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

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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

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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 (DiHDPA), 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 ± 0.5 °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 ($C_{20:4\ d8}$) and dried under N_2 flow. The dried film was dissolved in 50 µl of 100% methanol, and 5 µl 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 µm, 100 Å, 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 µl/min, 15–35 min linear gradient from 50% to 65% B at 25 µl/min, 40–55 min linear gradient from 65% to 80% B at 25 µl/min, 55–57 min linear gradient from 85% to 95% B at 25 µl/min, 57–65 min isocratic flow of 95% B at 25 µl/min, 65–70 min linear gradient from 95% to 50% B at 25 µl/min. The column was then re-equilibrated for 5 min with 50% B at 200 µl/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