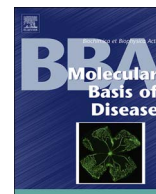




Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

Global assessment of oxidized free fatty acids in brain reveals an enzymatic predominance to oxidative signaling after trauma

Tamil S. Anthony^{a,b,c}, Elizabeth M. Kenny^{a,b,c}, Andrew A. Amoscato^d, Jesse Lewis^{a,b,c}, Patrick M. Kochanek^{a,b,e}, Valerian E. Kagan^{c,d}, Hülya Bayır^{a,b,c,d,e,*}^a Department of Critical Care Medicine, University of Pittsburgh, Pittsburgh, PA 15261, United States^b Safar Center for Resuscitation Research, University of Pittsburgh, Pittsburgh, PA 15224, United States^c Center for Free Radical and Antioxidant Health, University of Pittsburgh, Pittsburgh, PA 15219, United States^d Department of Environmental and Occupational Health, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA 15219, United States^e Children's Hospital of Pittsburgh of UPMC, University of Pittsburgh, Pittsburgh, PA 15224, United States

ARTICLE INFO

Keywords:

Lipid peroxidation

Docosanoids

Eicosanoids

Octadecanoids

Cardiolipin

Pro-resolving mediators

ABSTRACT

Traumatic brain injury (TBI) is a major health problem associated with significant morbidity and mortality. The pathophysiology of TBI is complex involving signaling through multiple cascades, including lipid peroxidation. Oxidized free fatty acids, a prominent product of lipid peroxidation, are potent cellular mediators involved in induction and resolution of inflammation and modulation of vasomotor tone. While previous studies have assessed lipid peroxidation after TBI, to our knowledge no studies have used a systematic approach to quantify the global oxidative changes in free fatty acids. In this study, we identified and quantified 244 free fatty acid oxidation products using a newly developed global liquid chromatography tandem-mass spectrometry (LC–MS/MS) method. This methodology was used to follow the time course of these lipid species in the contusional cortex of our pediatric rat model of TBI. We show that oxidation peaked at 1 h after controlled cortical impact and was progressively attenuated at 4 and 24 h time points. While enzymatic and non-enzymatic pathways were activated at 1 h post-TBI, enzymatic lipid peroxidation was the predominant mechanism with 15-lipoxygenase (LOX) contributing to the majority of total oxidized fatty acid content. Pro-inflammatory lipid mediators were significantly increased at 1 and 4 h after TBI with return to basal levels by 24 h. Anti-inflammatory lipid mediators remained significantly increased across all three time points, indicating an elevated and sustained anti-inflammatory response following TBI.

1. Introduction

Traumatic brain injury (TBI) is a common worldwide health problem associated with significant morbidity, mortality, and financial burden. TBI is a problem across all age and socioeconomic classes with peak incidence occurring in the 0–4, 5–19, and older than 75 years of age groups [1]. Like other traumatic injuries, TBI exhibits a male predilection across all ages [1]. While precise quantitation of TBI incidence poses several challenges, the Centers for Disease Control and Prevention estimates that in 2010, TBI accounted for approximately 2.5 million emergency department visits, 280,000 hospitalizations, and 52,000 deaths in the US [2]. The public health impact of TBI extends beyond acute injury, with estimates of 3.2 to 5.3 million people in the US living with TBI-related disability [3,4]. Long-term sequelae of TBI include cognitive, behavioral, emotional, motor, endocrine, and autonomic

dysfunction and deficits that result in significant functional limitation and reduced quality of life [5]. Furthermore, TBI increases long-term mortality and reduces life expectancy [5]. In addition to the morbidity and mortality associated with TBI, the conservative estimates of annual direct and indirect medical costs of TBI in the US approach \$76.5 billion [6,7]. Severe TBI resulting in hospitalization or death accounts for approximately 90% of total TBI-related medical spending [6,7].

TBI is a heterogeneous disease, but the pathophysiology consists of primary and secondary injury. Primary injury occurs at the time of trauma and is a result of mechanical forces including direct impact, penetration, rapid acceleration or deceleration, or blast waves [8]. Primary mechanical injury results in structural damage including axonal shearing, microhemorrhage, hematoma, and contusion formation [8,9]. Secondary injury is initiated at the time of trauma and progresses for hours to days post-injury expanding the volume of damage. Multiple

* Corresponding author at: Department of Critical Care Medicine, Department of Environmental and Occupational Health, Pediatric Critical Care Medicine, Center for Free Radical and Antioxidant Health, Safar Center for Resuscitation Research, University of Pittsburgh, United States.

E-mail address: bayirh@ccm.upmc.edu (H. Bayır).

<http://dx.doi.org/10.1016/j.bbadis.2017.03.015>

Received 19 December 2016; Received in revised form 15 March 2017; Accepted 23 March 2017

0925-4439/ © 2017 Elsevier B.V. All rights reserved.

molecular pathways are triggered by trauma and contribute to secondary brain injury including: neurotransmitter-mediated excitotoxicity, membrane permeability and electrolyte imbalance, calcium perturbations, free-radical generation, mitochondrial dysfunction, lipid peroxidation, inflammation, and cerebrovascular disturbances with secondary tissue hypoxia and blood brain barrier (BBB) dysfunction [8–10].

Lipid peroxidation is part of the normal signaling machinery for homeostatic central nervous system physiology. Oxidized free fatty acids (FFA) are important signaling molecules involved in numerous cellular responses: maintenance of low levels of endogenous FFA [11], stimulation and resolution of the inflammatory response [12], and mediation of the vascular response [13]. While lipid peroxidation has normal physiologic roles, the uncontrolled and excessive production of oxidized lipids plays a key role in secondary injury after TBI [14]. Unsaturated lipids such as polyunsaturated fatty acids (PUFAs) have a highly oxidizable structure [15]. PUFAs are particularly abundant in brain phospholipids and thus a target for lipid peroxidation following TBI, generating numerous oxidized FFA [16]. A growing body of evidence reports the accumulation of lipid peroxidation products following TBI that correlate with both injury severity and mortality in humans and also play a role in the pathophysiology of TBI [17,18].

Lipid peroxidation following TBI can occur via enzymatic and non-enzymatic mechanisms. A number of enzymes have been reported to function as peroxidases or oxygenases in the setting of TBI – most notably cyclooxygenases (COX), lipoxygenases (LOX), cytochrome (Cyt) P450, and cytochrome (Cyt) C [19]. Non-enzymatic peroxidation occurs largely through interactions of transition metals with oxygen and reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) [20]. In addition, TBI results in depletion of antioxidant defenses that protect cells from accumulation of toxic lipid peroxidation products [20].

While lipid peroxidation after TBI has been studied for decades [21,22] and therapeutically targeted in various clinical trials [23,24], precise identification and quantification of both the products and temporal course of lipid peroxidation post-injury are lacking. Though targeting lipid peroxidation in animal models of TBI has shown protective effects, inhibition of lipid peroxidation following TBI has proven unsuccessful in clinical trials. Moreover, recent studies have identified lipid oxidation products generated after TBI that exert protective effects on various neurological pathways. Together these data indicate a dichotomized role of lipid peroxidation in TBI pathology with generation of both neurotoxic and neuroprotective species. Identifying the various lipid peroxidation products generated after TBI and understanding their time course and role in TBI pathophysiology are likely crucial to develop therapies that inhibit the pathologic production and promote the protective production of oxidized lipid species.

In this study, 244 possible FFA oxidation products were identified and quantified using a newly developed global liquid chromatography tandem-mass spectrometry (LC-MS/MS) method. The temporal course of FFA oxidation following TBI was evaluated at 1, 4, and 24 h after injury. Here we show an immediate surge in FFA oxidation following TBI involving mostly enzymatic lipid peroxidation mechanisms. Accumulation of oxidized products peaked at 1 h after injury, with subsequent reduction over time. Our results indicate that pro-inflammatory lipid mediators are increased at 1 and 4 h after injury with return to basal levels by 24 h. In contrast, anti-inflammatory signaling remained elevated even at 24 h following TBI.

2. Materials and methods

2.1. Materials

All solvents were HPLC- or LC/MS-grade and purchased from Fisher Scientific (San Jose, CA). The following lipid standards were purchased from Cayman Chemicals (Ann Arbor, MI): 15-hydroperoxy-eicosatetraenoic (HpETE) acid, 13-hydroxyoctadecadienoic (HODE) acid, 9-

hydroperoxy-octadecadienoic (HpODE) acid, 5-HpETE, 12-hydroxyeicosatetraenoic (HETE) acid, 8,9-epoxyeicosatrienoic (EET) acid, 12-HpETE, 15-HETE, 10,13-dihydroxyoctadecadienoic (DiHODE) acid, 13-oxo-octadecadienoic (KODE) acid, 14,15-epoxy eicosatetraenoic (EpETE) acid, 13-HpODE, 13-epoxy octadecenoic (EpOME) acid, 13-HODE, 11,12-dihydroxyeicosatrienoic (DiHETRe) acid, docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), linoleic acid, arachidonic acid (AA), prostaglandin B2 (PGB2), PGF2, PGI2, leukotriene B4 (LTB4), PGD2, PGJ2, hepoxilin A3, 7,17-dihydroxydocosapentaenoic acid (DiHDDPA), resolvin D1, resolvin D2, resolvin D3, maresin, *epi* maresin, 17-hydroxydocosahexaenoic acid (HDHA), and 13,14-epoxydocosapentaenoic acid (EpDPA).

2.2. Controlled cortical impact model

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Male postnatal day (PND) 17 Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). An established controlled cortical impact (CCI) model of TBI was performed as previously described [25]. Briefly, anesthesia was induced with 3.5% isoflurane and maintained with 2% isoflurane with N_2O/O_2 (2:1) via nose cone. Rats were placed in a stereotaxic frame and temperature was allowed to reach $37 \pm 0.5^\circ C$. Temperature was maintained for 5 min before using a high-speed dental drill to remove the bone overlying the left parietal cortex. A vertically-directed CCI was delivered using a flat 6 mm pneumatically-driven impactor tip (4.0 ± 0.2 m/s, 50 ms dwell time, 2.5 mm depth). After injury, the bone flap was replaced and sealed with dental cement, and the scalp incision was closed. Anesthesia was discontinued and rats were monitored with supplemental O_2 for 1 h before returning to their cages. Rats were sacrificed at 1, 4, or 24 h post-injury ($n = 4$ /group for naïve and 1 h rats, $n = 3$ /group for 4 and 24 h rats). Brains were perfused with heparinized saline, the contusional cortex was dissected, and samples were snap-frozen in liquid nitrogen for lipid extraction.

2.3. Lipid extraction and mass spectrometry analysis

The contusional cortex (approximately 30% of the left cortex by weight) was dissected and subjected to lipid extraction. Lipid extraction was achieved using the Folch method [26]. Total phosphate content of the lipid extracts was quantified using a previously reported method [25]. Lipid extract (50 nmol of total phospholipid) was added to a glass tube with 50 pmol of deuterated-eicosatetraenoic acid internal standard ($C_{20:4} d_8$) and dried under N_2 flow. The dried film was dissolved in 50 μ l of 100% methanol, and 5 μ l of the reconstituted sample was injected into the LC-MS/MS system for FFA analysis. LC/MS analysis was performed using a Dionex UltiMate 3000 RSLCnano System coupled online to a Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA) using a reverse-phase C18 column (Luna 3 μ m, 100 \AA , 150×0.5 mm) (Phenomenex, Torrance, CA). A multi-step binary gradient with solvents A (methanol:water, 20:80 v/v containing 5 mM ammonium acetate) and B (methanol:water, 90:10 v/v containing 5 mM ammonium acetate) was used as follows: 0–15 min isocratic flow of 50% B at flow rate of 25 μ l/min, 15–35 min linear gradient from 50% to 65% B at 25 μ l/min, 40–55 min linear gradient from 65% to 80% B at 25 μ l/min, 55–57 min linear gradient from 85% to 95% B at 25 μ l/min, 57–65 min isocratic flow of 95% B at 25 μ l/min, 65–70 min linear gradient from 95% to 50% B at 25 μ l/min. The column was then re-equilibrated for 5 min with 50% B at 200 μ l/min. The mass spectra were acquired in a data-dependent acquisition with a negative-ion mode from 0 to 57 min. The spray voltage was set as 3.2 kV with a sheath gas flow rate of 8 units. The spectra were recorded at 140,000 full width at half maximum (FWHM) resolution between 290 and 600 m/z range. The top 5 abundant ions from the provided inclusion list were selected for fragmentation. To

fragment the species, higher energy collisional dissociation (HCD) fragmentation with 30 normalized collision energy (NCE) was used, and the ions were isolated at ± 1.5 m/z isolation window.

2.4. Data analysis

The mass spectrometry data was analyzed with SIEVE 2.1 software (Thermo Fischer Scientific, San Jose, CA) to identify oxidized and non-oxidized fatty acids using a database developed in-house. The extracted ion chromatogram for each m/z value corresponding to oxidized FFA was obtained, representing all the isobaric species for each m/z . Individual species were then identified manually using the following parameters: (i) signal-to-noise (S/N) ratio > 3 ; (ii) well-defined local maxima and minima; (iii) peak width > 0.5 min; (iv) peak presence in > 3 samples. The species were then quantified using the calibration values obtained from a closely related oxidized fatty acid. All values were compared to naïve using Student's *t*-test with statistical significance set at $p < 0.05$.

3. Results

Lipidomics profiling of oxidized fatty acids from biological tissues and fluids is challenging due to the low abundance and difficult separation of structurally similar species [27]. For this reason, lipidomics profiling is often carried out for a select number of species, known as a targeted approach [28,29]. While this targeted approach to LC/MS-MS is useful for identifying a subset of oxidized fatty acids, it fails to address the global spectrum of lipid changes including identification of all possible lipid species. As TBI results in peroxidation of numerous lipid species, we developed a global LC-MS/MS method to identify oxidized and non-oxidized FFA post-injury. As expected, many ions corresponding to the m/z of possible oxidized fatty acids were observed in the post-TBI samples, especially at 1 h after injury (Fig. 1a). The extracted ion chromatogram indicated that multiple potential oxidized FFA species were present for each m/z (Fig. 1b). The number of distinct lipid species observed for each m/z value corresponding to oxidized FFA is shown in Fig. 1c.

Among the 244 identified oxidized FFA, 25 octadecanoids (18-carbon length), 93 eicosanoids (20-carbon length), 96 docosanoids (22-carbon length), and 29 tetracosanoids (24-carbon length) were observed. As expected, $C_{22:6} + 2O$ (m/z 359.223) exhibited a high number of isobaric species with 21 individual species identified. HCD resulted in fragmentation and generation of the MS^2 spectra to further identify and characterize 40 species (Supplementary Fig. 1). An increase in non-oxidized FFA was also observed after TBI (Supplementary Fig. 2).

3.1. Octadecanoid production after TBI

Octadecanoids are the oxidized products of PUFAs containing 18 carbons such as linoleic ($C_{18:2}$) and α -linolenic ($C_{18:3}$) acids. Production of octadecanoids after TBI has been reported [30]. In this study, 25 octadecanoids were identified in the contusional cortex after TBI (Fig. 1c), and 13 were confirmed through fragmentation (Fig. 2, Supplementary Fig. 1). Sixteen octadecanoids were significantly increased at 1 h after TBI, including 9-HODE, 13-HODE, 9, 10-DiHODE, 9, 14-DiHODE, 12, 13-DiHODE, 8, 13-DiHODE, 9-HpODE, and 13-HpODE (Fig. 2a, d). The di-oxygenated species (9, 10-DiHODE, 12, 13-DiHODE, and 9-HpODE) exhibited the greatest increase relative to naïve with 11.2-, 12.8-, and 12.5-fold change, respectively (Fig. 2d). At 4 h post-injury, only 13-HODE, 12, 13-DiHODE, and 9-HpODE were significantly increased with 2.7-, 2.8-, and 1.6-fold change, respectively (Fig. 2b, d). No significant elevation in octadecanoids was observed at 24 h after TBI with respect to naïve (Fig. 2c, d). In terms of absolute quantitation, 13-HODE was the most increased octadecanoid (2.8 pmol per nmol of phospholipid) at 1 h after injury (Fig. 2e).

3.2. Eicosanoid production after TBI

Eicosanoids are one of the most studied and well-understood oxidized fatty acid products. Eicosanoids are derived from 20-carbon chain PUFAs such as eicosadienoic acid ($C_{20:2}$), eicosatrienoic acid ($C_{20:3}$), eicosatetraenoic acid ($C_{20:4}$), and eicosapentaenoic acid ($C_{20:5}$). As the result of increased COX and LOX activity, multiple eicosanoids are reported to increase after TBI [31–33]. This study identified 93 eicosanoids containing 1 to 4 oxygens (Fig. 3). Of the 93 identified eicosanoids, 89 species (96%) increased significantly at 1 h after TBI (Fig. 3a). Significant increases in 47 species were observed at 4 h post-injury, and 14 species at 24 h (Fig. 3b, c). Analysis of the fragmentation pattern identified 3 products oxidized by COX, 2 by Cyt P450, 16 by LOX, and 11-HETE produced by a non-enzymatic mechanism. All of these 22 eicosanoids confirmed by fragmentation were significantly increased immediately after TBI (1 h). With respect to naïve, 8,9-EET, 15-HETE, hepxilin A3 (HXA3), 5,12-DiHETE, 8,15-DiHETE, lipoxin A4, hepxilin B3, LTB4, and 15-HpETE were the most elevated species at 1 h. At 4 h after TBI, 20 of 22 identified eicosanoids remained significantly increased. The most elevated species at this time point included 12-HpETE, followed by LTB4 and HXA3. At 24 h after TBI, 9 eicosanoids remained significantly elevated, namely 8-HETrE, 12-HETrE, 8, 9-EET, 15-HETE, LTB4, HXB3, HXA3, 15-HpETE, and lipoxin A4 (Fig. 3d). While 15-HpETE exhibited the highest fold-change increase, its reduced product 15-HETE showed the highest quantitative increase at all three time points with respect to naïve (19, 3.5, and 1.1 pmol per nmol of phospholipid at 1, 4, and 24 h, respectively) (Fig. 3e). Non-enzymatic product, 11-HETE, was the next most elevated species with 2.2, 0.39, and 0.09 pmol per nmol of phospholipid observed at 1, 4, and 24 h, respectively.

3.3. Docosanoid production after TBI

Brain lipids are enriched in DHA, which are the primary source of docosanoid production [34]. In addition to DHA ($C_{22:6}$), other 22-carbon containing PUFAs such as DPA ($C_{22:5}$) also serve as docosanoid precursors. The evaluation of docosanoids in TBI is very limited. Our LC-MS/MS approach identified 96 possible oxidized docosanoids, containing 1 to 5 oxygens (Fig. 4). All 96 possible docosanoids were significantly increased at 1 h after TBI. Of the 96 species, 55 and 17 docosanoids remained significantly elevated at 4 and 24 h, respectively (Fig. 4a–c). Our analysis identified 8 specialized pro-resolving lipid mediators. All 8 mediators [14-HDHA, 17-HDHA, neuroprotectin D1, protectin DX1, resolvin D5, 17-hydroperoxy docosahexaenoic (HpDHA) acid, 14-HpDHA, and resolvin D1] were significantly increased at all three time points after TBI (Fig. 4d). Quantitatively, the mono-oxygenated DHA species (17-HDHA and 14-HDHA) exhibited the greatest increase at all three time points with 1.97, 0.31, and 0.10 pmol of 17-HDHA per nmol of phospholipid and 0.45, 0.10, and 0.03 pmol of 14-HDHA per nmol of phospholipid measured at 1, 4, and 24 h, respectively (Fig. 4e).

3.4. Changes in other oxidized fatty acids after TBI

Apart from the 18-, 20-, and 22-carbon containing fatty acids, brain tissue is enriched in ultra-long chain (ULC)-PUFAs with chains containing 24 or more carbons and more than 2 unsaturated carbon-carbon bonds [35,36]. We identified 29 species with the exact mass and expected retention time of these PUFAs. Interestingly, these species shared a similar temporal profile with the other identified oxidized fatty acids (Fig. 5). Twenty of the 29 species showed a significant increase at 1 h post-injury with respect to naïve (Fig. 5a, d). Significant elevation of 10 species was observed at 4 h after TBI (Fig. 5b, d). Only 1 lipid was significantly increased at 24 h (Fig. 5c, d). $C_{24:4} + 3O$ (m/z 407.280 at 41 min) was the only species that was significantly increased at all time points.

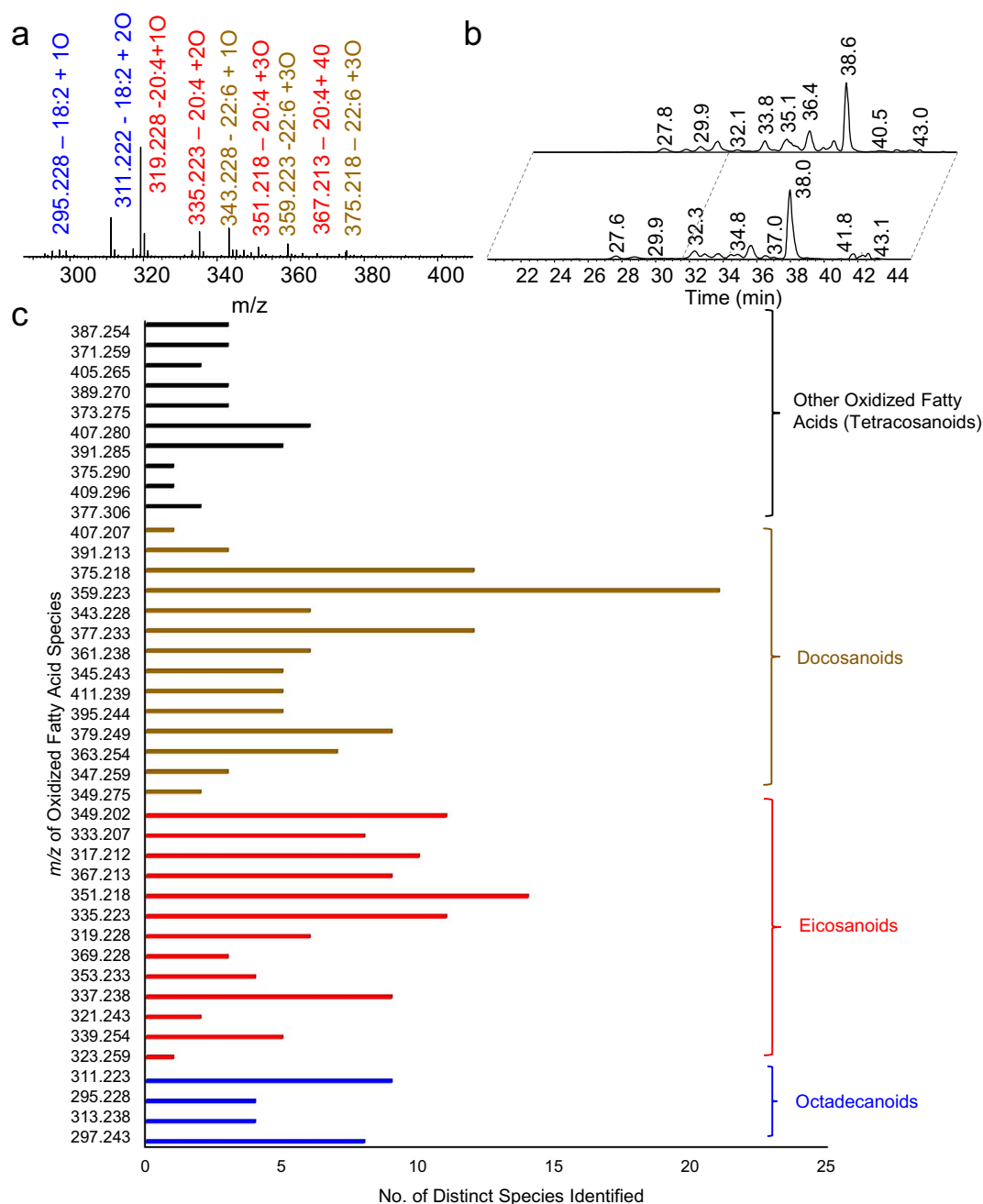


Fig. 1. A new global LC-MS/MS method for oxidized FFA analysis. (a) Representative spectrum showing oxidized FFA identified using the newly developed LC-MS/MS method. (b) Extracted ion chromatogram of m/z 359.22 (DHA + 2O) – top layer – and m/z 335.222 (arachidonic acid + 2O) – bottom layer – showing multiple lipid species. (c) Number of species identified for each m/z based on the exact mass and retention time. With this methodology, 244 distinct oxidized FFA were identified. The identity of 40 species was confirmed by MS^2 analysis.

3.5. Enzymatic and non-enzymatic production of oxidized fatty acids after TBI

After identifying the oxidized fatty acid changes following TBI, we sought to determine the contribution of enzymatic and non-enzymatic mechanisms to their production. Oxidized fatty acids were categorized as 5-LOX, 8-LOX, 12-LOX, 15-LOX, multiple LOX, COX, Cyt P450, or non-enzymatic lipid peroxidation products. The amount of oxidized products generated by 5-LOX, 8-LOX, 12-LOX, 15-LOX, multiple LOX, COX, Cyt P450, or non-enzymatic mechanisms was quantified (Fig. 6a). The quantity (pmol per nmol of phospholipid) of oxidized lipids generated by non-enzymatic mechanisms at 1 h (2.2 ± 0.9), 4 h (2.3 ± 0.3), and 24 h (2.2 ± 0.2) after TBI was similar to that of naïve (1.7 ± 0.4). Products of 5-LOX activity increased after TBI,

albeit only moderately with 2.6-, 1.3-, and 0.9-fold change at 1, 4, and 24 h after injury, respectively. At 1 h after injury, the greatest fold changes in observed oxidation products were generated from Cyt P450 (88-fold), 15-LOX (64-fold), and 8-LOX (51-fold) activity. These enzymatic products were the predominant species at 4 h as well, with 15-, 11-, and 12-fold changes in Cyt P450, 15-LOX, and 8-LOX products, respectively. At 24 h post-TBI, COX products exhibited the greatest fold change differences (3.6-fold), followed by 15-LOX (3.5-fold) and 8-LOX (3.4-fold). While Cyt P450 products showed the greatest fold change at 1 h post-TBI, the oxidation products generated by 15-LOX were the predominant species accounting for 70% of total oxidized fatty acids. Quantitatively, the next most abundant oxidized products came from 12-LOX (16%). Oxidized FFA products generated from other enzymes accounted for < 1% of the total content. At 4 h after TBI, 15-LOX

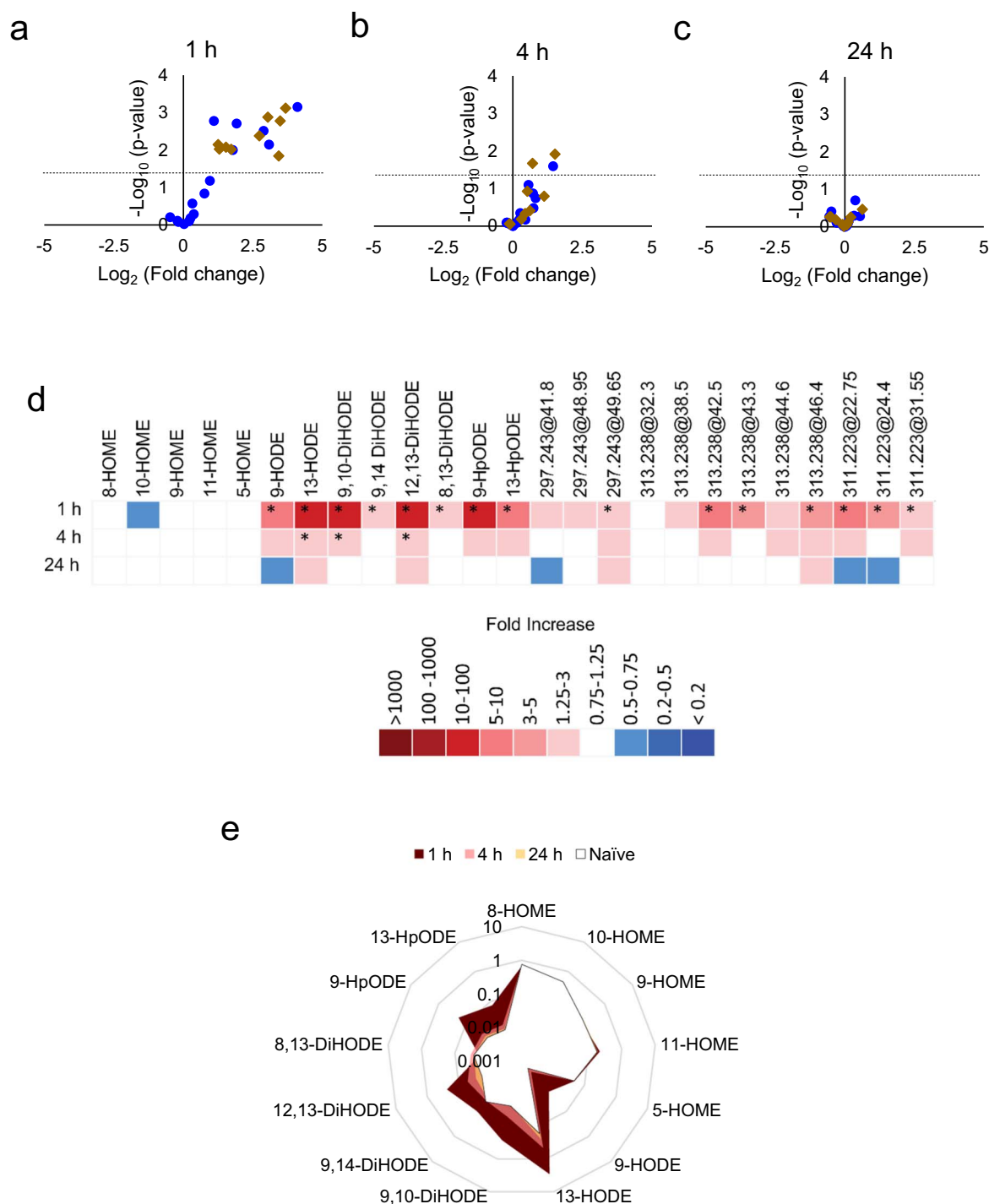


Fig. 2. Octadecanoid production and temporal profile after TBI. Volcano plots showing changes in octadecanoids at 1 h (a), 4 h (b), and 24 h (c) after TBI with respect to naïve. The species above the dotted line are significantly increased ($p < 0.05$). (d) Heat map showing fold increase of octadecanoids at 1, 4, and 24 h after TBI compared to naïve. Species confirmed with MS² analysis are listed by name, whereas species that did not undergo fragmentation are marked with m/z at the aligned retention time ($p < 0.05$). (e) Radar plot showing the quantitation of confirmed octadecanoids after TBI. The radial axis is shown in \log_{10} scale of pmol per nmol of phospholipid.

(51%), non-enzymatic (27%), 12-LOX (15%), and multiple LOX (4%) products accounted for the majority of identified oxidized FFA species. At 24 h following TBI, products generated by non-enzymatic reactions accounted for 44% of the total oxidized FFA content, while 15-LOX, 12-LOX, and multiple LOX contributed 36%, 13%, and 3%, respectively.

3.6. Pro- and anti-inflammatory oxidized FFA after TBI

As oxidized FFA have well known roles in mediation of

inflammatory response, we next investigated the overall changes in pro- and anti-inflammatory signaling by oxidized FFA. Based on the literature, 9-HODE, 9, 10-DiHODE, 9, 14-DiHODE, 12, 13-DiHODE, 8, 13-DiHODE, 9-HpODE, PGF2 α , 5-HETE, LTB4, PGH2, and PGD2 were grouped as pro-inflammatory mediators. The following oxidized products were grouped as anti-inflammatory mediators: 13-HODE, 13-HpODE, 15-HEDE, 8-HETRe, 12-HETRe, 14,15-EET, 8,9-EET, 8-HETE, 12-HETE, 11-HETE, 15-HETE, 8,15-DiHETE, 5,12-DiHETE, hepoxilin A3, hepoxilin B3, 12-HpETE, 15-HpETE, 14-HDHA, 17-HDHA, 4-

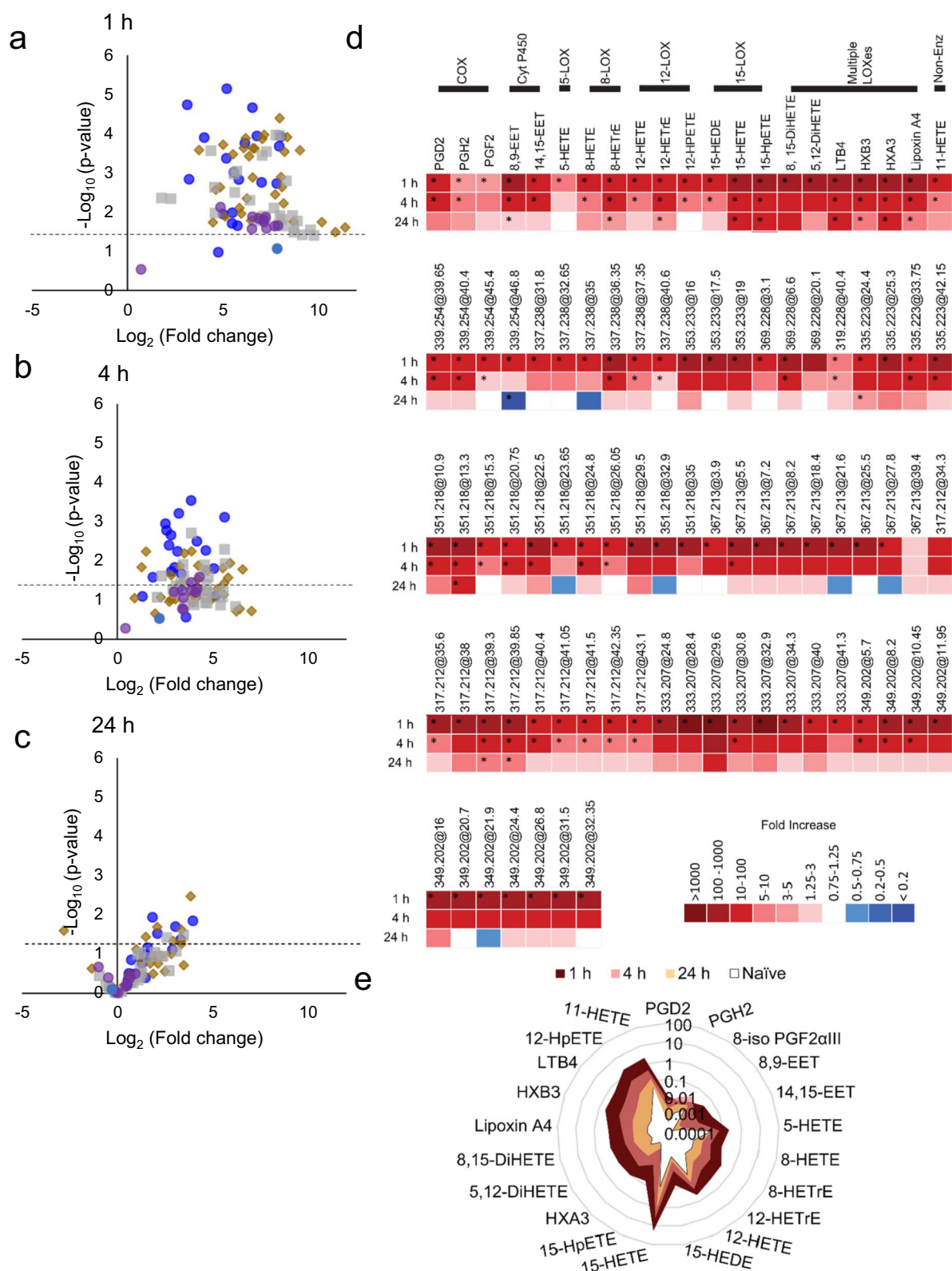


Fig. 3. Eicosanoid production and temporal profile after TBI. Volcano plots showing changes in eicosanoids at 1 h (a), 4 h (b), and 24 h (c) after TBI with respect to naïve. The species above the dotted line are significantly increased ($p < 0.05$). (d) Heat map showing fold increase of eicosanoids at 1, 4, and 24 h after TBI compared to naïve. Species confirmed with MS² analysis are listed by name, whereas species that did not undergo fragmentation are marked with m/z at the aligned retention time ($p < 0.05$). (e) Radar plot showing the quantitation of confirmed eicosanoids after TBI. The radial axis is shown in log₁₀ scale of pmol per nmol of phospholipid.

HDHA, resolvin D5, neuroprotectin DX1, 17-HpDHA, 14-HpDHA, and resolvin D1. Using these groupings, 0.22 pmol per nmol of phospholipid of the pro-inflammatory mediators and 0.43 pmol per nmol of

phospholipid of the anti-inflammatory mediators were detected in naïve animals (Fig. 6b). Pro- and anti-inflammatory oxidized FFA products increased to 1.8 and 29 pmol per nmol of phospholipid,

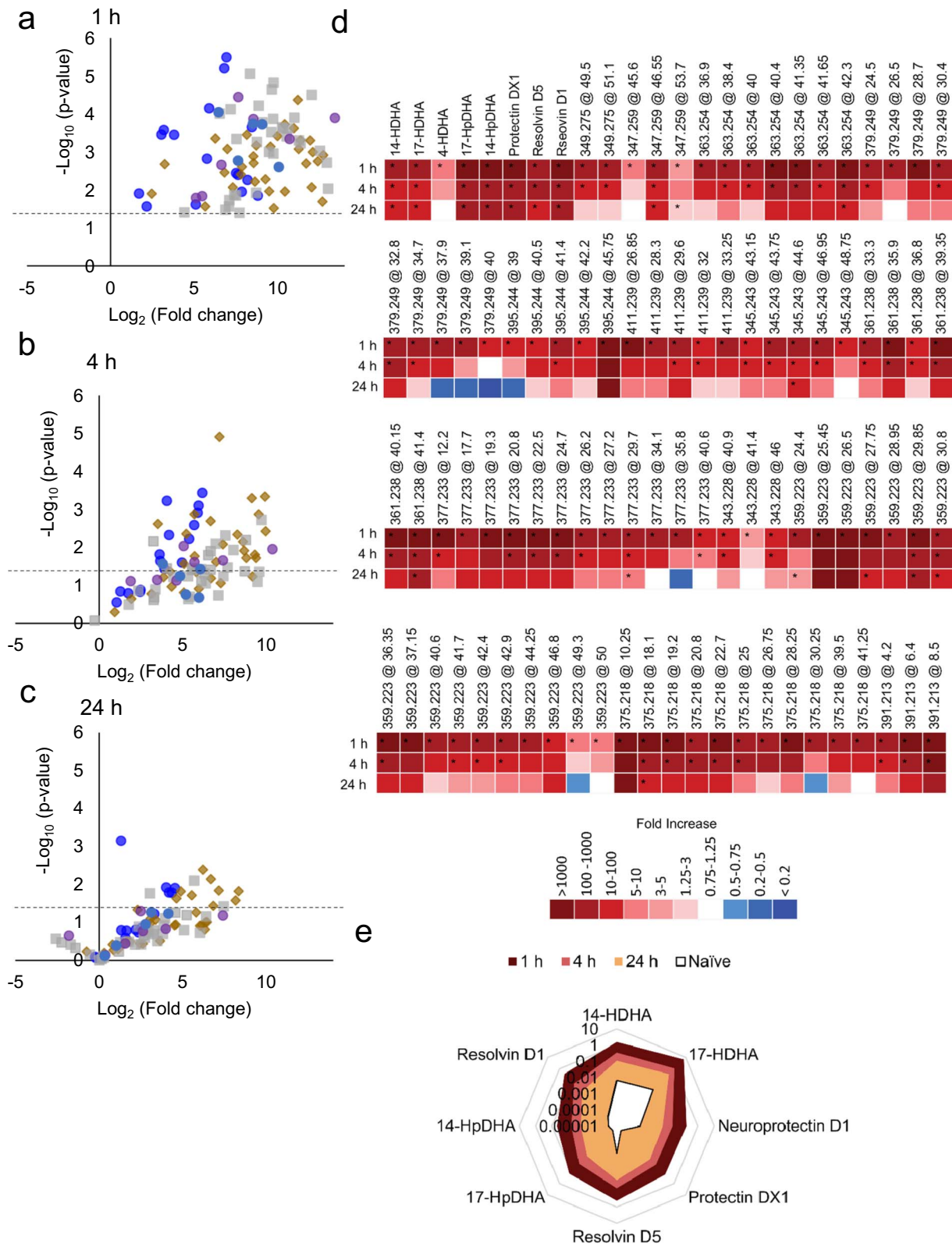


Fig. 4. Docosanoid production and temporal profile after TBI. Volcano plots showing changes in docosanoids at 1 h (a), 4 h (b), and 24 h (c) after TBI with respect to naïve. The species above the dotted line are significantly increased ($p < 0.05$). (d) Heat map showing fold increase of docosanoids at 1, 4, and 24 h after TBI compared to naïve. Species confirmed with MS² analysis are listed by name, whereas species that did not undergo fragmentation are marked with m/z at the aligned retention time ($p < 0.05$). (e) Radar plot showing the quantitation of confirmed docosanoids after TBI. The radial axis is shown in \log_{10} scale of pmol per nmol of phospholipid.

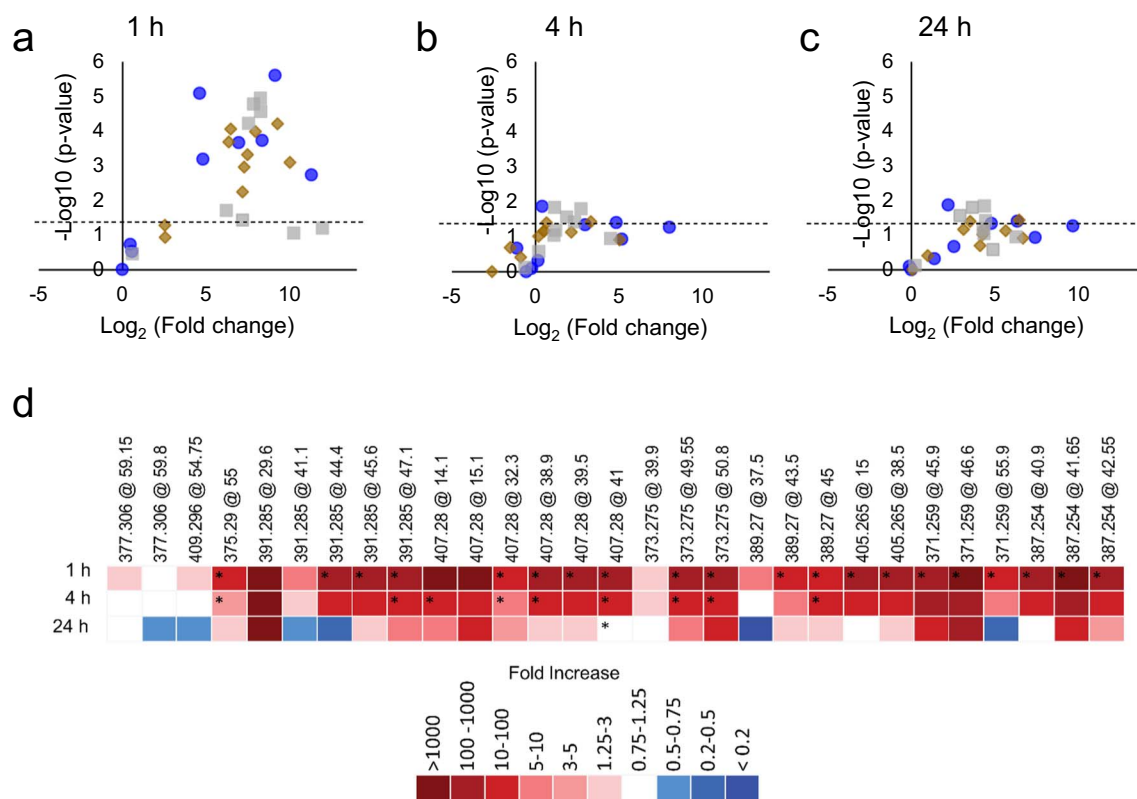


Fig. 5. Production and temporal course of other oxidized fatty acids after TBI. Volcano plots showing changes in other oxidized fatty acids at 1 h (a), 4 h (b), and 24 h (c) after TBI with respect to naïve. The species above the dotted line are the significantly increased ($p < 0.05$). (c) Heat map showing fold increase of other oxidized fatty acids at 1, 4, and 24 h after TBI compared to naïve. Individual species are marked with m/z at the aligned retention time ($p < 0.05$).

respectively, at 1 h after injury. By 4 h, the amounts of both the pro- and anti-inflammatory oxidized lipid products were decreased (0.5 and 5.3 pmol per nmol of phospholipid, respectively). Pro-inflammatory mediators returned to baseline levels (0.2 pmol per nmol of phospholipid) at 24 h after injury, while anti-inflammatory mediators remained elevated at this time point (1.6 pmol per nmol of phospholipid, 3.8-fold change vs naïve).

4. Discussion

Lipid peroxidation is a major component of secondary injury after TBI [10]. The role of lipid peroxidation in the pathophysiology of TBI stems from several lines of evidence: generation of lipid peroxidation products following injury [22], association of lipid peroxidation products with outcome [31], neuroprotection from TBI with chemical or genetic modification of the peroxidases, and success of pre-clinical

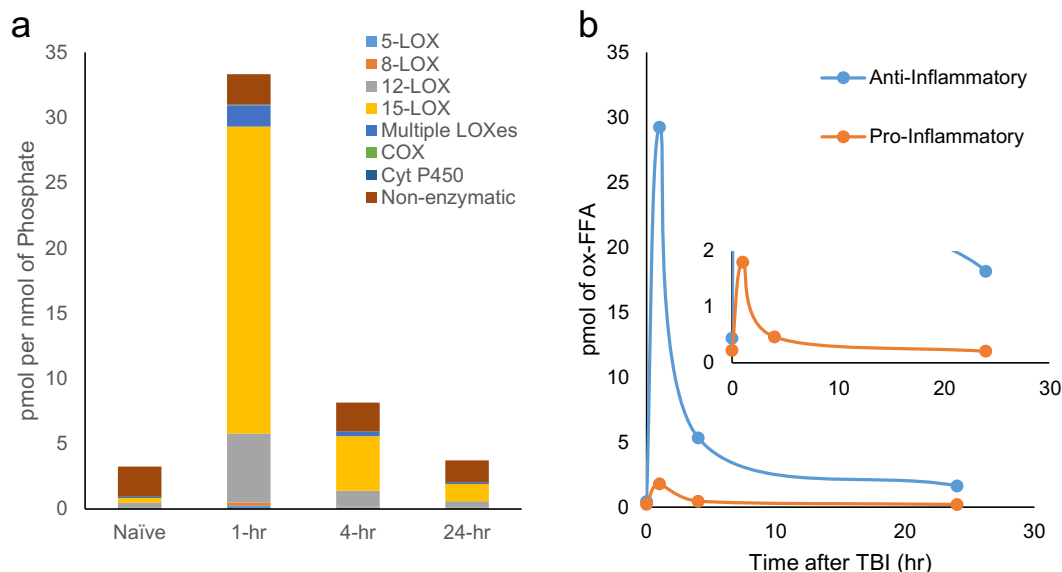


Fig. 6. Source and inflammatory signaling of oxidized fatty acids after TBI. (a) Bar graph showing the amount of oxidized fatty acids (pmol per nmol of phospholipid) generated from enzymatic (COX, LOX, Cyt P450) or non-enzymatic mechanisms. (b) Time course quantifying pro- and anti-inflammatory oxidized free fatty acids after TBI. Inset showing increased anti-inflammatory lipid mediators at 24 h compared to naïve with a return of pro-inflammatory lipid mediators to basal levels at this time point.

trials targeting lipid peroxidation in TBI. Despite strong evidence that lipid peroxidation is involved in the secondary injury of TBI, clinical trials have failed to provide significant benefit. Although many reasons have been suggested to explain the failure of clinical trials targeting TBI, for therapies targeting oxidative stress, our inadequate understanding of the sources of lipid peroxidation products and their normal signaling roles, and untargeted inhibition of lipid oxidation are likely important to those failures. Here we systematically studied the temporal course of lipid peroxidation after TBI by studying one of the prominent products – oxidized FFA. Our analysis identified several major features of FFA peroxidation following TBI. (i) FFA oxidation began immediately after injury. Oxidized FFA levels peaked at 1 h post-TBI with progressive attenuation of lipid peroxidation products at 4 and 24 h time points. (ii) After TBI, enzymatic lipid peroxidation predominated, contributing to the production of the majority of observed oxidized FFA products. (iii) FFA oxidation simultaneously produced both pro-inflammatory and anti-inflammatory signals. The majority of pro-inflammatory mediators were cleared at 4 h after TBI, whereas the levels of anti-inflammatory, pro-resolving mediators remained elevated up to 24 h. (iv) FFA oxidation after TBI generated products from very long chain fatty acids such as 24-carbon containing fatty acids. Further experimentation is needed to confirm and characterize such products and evaluate their physiologic effects.

4.1. Mechanisms of FFA oxidation after TBI

Two major pathways are implicated in the production of oxidized fatty acids after TBI: (1) calcium-dependent, and (2) mitochondrial-based, calcium-independent pathways (Fig. 7) [30]. The former involves the hydrolysis of PUFAs from phospholipid precursors via calcium-dependent phospholipase A2 (PLA2) [37] and subsequent peroxidation by COX [38], LOX [39], and Cyt P450 [40]. While the calcium-

dependent lipid peroxidation pathway uses fatty acids derived from all cellular and organellar membranes, the calcium-independent pathway takes place exclusively at the mitochondrial membrane. During this process the mitochondrial-specific phospholipid, cardiolipin (CL), forms a complex with Cyt C and is subsequently oxidized by the CL/Cyt C peroxidase complex utilizing H_2O_2 as an oxidizing equivalent [41,42]. The oxidized, esterified fatty acids within CL are then hydrolyzed by the calcium-independent lipid hydrolase enzyme, iPLA2 γ [30]. Brain CL exhibits diversity in fatty acyl chains unseen in other tissues. The highly unsaturated fatty acyl chains of brain CL thus can function as a rich source for the production of lipid mediators [30]. Although this analysis cannot distinguish between the lipid mediators produced by these two pathways, an increase in CL oxidation products similar to FFA oxidation was observed after TBI [30]. Moreover, the previously reported lipid mediators from CL peroxidation were also increased in this setting. These results are consistent with previous reports that both calcium-dependent and calcium-independent pathways occur simultaneously after TBI.

Whether generated through calcium-dependent or calcium-independent mechanisms, degradation of peroxy-lipids can happen spontaneously as a result of their highly unstable structure [43] or through lipid peroxide-reducing enzymes including the glutathione peroxidase family [44]. Because of the instability of peroxidized lipids, many previous studies regarding lipid peroxidation after TBI attempted instead to quantify the more stable breakdown products of this process [18,33]. Our study is one of the first attempts to systematically analyze and identify both immediate (peroxy-FFA) as well as end (hydroxy-FFA) products of lipid peroxidation following TBI. Here we show increases in the oxidized products of COX, LOX and Cyt P450 enzymes involved in the calcium-dependent pathway of lipid peroxidation after TBI, demonstrating the complex nature of this disease process.

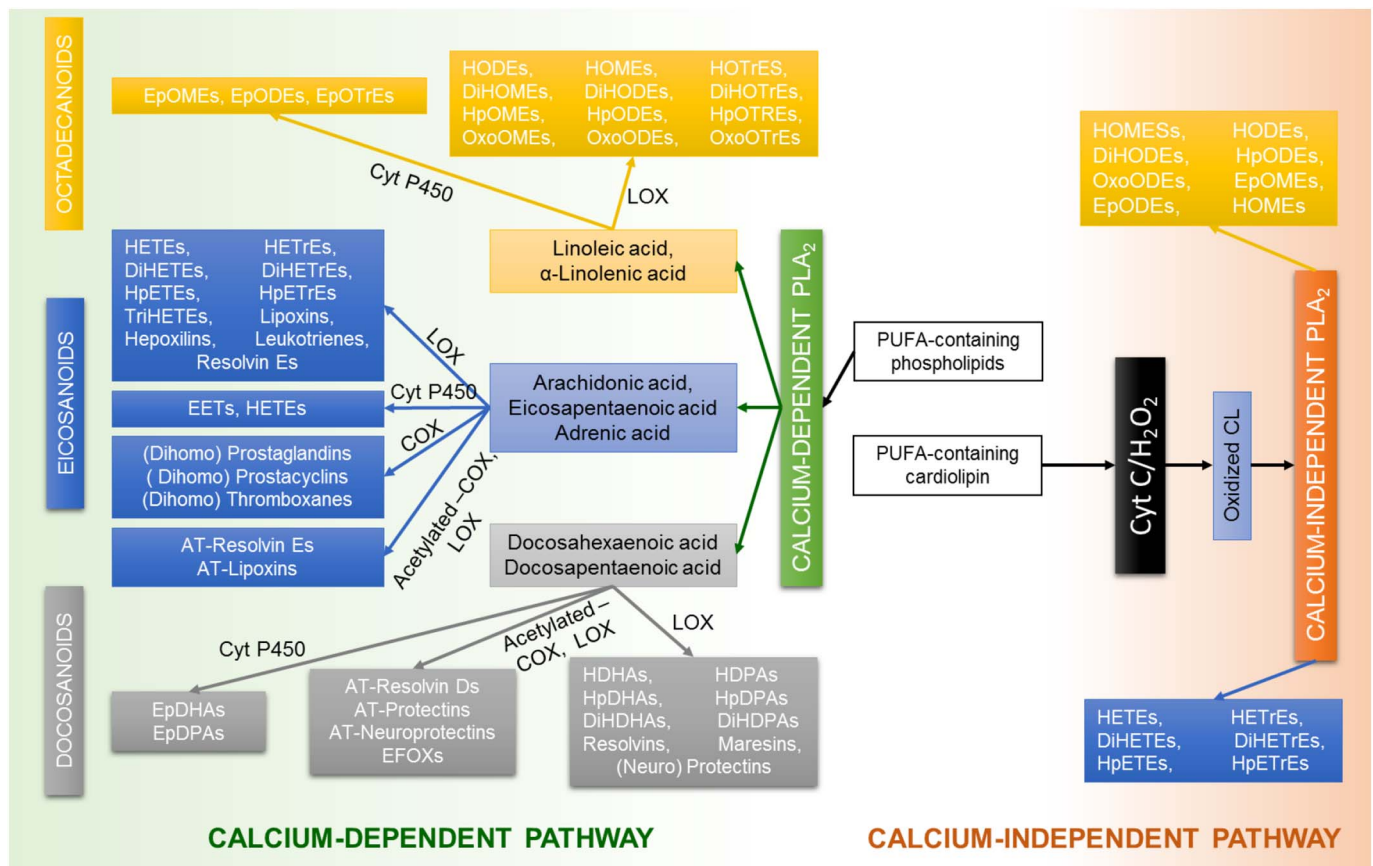


Fig. 7. Pathways involved in oxidized FFA production after TBI.

4.2. Oxidized fatty acids in TBI pathophysiology

Though oxidized fatty acids have purported roles in multiple physiological pathways, two signaling functions are most relevant to TBI pathophysiology: amplification and resolution of inflammation and regulation of cerebral blood flow (CBF). In TBI, development of the inflammatory response is very rapid, beginning with activation of resident microglia and polymorphonuclear leukocytes (PMN) [45]. Activated microglia are known to produce PGD2 [46] and LOX products in an iron-dependent manner [47]. In this study, we showed substantial FFA oxidation predominantly mediated by LOX at 1 h post-TBI. Because microglia and PMN are activated at this time point and have been shown to generate oxidized lipid mediators, it is possible that these cell types may be in part responsible for the profound oxidation and subsequent inflammatory response observed in our model. Pro-inflammatory cytokines such as IL-1, IL-6, GCSF, TNF- α , and IL-8 are upregulated immediately after injury [45]. Anti-inflammatory cytokines such as TGF- β and IL-10 are increased in cerebrospinal fluid in both animal models and patients with TBI [48]. In animal models of TBI, a biphasic course of TGF- β production has been reported. An initial peak of TGF- β was seen at 30 min post-injury and a second peak at 48 h time when pro-inflammatory cytokine signaling had resolved [49] [55]. After the initial cytokine response and microglia and PMN activation, TBI results in increased circulating neutrophils as early as 3.5 h after impact [50,51]. BBB damage after TBI allows peripheral neutrophils and monocytes to infiltrate the site of cerebral injury by approximately 24 h following injury [52]. Monocytes are known to produce active lipid mediators including specialized pro-resolving mediators (SPM) [53,54]. As we observed elevated SPM at 24 h after injury when monocytes are able to infiltrate the contusional area, it is possible that these cells play a role in the pro-resolving inflammatory signaling observed in our model.

In addition to their roles in inflammatory response oxidized FFA play important roles in vascular tone and blood flow [55]. CBF and CBF autoregulation are often impaired or lost following TBI [10,56]. CBF autoregulation is controlled by vasoactive mediators such as nitric oxide, adenosine, and oxidized FFA [55]. Multiple oxidized FFA including EETs, 13-HODE, 13-HpODE, and prostaglandins have been shown to stimulate vasodilation, while 20-HETE has reported vasoconstrictive effects [57–62]. EETs' vasodilatory actions are mediated through inhibition of K_{Ca} channels resulting in hyperpolarization of vascular smooth muscle (VSM) cells [58]. The COX products, PGI2 and PGE2, stimulate vasodilation through increased cAMP production with subsequent VSM relaxation [60,63]. The oxidized products of linoleic acid, 13-HODE and 13-HpODE, induce vasodilation through activation of the Na^+/K^+ -ATPase pump [61]. The vasoconstrictive actions of 20-HETE are mediated through inhibition of the K_{Ca} channel with subsequent VSM depolarization [57,62].

4.3. Octadecanoids after TBI

Different PUFAs present in brain phospholipids can be oxidized, generating thousands of structurally and stereochemically distinct species [23]. The rapid surge and subsequent elimination of a vast number of oxidized FFA seen after injury are indicative of the complex nature of TBI pathophysiology. Oxidized FFA are classified into three main groups based on the carbon chain length: octadecanoids (18 carbons), eicosanoids (20 carbons), and docosanoids (22 carbons) [55]. The lipid peroxidation enzymes generate oxidized products with differing affinity and reaction kinetics. Nevertheless, almost all of the enzymes involved in lipid peroxidation can generate any of the oxidized lipid products [56,57]. Octadecanoids are the oxidation products of 18-carbon containing fatty acids such as HODE, HOME, and HOTrE. While the function of many of the octadecanoids are still unclear, the signaling roles of 13-HODE and 9-HODE have been better elucidated. Both 13-HODE and 9-HODE are reported to increase apoptosis by binding

PPAR γ receptor, testicular orphan receptor 4 (TR4), or capsaicin receptor TRPV1 [64,65]. 13-HODE, is reported to function as an anti-inflammatory signal whereas structurally similar 9-HODE, produced by the same system (12/15-LOX), acts as a pro-inflammatory mediator [66]. Recently, the ratio of 13-HODE to 9-HODE was identified as a biomarker for determining immune status during active influenza infection, where lower values indicated an active pro-inflammatory process [67]. In this study we identified significantly increased pro-inflammatory octadecanoids as early as 1 h after TBI, indicating an early onset of inflammation in this disease process.

4.4. Eicosanoids after TBI

Eicosanoids are the oxidized products of arachidonic acid and related PUFAs. Eicosanoids are involved in a number of processes: vasodilation and vascular permeability, regulation of cytokine expression, hyperalgesia, fever, adipogenesis, mast cell maturation, neuroprotection, eosinophil recruitment and allergic response, smooth muscle contraction, platelet aggregation, and neutrophil recruitment [12]. Additionally, eicosanoids serve as the precursor of anti-inflammatory lipoxin production. Lipoxins and docosanoids such as resolvins and protectins are collectively known as SPM and represent a form of eicosanoid class-switching that signals as a stop message for the acute inflammatory response. SPM act differently than other anti-inflammatory mediators by preventing further neutrophil infiltration [39]. Of the many possible pro-inflammatory lipid mediators [67], this study identified significantly increased production of PGD2, LTB4, and 5-HETE as early as 1 h after TBI. Of the many eicosanoid-derived anti-inflammatory mediators, our study showed significant increases in 8-HETrE, 12-HETrE, 15-HETE, and HXB3 at 1 h, 4 h and 24 h post-TBI. This indicates that anti-inflammatory signaling is sustained for a longer duration than pro-inflammatory signaling. Similarly, vasodilatory eicosanoids such as 8,9-EET and 14,15-EET peaked at 1 h after injury and remained elevated at 24 h indicating an immediate and prolonged vasodilatory response following TBI.

4.5. Docosanoids after TBI

DHA, along with other omega-3 fatty acids, has been used in clinical trials evaluating protective therapies for TBI [68]. Though there is evidence for the involvement of DHA in ion channel and receptor modulation, the most important neuroprotective function of DHA stems from its role as a precursor for the production of lipid mediators. DHA serves as a precursor for the production of resolvins, protectins [69], and maresins [70] which contribute significantly to the resolution of inflammation. Resolvins are trihydroxy omega-3 fatty acids which can be further classified as resolvin E (EPA-derived) or D (DHA-derived) based on the precursor fatty acid [54]. Protectins are dihydroxy omega-3 fatty acids with a similar classification schema as resolvins. While resolvins and protectins are produced by the action of two LOX enzymes, another class of similar molecules known as aspirin-triggered (AT)-resolvins and -protectins are generated by acetylated-COX2 and acetylated-LOX enzymes [71]. In the central nervous system, docosanoids were first identified in a model of ischemic stroke. In this study, neuroprotection D1 (NPD1) was identified at 1 h and peaked at 8 h after injury [72,73]. Studies in HN cells (primary co-culture of human neurons and glia) suggest that the neuroprotective effects of NPD1 in stroke occur through inhibition of glutamate-induced cytotoxicity by regulation of critical cell-survival proteins, such as Bcl-2 [72]. To our knowledge, our study is the first report of the production of 4 SPM (neuroprotectin D1, protectin DX1, resolvin D5, and resolvin D1) and the production of 4 SPM intermediates (14-HDHA, 17-HDHA, 17-HpDHA, and 14-HpDHA) in TBI. Consistent with their anti-inflammatory and pro-resolving roles, these SPM remained elevated up to 24 h after injury.

4.6. Other considerations of oxidized FFA in TBI

Quantitation of various lipid peroxidation products and analysis of their time course can provide insight to the regulation of both peroxidase enzymes and inflammatory cascades. The greatest quantitative increases in octadecanoids, eicosanoids, and docosanoids were seen in 13-HODE, 15-HETE, and 17-HDHA, respectively. Interestingly, all of these oxidized fatty acid metabolites are end products of 15-LOX activity. This indicates that 15-LOX is the most active lipid peroxidation enzyme after TBI. This is consistent with previous reports demonstrating a 4-fold increase in 15-LOX gene expression after TBI [74]. Analysis of lipid mediators with pro- and anti-inflammatory functions exhibited an interesting pattern. Oxidized FFA identification at 1 h post-TBI showed increases in pro-inflammatory lipids, anti-inflammatory lipids, and lipids with unknown roles in inflammatory response. At 4 h after TBI, many of the identified oxidized lipids decreased significantly from the 1 h time point; however, many known pro-inflammatory and anti-inflammatory signals remained significantly elevated at 4 h. By 24 h after injury, almost all pro-inflammatory signals were cleared from the contusional cortex, while the anti-inflammatory and resolution signals remained significantly elevated. This pattern may reflect a progressive change in the contusional cortex from pro- to anti-inflammatory signaling.

We utilized a developmental model of TBI for our studies. Due to the increased susceptibility of the immature brain to oxidative injury [20], the changes in FFA oxidation seen in our pediatric CCI model may have been magnified compared to adult brain. The immature brain is more susceptible to oxidative stress after TBI for several reasons: greater fatty acid content, relatively higher oxygen utilization [75], and compromised oxidative defense mechanisms relative to the adult brain [20]. Enzymes involved in oxidative defense mechanisms such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) have lower expression in the immature brain compared to that of adult [76,77]. While both of these enzymes are involved in hydrogen peroxide removal, the latter can also reduce lipid peroxides. Though FFA oxidation may not be as extensive in the adult brain, our newly developed methods offer an obvious advantage in identifying, tracking, and quantifying lipid peroxidation products to better understand their signaling functions after TBI. Moreover, as TBI is a major cause of death and disability in the pediatric population, these results are relevant to the development of therapies specific for the immature brain.

The low stability of hydroperoxy-lipids formed as the primary products of peroxidation leads to the formation of numerous electrophiles including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) [78]. MDA levels are reported to increase by 1 h to 2 days [79], whereas 4-HNE levels increase by 3 h with a peak by day 2 after injury [80–82]. Although these species are more associated with free radical-mediated non-enzymatic lipid peroxidation, it is also possible that the oxidized lipids identified in this study can be degraded to form such products.

There are a few limitations in our study. Although secondary injury after TBI can continue for weeks following injury, our study is limited to the changes in FFA and their oxidized products within the first 24 h post-CCI. While our study is designed to assess immediate oxidative changes in FFA, we cannot make conclusions about the lipid peroxidation process beyond this time frame. It is possible that a second wave of FFA oxidation could occur in a more delayed fashion. We did not include sham-operated animals in the study design since previous studies have shown that craniotomy performed by a dental drill can induce a mild TBI with local inflammatory response [83]. It is possible that anesthesia and craniotomy could also contribute to the severe CCI-induced lipid peroxidation response we observed. Future studies that include both sham-operated animals as well as naïve controls could more specifically assign the observed lipid peroxidation response to severe TBI.

In conclusion, TBI results in lipid peroxidation that not only

correlates with patient morbidity and mortality, but contributes to the pathophysiology of secondary injury. Oxidized FFA are an important component of lipid peroxidation following TBI, but the existing literature uses a targeted approach to lipidomics that fails to address changes in the global lipidome. Here we employed a novel global LC-MS/MS methodology for oxidized FFA analysis that resulted in the identification of 244 potential oxidized products. Furthermore, we evaluated the temporal profile of oxidized FFA changes at 1, 4, and 24 h post-injury, revealing an interesting pattern in oxidative changes. Immediate injury resulted in increases across all classes of oxidized lipid products. At 4 h after TBI, both pro- and anti-inflammatory lipid mediators were significantly elevated. By 24 h, the oxidized lipid profile shifted towards a predominant anti-inflammatory response. Lipid signaling in the setting of TBI is a complex process, but understanding the global lipid changes is key to developing targeted neuroprotective therapies.

Transparency document

The <http://dx.doi.org/10.1016/j.bbadis.2017.03.015> associated with this article can be found, in online version.

Acknowledgement

The authors acknowledge Henry Alexander for technical assistance in performing CCI experiments. The work is supported, in part, by grants from the NIH (NS061817, U19AI068021, NS076511 and NS084604).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbadis.2017.03.015>.

References

- [1] M. Faul, L. Xu, M.M. Wald, V. Coronado, N.C.o.I.P.a.C. Centers for Disease Control and Prevention (Ed.), *Traumatic Brain Injury in the United States: Emergency Department Visits, Hospitalizations and Deaths, 2002–2006*, 2010 (Atlanta, GA).
- [2] C.f.D.C.a. Prevention, N.C.f.I.P.a.C.D.o.U.I. Prevention (Ed.), *Report to Congress on Traumatic Brain Injury in the United States: Epidemiology and Rehabilitation*, 2015 (Atlanta, GA).
- [3] D.J. Thurman, C. Alverson, K.A. Dunn, J. Guerrero, J.E. Snizek, *Traumatic brain injury in the United States: a public health perspective*, *J. Head Trauma Rehabil.* 14 (1999) 602–615.
- [4] E. Zaloshnja, T. Miller, J.A. Langlois, A.W. Selassie, *Prevalence of long-term disability from traumatic brain injury in the civilian population of the United States, 2005*, *J. Head Trauma Rehabil.* 23 (2008) 394–400.
- [5] B.E. Masel, D.S. DeWitt, *Traumatic brain injury: a disease process, not an event*, *J. Neurotrauma* 27 (2010) 1529–1540.
- [6] E. Finkelstein, P.S. Corso, T.R. Miller, *The Incidence and Economic Burden of Injuries in the United States*, Oxford University Press, USA, 2006.
- [7] V.G. Coronado, L.C. McGuire, M. Faul, D.E. Sugerman, W.S. Pearson, *Traumatic brain injury epidemiology and public health issues*, *Brain Injury Medicine: Principles and Practice*, 84 2012.
- [8] A.I. Maas, N. Stocchetti, R. Bullock, *Moderate and severe traumatic brain injury in adults*, *Lancet Neurol.* 7 (2008) 728–741.
- [9] Y. Xiong, A. Mahmood, M. Chopp, *Animal models of traumatic brain injury*, *Nat. Rev. Neurosci.* 14 (2013) 128–142.
- [10] C. Werner, K. Engelhard, *Pathophysiology of traumatic brain injury*, *Br. J. Anaesth.* 99 (2007) 4–9.
- [11] G. Pérez-Chacón, A.M. Astudillo, D. Balgoma, M.A. Balboa, J. Balsinde, *Control of free arachidonic acid levels by phospholipases A2 and lysophospholipid acyltransferases*, *Biochim. Biophys. Acta (BBA) – Mol. Cell Biol. Lipids* 1791 (2009) 1103–1113.
- [12] E.A. Dennis, P.C. Norris, *Eicosanoid storm in infection and inflammation*, *Nat. Rev. Immunol.* 15 (2015) 511–523.
- [13] C.D. Funk, *Prostaglandins and leukotrienes: advances in eicosanoid biology*, *Science* 294 (2001) 1871–1875.
- [14] E. Niki, *Lipid peroxidation: physiological levels and dual biological effects*, *Free Radic. Biol. Med.* 47 (2009) 469–484.
- [15] E. Niki, Y. Yoshida, Y. Saito, N. Noguchi, *Lipid peroxidation: mechanisms, inhibition, and biological effects*, *Biochem. Biophys. Res. Commun.* 338 (2005) 668–676.
- [16] R.P. Bazinet, S. Layé, *Polyunsaturated fatty acids and their metabolites in brain function and disease*, *Nat. Rev. Neurosci.* 15 (2014) 771–785.
- [17] K.I. Williams, G.A. Higgs, *Eicosanoids and inflammation*, *J. Pathol.* 156 (1988)

- 101–110.
- [18] H.A. Kasprzak, A. Wozniak, G. Drewa, B. Wozniak, Enhanced lipid peroxidation processes in patients after brain contusion, *J. Neurotrauma* 18 (2001) 793–797.
 - [19] T.S. Anthonyamuthu, E.M. Kenny, H. Bayir, Therapies targeting lipid peroxidation in traumatic brain injury, *Brain Res.* 1640 (2016) 57–76.
 - [20] H. Bayir, P.M. Kochanek, V.E. Kagan, Oxidative stress in immature brain after traumatic brain injury, *Dev. Neurosci.* 28 (2006) 420–431.
 - [21] P.H. Chan, R.A. Fishman, Transient formation of superoxide radicals in polyunsaturated fatty acid-induced brain swelling, *J. Neurochem.* 35 (1980) 1004–1007.
 - [22] H. Bayir, D.W. Marion, A.M. Puccio, S.R. Wisniewski, K.L. Janesko, R.S. Clark, P.M. Kochanek, Marked gender effect on lipid peroxidation after severe traumatic brain injury in adult patients, *J. Neurotrauma* 21 (2004) 1–8.
 - [23] J.P. Muizelaar, A. Marmarou, H.F. Young, S.C. Choi, A. Wolf, R.L. Schneider, H.A. Kontos, Improving the outcome of severe head injury with the oxygen radical scavenger polyethylene glycol-conjugated superoxide dismutase: a phase II trial, *J. Neurosurg.* 78 (1993) 375–382.
 - [24] R.K. Narayan, M.E. Michel, B. Ansell, A. Baethmann, A. Biegon, M.B. Bracken, M.R. Bullock, S.C. Choi, G.L. Clifton, C.F. Contant, Clinical trials in head injury, *J. Neurotrauma* 19 (2002) 503–557.
 - [25] H. Bayir, V.A. Tyurin, Y.Y. Tyurina, R. Viner, V. Ritov, A.A. Amoscato, Q. Zhao, X.J. Zhang, K.L. Janesko-Feldman, H. Alexander, L.V. Basova, R.S. Clark, P.M. Kochanek, V.E. Kagan, Selective early cardioplin peroxidation after traumatic brain injury: an oxidative lipidomics analysis, *Ann. Neurol.* 62 (2007) 154–169.
 - [26] J. Folch, M. Lees, G. Sloane-Stanley, A simple method for the isolation and purification of total lipids from animal tissues, *J. Biol. Chem.* 226 (1957) 497–509.
 - [27] H. Yue, S.A. Jansen, K.I. Strauss, M.R. Borenstein, M.F. Barbe, L.J. Rossi, E. Murphy, A liquid chromatography/mass spectrometric method for simultaneous analysis of arachidonic acid and its endogenous eicosanoid metabolites prostaglandins, dihydroxyeicosatrienoic acids, hydroxyeicosatetraenoic acids, and epoxyeicosatrienoic acids in rat brain tissue, *J. Pharm. Biomed. Anal.* 43 (2007) 1122–1134.
 - [28] D.S. Dumlao, M.W. Buczynski, P.C. Norris, R. Harkewicz, E.A. Dennis, High-throughput lipidomic analysis of fatty acid derived eicosanoids and N-acylethanolamines, *Biochim. Biophys. Acta (BBA)-Mol. Cell Biol. Lipids* 1811 (2011) 724–736.
 - [29] G. Dasilva, M. Pazos, J.M. Gallardo, I. Rodríguez, R. Cela, I. Medina, Lipidomic analysis of polyunsaturated fatty acids and their oxygenated metabolites in plasma by solid-phase extraction followed by LC-MS, *Anal. Bioanal. Chem.* 406 (2014) 2827–2839.
 - [30] Y.Y. Tyurina, S.M. Poloyac, V.A. Tyurin, A.A. Kapralov, J. Jiang, T.S. Anthonyamuthu, V.I. Kapralova, A.S. Vikulina, M.Y. Jung, M.W. Epperly, D. Mohammadyani, J. Klein-Seetharaman, T.C. Jackson, P.M. Kochanek, B.R. Pitt, J.S. Greenberger, Y.A. Vladimirov, H. Bayir, V.E. Kagan, A mitochondrial pathway for biosynthesis of lipid mediators, *Nat. Chem.* 6 (2014) 542–552.
 - [31] S.E. Farias, K.A. Heidenreich, M.V. Wohlauser, R.C. Murphy, E.E. Moore, Lipid mediators in cerebral spinal fluid of traumatic brain injured patients, *J. Trauma Acute Care Surg.* 71 (2011) 1211–1218.
 - [32] R.W. Hickey, P.D. Adelson, M.J. Johnnides, D.S. Davis, Z. Yu, M.E. Rose, Y.-F. Chang, S.H. Graham, Cyclooxygenase-2 activity following traumatic brain injury in the developing rat, *Pediatr. Res.* 62 (2007) 271–276.
 - [33] E.F. Ellis, R.J. Police, L.Y. Rice, M. Grabeel, S. Holt, Increased plasma PGE₂, 6-keto-PGF_{1α}, and 12-HETE levels following experimental concussive brain injury, *J. Neurotrauma* 6 (1989) 31–37.
 - [34] S.C. Dyall, Long-chain omega-3 fatty acids and the brain: a review of the independent and shared effects of EPA, DPA and DHA, *Front. Aging Neurosci.* 7 (2015) 52.
 - [35] T. Sassa, A. Kihara, Metabolism of very long-chain fatty acids: genes and pathophysiology, *Biomol. Ther.* 22 (2014) 83–92.
 - [36] B.S. Robinson, D.W. Johnson, A. Poulos, Unique molecular species of phosphatidylcholine containing very-long-chain (C24-C38) polyenoic fatty acids in rat brain, *Biochem. J.* 265 (1990) 763–767.
 - [37] A.A. Farooqui, L.A. Horrocks, Phospholipase A₂-generated lipid mediators in the brain: the good, the bad, and the ugly, *Neuroscientist* 12 (2006) 245–260.
 - [38] W.L. Smith, D.L. DeWitt, R.M. Garavito, Cyclooxygenases: structural, cellular, and molecular biology, *Annu. Rev. Biochem.* 69 (2000) 145–182.
 - [39] C.W. Parker, Lipid mediators produced through the lipoxygenase pathway, *Annu. Rev. Immunol.* 5 (1987) 65–84.
 - [40] P. Ortiz de Montellano, J. Voss, Substrate oxidation by cytochrome P450 enzymes, *Cytochrome P450* (2005) 183–245.
 - [41] V.E. Kagan, V.A. Tyurin, J. Jiang, Y.Y. Tyurina, V.B. Ritov, A.A. Amoscato, A.N. Osipov, N.A. Belikova, A.A. Kapralov, V. Kini, I.I. Vlasova, Cytochrome c acts as a cardioplin oxygenase required for release of proapoptotic factors, *Nat. Chem. Biol.* 1 (4) (2005) 223–232.
 - [42] J. Ji, A.E. Kline, A. Amoscato, A.K. Samhan-Arias, L.J. Sparvero, V.A. Tyurin, Y.Y. Tyurina, B. Fink, M.D. Manole, A.M. Puccio, Lipidomics identifies cardioplin oxidation as a mitochondrial target for redox therapy of brain injury, *Nat. Neurosci.* 15 (2012) 1407–1413.
 - [43] B. Halliwell, S. Chirico, Lipid peroxidation: its mechanism, measurement, and significance, *Am. J. Clin. Nutr.* 57 (1993) 715S–724S.
 - [44] H. Imai, Y. Nakagawa, Biological significance of phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells, *Free Radic. Biol. Med.* 34 (2003) 145–169.
 - [45] J.M. Ziebell, M.C. Morganti-Kossmann, Involvement of pro-and anti-inflammatory cytokines and chemokines in the pathophysiology of traumatic brain injury, *Neurotherapeutics* 7 (2010) 22–30.
 - [46] R.J. Hariri, J.B. Ghajar, K.B. Pomerantz, D.P. Hajjar, R.F. Giannuzzi, E. Tomich, D.W. Andrews, R.H. Patterson Jr., Human glial cell production of lipoxygenase-generated eicosanoids: a potential role in the pathophysiology of vascular changes following traumatic brain injury, *J. Trauma* 29 (1989) 1203–1210.
 - [47] T. Yoshida, M. Tanaka, A. Sotomatsu, S. Hirai, K. Okamoto, Activated microglia cause iron-dependent lipid peroxidation in the presence of ferritin, *Neuroreport* 9 (1998) 1929–1933.
 - [48] E. Csuka, M.C. Morganti-Kossmann, P.M. Lenzlinger, H. Joller, O. Trentz, T. Kossmann, IL-10 levels in cerebrospinal fluid and serum of patients with severe traumatic brain injury: relationship to IL-6, TNF-α, TGF-β1 and blood-brain barrier function, *J. Neuroimmunol.* 101 (1999) 211–221.
 - [49] A.-C. Rimioli, D. Lekiéffre, A. Serrano, A. Masson, J. Benavides, F. Zavala, Biphasic transforming growth factor-β[alpha] production flanking the pro-inflammatory cytokine response in cerebral trauma, *Neuroreport* 7 (1995) 133–136.
 - [50] A. Subramanian, D. Agrawal, R.M. Pandey, M. Nimiya, V. Albert, The leukocyte count, immature granulocyte count and immediate outcome in head injury patients, *Brain Injury—Pathogenesis, Monitoring, Recovery and Management*, InTech, Rijeka, Croatia, 2012, pp. 139–152.
 - [51] P.G. Popovich, P. Wei, B.T. Stokes, Cellular inflammatory response after spinal cord injury in Sprague-Dawley and Lewis rats, *J. Comp. Neurol.* 377 (1997) 443–464.
 - [52] R.S. Clark, J.K. Schiding, S.L. Kaczorowski, D.W. Marion, P.M. Kochanek, Neutrophil accumulation after traumatic brain injury in rats: comparison of weight drop and controlled cortical impact models, *J. Neurotrauma* 11 (1994) 499–506.
 - [53] P.C. Norris, E.A. Dennis, A lipidomic perspective on inflammatory macrophage eicosanoid signaling, *Adv. Biol. Regul.* 54 (2014) 99–110.
 - [54] C.N. Serhan, Pro-resolving lipid mediators are leads for resolution physiology, *Nature* 510 (2014) 92–101.
 - [55] D. Attwell, A.M. Buchan, S. Charpak, M. Lauritzen, B.A. MacVicar, E.A. Newman, Glial and neuronal control of brain blood flow, *Nature* 468 (2010) 232–243.
 - [56] L. Rangel-Castilla, J. Gasco, H.J. Nauta, D.O. Okonkwo, C.S. Robertson, Cerebral pressure autoregulation in traumatic brain injury, *Neurosurg. Focus* 25 (2008) E7.
 - [57] D. Gebremedhin, A.R. Lange, T.F. Lowry, M.R. Taheri, E.K. Birks, A.G. Hudezt, J. Narayanan, J.R. Falck, H. Okamoto, R.J. Roman, Production of 20-HETE and its role in autoregulation of cerebral blood flow, *Circ. Res.* 87 (2000) 60–65.
 - [58] D. Gebremedhin, Y. Ma, J.R. Falck, R.J. Roman, M. VanRollins, D.R. Harder, Mechanism of action of cerebral epoxyeicosatrienoic acids on cerebral arterial smooth muscle, *Am. J. Phys. Heart Circ. Phys.* 263 (1992) H519–H525.
 - [59] X. Liu, C. Li, J.R. Falck, R.J. Roman, D.R. Harder, R.C. Koehler, Interaction of nitric oxide, 20-HETE, and EETs during functional hyperemia in whisker barrel cortex, *Am. J. Phys. Heart Circ. Phys.* 295 (2008) H619–H631.
 - [60] S.T. Davidge, Prostaglandin H synthase and vascular function, *Circ. Res.* 89 (2001) 650–660.
 - [61] S.I. Pomposiello, M. Alva, D.W. Wilde, O.A. Carretero, Linoleic acid induces relaxation and hyperpolarization of the pig coronary artery, *Hypertension* 31 (1998) 615–620.
 - [62] A. Lange, D. Gebremedhin, J. Narayanan, D. Harder, 20-Hydroxyeicosatetraenoic acid-induced vasoconstriction and inhibition of potassium current in cerebral vascular smooth muscle is dependent on activation of protein kinase C, *J. Biol. Chem.* 272 (1997) 27345–27352.
 - [63] T.D. Giles, G.E. Sander, B.D. Nossaman, P.J. Kadowitz, Impaired vasodilation in the pathogenesis of hypertension: focus on nitric oxide, endothelial-derived hyperpolarizing factors, and prostaglandins, *J. Clin. Hypertens.* 14 (2012) 198–205.
 - [64] V.N. Vangaveti, V.M. Shashidhar, C. Rush, U.H. Malabu, R.R. Rasalam, F. Collier, B.T. Baune, R.L. Kennedy, Hydroxyoctadecadienoic acids regulate apoptosis in human THP-1 cells in a PPARγ-dependent manner, *Lipids* 49 (2014) 1181–1192.
 - [65] V. Vangaveti, B.T. Baune, R.L. Kennedy, Hydroxyoctadecadienoic acids: novel regulators of macrophage differentiation and atherogenesis, *Ther. Adv. Endocrinol. Metab.* 1 (2010) 51–60.
 - [66] G. Chinetti, J.-C. Fruchart, B. Staels, Peroxisome proliferator-activated receptors (PPARs): nuclear receptors at the crossroads between lipid metabolism and inflammation, *Inflamm. Res.* 49 (2000) 497–505.
 - [67] V.C. Tam, O. Quehenberger, C.M. Oshansky, R. Suen, A.M. Armando, P.M. Treuting, P.G. Thomas, E.A. Dennis, A. Aderem, Lipidomic profiling of influenza infection identifies mediators that induce and resolve inflammation, *Cell* 154 (2013) 213–227.
 - [68] A.T. Michael-Titus, J.V. Priestley, Omega-3 fatty acids and traumatic neurological injury: from neuroprotection to neuroplasticity? *Trends Neurosci.* 37 (2014) 30–38.
 - [69] J.M. Schwab, N. Chiang, M. Arita, C.N. Serhan, Resolvin E1 and protectin D1 activate inflammation-resolution programmes, *Nature* 447 (2007) 869–874.
 - [70] C.N. Serhan, R. Yang, K. Martinod, K. Kasuga, P.S. Pillai, T.F. Porter, S.F. Oh, M. Spite, Maresins: novel macrophage mediators with potent antiinflammatory and proresolving actions, *J. Exp. Med.* 206 (2009) 15–23.
 - [71] C. Chen, Lipids: COX-2's new role in inflammation, *Nat. Chem. Biol.* 6 (2010) 401–402.
 - [72] N.G. Bazan, Omega-3 fatty acids, pro-inflammatory signaling and neuroprotection, *Curr. Opin. Clin. Nutr. Metab. Care* 10 (2007) 136–141.
 - [73] V.L. Marcheselli, S. Hong, W.J. Lukiw, X.H. Tian, K. Gronert, A. Musto, M. Hardy, J.M. Gimenez, N. Chiang, C.N. Serhan, Novel docosanoids inhibit brain ischemia-reperfusion-mediated leukocyte infiltration and pro-inflammatory gene expression, *J. Biol. Chem.* 278 (2003) 43807–43817.
 - [74] M. Birnie, R. Morrison, R. Camara, K.I. Strauss, Temporal changes of cytochrome P450 (Cyp) and eicosanoid-related gene expression in the rat brain after traumatic brain injury, *BMC Genomics* 14 (2013) 1.
 - [75] R. Dringen, Metabolism and functions of glutathione in brain, *Prog. Neurobiol.* 62 (2000) 649–671.
 - [76] J.Y. Khan, S.M. Black, Developmental changes in murine brain antioxidant enzymes, *Pediatr. Res.* 54 (2003) 77–82.
 - [77] K. Tsuru-Aoyagi, M.B. Potts, A. Trivedi, T. Pfankuch, J. Raber, M. Wendland,

- C.P. Claus, S.E. Koh, D. Ferrero, L.J. Noble-Haeusslein, Glutathione peroxidase activity modulates recovery in the injured immature brain, *Ann. Neurol.* 65 (2009) 540–549.
- [78] E.N. Frankel, Secondary products of lipid oxidation, *Chem. Phys. Lipids* 44 (1987) 73–85.
- [79] L. Cristofori, B. Tavazzi, R. Gambin, R. Vagnozzi, C. Vivenza, A.M. Amorini, D. Di Pierro, G. Fazzina, G. Lazzarino, Early onset of lipid peroxidation after human traumatic brain injury, *J. Investig. Med.* 49 (2001) 450–458.
- [80] D.M. Miller, J.A. Wang, A.K. Buchanan, E.D. Hall, Temporal and spatial dynamics of nrf2-antioxidant response elements mediated gene targets in cortex and hippocampus after controlled cortical impact traumatic brain injury in mice, *J. Neurotrauma* 31 (2014) 1194–1201.
- [81] E.D. Hall, M.R. Detloff, K. Johnson, N.C. Kupina, Peroxynitrite-mediated protein nitration and lipid peroxidation in a mouse model of traumatic brain injury, *J. Neurotrauma* 21 (2004) 9–20.
- [82] R. Hill, I. Singh, J. Wang, J. Kulbe, E. Hall, Differing time courses of synaptic & non-synaptic mitochondrial dysfunction & oxidative damage following Tbi in young adult males, *J. Neurotrauma* 33 (2016) A117–A118.
- [83] J.T. Cole, A. Yarnell, W.S. Kean, E. Gold, B. Lewis, M. Ren, D.C. McMullen, D.M. Jacobowitz, H.B. Pollard, J.T. O'Neill, Craniotomy: true sham for traumatic brain injury, or a sham of a sham? *J. Neurotrauma* 28 (2011) 359–369.