrs by an experienced investigator. Homogenization, lipid extraction and hydrolysis of this number of samples requires ~ 3 hrs; Sep-Pac purifications takes ~ 2 hrs; drying, derivatization and TLC purification requires ~ 4 hrs; and drying and silylation requires ~ 3 hrs. Though compared to other assays of oxidative stress the time requirement for this assay is relatively large, it is noteworthy that the present assay has the greatest sensitivity and specificity for the detection of lipid peroxidation. Mass spectrometric analysis is automated and each sample requires ~ 10 min of instrument time. If the peak signal is low or if no peaks are detected by the mass spectrometer, the samples should be removed from the auto sampler vial, washed by ethyl acetate, dried under nitrogen and the conversion procedure to silylether derivative should be repeated. If the internal standard is detected at *m/z* 573, but

there is low or non-existent peak at 569 or 593, than levels of F₂-IsoPs or F₄-NeuroPs are below the limit of detection. The limit of detection for the assay is ~ 5 pg.

5. F₂-IsoPs or F₄-NeuroPs levels in neuronal tissues

We have explored cerebral oxidative damage in a several models of neurodegeneration including excitotoxicity generated by kainic acid (KA) (14, 17), neurotoxicity associated with anticholinesterase agents (18–20), metals (21,22) and innate immune activation by lipopolysaccharide (LPS) (23,24). For glial innate immune response we have used a single intracerebroventricular (ICV) injection of LPS, a major component of Gram negative bacterial cell walls. We have previously shown that LPS-activated glial innate immune response leads to indirect neuronal oxidative damage and synaptodendritic degeneration exclusively through a CD14-dependent mechanism free of behavioral or febrile response, and that LPS itself has no direct toxic effect on neurons (24,25). Control mice received ICV PBS, which led to no change in cerebral F₂-IsoPs over 24 h following ICV PBS vehicle; average (+S.D.) basal F₂-IsoPs = 3.2 + 0.3 ng/g. ICV LPS generated delayed cerebral oxidative damage with no significant increase in cerebral F₂-IsoPs even 10 h after injection. However, following this delay, cerebral oxidative damage peaked at 24 h after ICV LPS administration, returning to near baseline levels by 36 h (14).

In contrast to indirect neuronal damage induced with ICV LPS, there was a rapid onset of detectable oxidative damage upon ICV KA administration that was greatest at the earliest time point (30 min) following injection, returned to baseline by 60 min, and remained at baseline levels 24 h after injection (17). Kainate is a rigid analog of glutamate, and is a very potent stimulant of a subset of ligand-gated ion channels called KA receptors (14,26). While mice entered status epilepticus within a minute of ICV KA injection, KA-induced seizures did not lead to activated resident inflammatory cells in brain until several days (>4) after exposure, outside of the time frame used in our experiments. Thus, cerebral oxidative damage from these two toxins followed very different temporal profiles. KA led to the rapid onset of cerebral oxidative damage while LPS led to delayed oxidative damage. In both instances, the magnitude of cerebral oxidative damage achieved in these two models was equivalent and comparable to that observed in diseased regions of brain from patients with Alzheimer's disease (27).

Rat *in vivo* study with anticholinesterases showed that exposure to diisopropylphosphorofluoridate (DFP), a model compound for organophosphorus (OP) insecticides or nerve agents, induced significant increase in F₂-IsoPs (142%) and F₄-NeuroPs (225%) as early as 30 min after a single acute exposure to DFP (1.25 mg/kg, sc) (20) (Figure 3). Time-course analysis of these cerebral biomarkers of oxidative damage showed transient increase that reached maximum at the time of the most intensive seizure activity (i.e., 1 h after DFP) and returned toward basal levels by 6 h (Fig. 3). The selective increase in F₄-NeuroPs indicates that neurons are specifically targeted by this mechanism (Figure 3b). Thus, a one-time challenge with DFP produced a transient increase in both F₂-IsoPs and F₄-NeuroPs, which are sensitive and specific *in vivo* biomarkers of oxidative damage to AA and DHA, respectively.

We have also evaluated metal-induced alterations in biomarker of oxidative damage, F₂-IsoPs, in primary astrocytes and neuronal cultures, as well as the nervous system *in vivo*. Our data show that primary rat cortical neurons exposed to 1 mM of manganese for 2 hours showed structural damage to neurons and two-fold increase in F₂-IsoPs levels compared to controls (40.74 ± 5.4 pg/mg protein) (22). Following the same experimental condition, primary astrocytes cultures showed 233 % increase in F₂-IsoPs levels compared to control (137.3 pg/mg protein) (21). We have also investigated cerebral oxidative damage in a mouse model of Mn neurotoxicity. Results from this study corroborated *in vitro* findings and revealed that one-time challenge with Mn (100 mg/kg, ip) was sufficient to produce significant increase in mice cerebral biomarkers of oxidative damage, F₂-IsoPs (22). Our previous study also showed that *N,N*-diethyldithiocarbamate (DEDC) mediates lipid oxidation and elevation of total copper in peripheral nerve. F₂-isoprostane levels in rat sciatic nerve were significantly elevated in the 2-week DEDC-exposed group (1.9 ng/g of sciatic nerve) over controls (1.2 ng/g of sciatic nerve) (28). This relationship is consistent with copper-mediated oxidative stress contributing to the myelinopathy.

Combined, quantification of F₂-IsoPs and F₄-NeuroPs has extended our understanding the role of free radicals in physiological processes and has established the occurrence of oxidative stress in a wide variety of research models and disease states. The method is reproducible and reliable and serves as an excellent platform for future studies on the role of oxidative stress in mediating human disease. Furthermore, the sensitivity of the method offers unique potential for surveying F₂-IsoPs and F₄-NeuroPs concentrations as potential biomarkers of early disease, as they can be reliably and accurately measured in a plethora of biological media.

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Figure 1. Schematic representation of the procedure for the extraction, purification, derivatization and mass spectrometric analysis of F₂-IsoPs from neural tissues.

Figure 2. Chromatograms of F₂-IsoPs and F₄-NeuroPs from mouse cerebrum. Chromatograms plot abundance vs. time (min) with m/z 569 chromatogram showing F₂-IsoPs, m/z 593 chromatogram showing F₄-NeuroPs and m/z 573 chromatogram showing internal standard.

Figure 3. Cerebral concentrations of F₂-IsoPs (a) and F₄-NeuroPs (b) following DFP (1.25 mg/kg, s.c.) exposure in rats. Values represent mean±SEM (n=4–6). *One-way ANOVA had p<0.001 with Bonferroni's multiple comparison tests showed significant difference (p<0.05) for vehicle injected control vs. DFP treatment.