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OXIDIZED PHOSPHOLIPID SIGNALING IN TRAUMATIC BRAIN INJURY

Tamil S. Anthonymuthu^{1,2}, Elizabeth M. Kenny^{1,2}, Andrew M. Lamade^{1,2}, Valerian E. Kagan^{2,3}, and Hülya Bayır^{1,2,4,*}

¹Safar Center for Resuscitation Research, Department of Critical Care Medicine, University of Pittsburgh, Pittsburgh, PA 15260

²Center for Free Radical and Antioxidant Health, Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, PA 15260

³Laboratory of Navigational Redox Lipidomics in Biomedicine, Department of Human Pathology, IM Sechenov First Moscow State Medical University, Russian Federation

⁴Children's Hospital of Pittsburgh, Pittsburgh, PA, 15224

Abstract

Oxidative stress is a major contributor to secondary injury signaling cascades following traumatic brain injury (TBI). The role of lipid peroxidation in the pathophysiology of a traumatic insult to neural tissue is increasingly recognized. As the methods to quantify lipid peroxidation have gradually improved, so has the understanding of mechanistic details of lipid peroxidation and related signaling events in the injury pathogenesis. While free-radical mediated, non-enzymatic lipid peroxidation has long been studied, recent advances in redox lipidomics have demonstrated the significant contribution of enzymatic lipid peroxidation to TBI pathogenesis. Complex interactions between inflammation, phospholipid peroxidation, and hydrolysis define the engagement of different cell death programs and the severity of injury and outcome. This review focuses on enzymatic phospholipid peroxidation after TBI, including the mechanism of production, signaling roles in secondary injury pathology, and temporal course of production with respect to inflammatory response. In light of the newly identified phospholipid oxidation mechanisms, we also discuss possible therapeutic targets to improve neurocognitive outcome after TBI. Finally, we discuss current limitations in identifying oxidized phospholipids and possible methodologic improvements that can offer a deeper insight into the region-specific distribution and subcellular localization of phospholipid oxidation after TBI.

Graphical Abstract

***Corresponding author.** Hülya Bayır, MD Professor, Department of Critical Care Medicine Department of Environmental and Occupational Health Director of Research, Pediatric Critical Care Medicine Associate Director, Center for Free Radical and Antioxidant Health Safar Center for Resuscitation Research University of Pittsburgh Tel.: 412-383-7865. bayihx@ccm.upmc.edu.

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redox lipidomics; inflammation; apoptosis; ferroptosis; efferocytosis; lipid mediator

Traumatic brain injury (TBI) affects over 1.7 million individuals each year in the United States alone [1]. Fortunately, survival following TBI has modestly improved over the past several years. However, it remains that fewer than half of patients with severe TBI maintain moderate or good functional status in long-term follow-up [2–4]. This positive but limited success can be attributed to the implementation of early aggressive, standardized treatments at all stages of the acute care continuum [5]. Yet, the longitudinal burden of TBI remains multifaceted, negatively impacting societal participation, mood, quality of life, and financial stability for both the patient and caregivers [6, 7]. Therefore, therapies that ameliorate the chronic burden of TBI by reducing neuronal injury and improving functional outcomes are sorely needed. Neuronal cell death is a major contributor to functional outcome after TBI [8, 9]. Accordingly, the interdependent and multimodal nature of TBI pathogenesis necessitates comprehensive understanding of the factors that contribute to both neuronal survival and death in the pursuit of effective pharmacotherapy [10].

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TBI is a complex and dynamic process including both primary and secondary injury with multitude of small molecule, enzymatic, and lipid-based signaling mediators involved. The primary injury of TBI refers to damage incurred from the mechanical forces of trauma. Consequently, secondary injury is initiated at the time of the insult and develops in the post-injury period through signaling of multiple interconnected molecular pathways, including but not limited to: inflammation, excitotoxicity, ion/fluid imbalance, oxidative stress, and mitochondrial dysfunction [11, 12]. The pro-inflammatory and pro-oxidative states observed following TBI stand apart; innumerable studies in pre-clinical and clinical settings recognize their critical and interdependent contributions to secondary injury evolution and their role as prime targets for therapeutic intervention [13, 14]. Whilst the importance of generalized oxidative stress after TBI is well recognized, the role of the oxidized signaling mediators, particularly those enzymatically regulated, is not completely understood. Phospholipids (PLs) are the most abundant and diverse of all the various oxidizable lipids found in the brain and are subsequently a major source of oxidized lipid derivatives. Enzymatically regulated oxidation of PLs serve a critical and under acknowledged role in both the pathogenesis and resolution of injury after brain trauma. In this focused review, we discuss the identification, sources and temporal course of formation, and signaling roles of oxidized PL and their derivatives in cell survival and death following TBI while identifying past and prospective targets of lipid oxidation for neuroprotective intervention. In this focused review, we discuss the identification, sources and temporal course of formation, and signaling roles of oxidized PL and their derivatives in cell survival and death following TBI while identifying past and prospective targets of lipid oxidation for neuroprotective intervention.

TBI pathogenesis and the role of oxidative stress

TBI is a physical injury to the brain tissue due to a mechanical force caused by a jolt, blast, compression or penetrating external object. The primary damage caused by the mechanical force expands in time as a result of a number of secondary injury cascades that include disruption of ionic channels, release of excitatory neurotransmitters, oxidative stress, mitochondrial dysfunction, and inflammation [15]. The time course of these secondary injury mechanisms differ with oxidative stress and mitochondrial dysfunction lasting weeks after injury, thus presenting themselves as potential therapeutic targets. TBI can cause temporary or permanent damage to the brain function owing to a complex disease process. Despite improvement in the overall mortality owing to advances in emergency and critical care of severe TBI patients, a significant portion of them (50%) endure neurological deficits affecting multiple domains of function [4]. Due to the heterogeneity of TBI, its classification depends on the data availability and purpose of the classification. The mode of classification includes: 1) etiological, 2) symptomatic 3) prognostic and 4) pathoanatomic. These classifications can serve as waymarks towards treatment strategies and have been reviewed thoroughly in the literature [16]. To understand the pathophysiology of TBI, numerous animal models have been developed. These models used extensively in preclinical setup and generally mimic focal cortical contusion (such as controlled cortical impact) or diffuse axonal injury (such as weight drop model). A discussion of different TBI animal models is beyond the scope of this review and the reader is referred to excellent reviews on this subject [17, 18].

While the contribution of oxidative stress to TBI pathogenesis is well-established, initial studies focused on the realization of oxidative stress through three major non-enzymatic mechanisms [19]. 1) *Release of iron and other transition metals* [20]. Free iron, commonly released through heme oxygenase-mediated breakdown of heme, and other transition metals have potential for biological toxicity, primarily through the production of highly potent hydroxyl radicals via Haber-Weiss, Fenton-like, and Fenton reactions. [20, 21]. TBI leads to mishandling of transition metals. TBI-induced intracranial hemorrhage leading to erythrocyte lysis is a major source of free heme and Fe^{2+} . Though the vast majority of transition metals are sequestered by storage proteins, such as ferritin and metallothioneins, to minimize the redox-related toxicity of unbound transition metals [22–25]. Highly reactive hydroxyl radicals, formed by the interaction of transition metals and soluble hydroperoxides such as hydrogen peroxide (H_2O_2), initiate a chain reaction of lipid peroxidation [21]. 2) *Generation of superoxide and peroxynitrite*. Dysregulation of the electron transport chain following TBI results in the partial reduction of oxygen and serves as the major source of superoxide generation [26]. Superoxide by itself is relatively nonreactive, however it can interact with nitric oxide (NO) to produce peroxynitrite at a rate an order of magnitude higher than its dismutation to hydrogen peroxide (H_2O_2) [27, 28]. Furthermore, inducible NO synthase is activated after TBI and generates NO in a calcium-independent fashion fueling formation of peroxynitrite [29]. 3) *Decrease in antioxidant defenses*. Cellular protection against superoxide involves superoxide dismutases (SOD) and antioxidants such as vitamin C and E [30]. H_2O_2 formed by enzymatic or non-enzymatic dismutation of superoxide is efficiently removed by catalase or glutathione peroxidase (GPx) [30]. Despite the inherent protective capacities of healthy cells, TBI-induced pro-oxidative processes can overrun innate antioxidant reserves and other defense systems against transition metals [31]. Resultant oxidative stress leads to injurious oxidative and nitrative modifications of DNA, protein, and lipids that disrupt normal function [32, 33]. In addition to alteration of membrane fluidity and integrity, oxidation of lipids produces a diverse range of signaling molecules.

While non-enzymatic oxidation mechanisms contribute to the overall oxidative burden immediately following TBI, the role of these non-enzymatic mechanisms beyond the acute post-traumatic period is unclear. More recently, the contribution of specific, enzymatic oxidation to oxidative stress following TBI has been increasingly appreciated. Adding to the burden of lipid oxidation in this setting is the overexpression of lipid peroxidation enzymes after brain trauma - namely members of the cyclooxygenase (COX) [34] family, lipoxygenase (LOX) [35] family, cytochrome (cyt) p450 [36], and cyt c [37]. Unlike non-enzymatic lipid oxidation which occurs immediately following injury, enzymatic oxidation of lipids results in delayed and persistent signaling. In the next section, we will briefly review the commonly studied markers of lipid peroxidation in clinical and experimental TBI and the significance of lipid peroxidation for clinical outcome.

Detection of lipid peroxidation after TBI

Various markers of oxidative stress have been studied in TBI (**Table 1**). In this review, we will focus on lipid peroxidation markers. A number of lipid oxidation markers have been shown to increase in brain tissue, serum, and cerebrospinal fluid (CSF) after experimental

and clinical TBI. The primary product of lipid oxidation is hydroperoxides, which have a short half-life. Thus, the study of lipid peroxidation in TBI has largely relied on the detection of more stable reaction end products including isoprostanes, malondialdehyde (MDA), and hydroxynonenal (HNE) [38]. Increased 4-HNE or HNE-modified proteins have been detected in brain tissue using various experimental models of TBI. Numerous clinical studies have observed an increase in F₂-isoprostanes and MDA in plasma or CSF following severe TBI [31, 39–43]. In addition to providing information on the extent of oxidation in the brain, elevations in F₂-isoprostanes [43] and thiobarbituric acid (TBA)-reactive substances (TBARS) [42, 44–46] - a surrogate marker of MDA - have been correlated with neurological outcome and mortality following TBI in humans.

While isoprostanes, MDA, and HNE are the most commonly evaluated markers of lipid oxidation in clinical samples and can provide generalized information on the extent of oxidation and neurological outcome, these methodologies are not without flaws. First, there are limitations to the methods used to detect lipid peroxidation end products. For example, detection of MDA continues to rely upon reaction with TBA for derivatization; however, TBA can react non-specifically with other aldehydes including carbohydrates, thus confounding the measurement of MDA [63, 64]. This is particularly problematic in diseases like TBI, where hyperglycemia is frequently seen after injury and is associated with neurological outcome and mortality [65]. Although separation of MDA-TBA from other TBARS using high-performance liquid chromatography (HPLC) can improve assay specificity [66], methods which detect end products of lipid peroxidation have an additional inherent flaw. Though measurement of end products of lipid peroxidation provides useful information about general lipid oxidation after TBI, important information about the species' origin and time course of their generation are lost. Through advances in HPLC and mass spectrometry-based oxidative lipidomics, hydroperoxy lipid species can now be detected and quantified [38, 48, 51, 67]. The advent of oxidative lipidomics [68] has begun to reveal the diversity of lipid oxidation after TBI and elucidate enzymatic mechanisms of lipid oxidation that could potentially be targeted. Understanding the source and signaling associated with lipid oxidation is important in therapeutic targeting and may explain why general antioxidative treatment strategies have so far failed in clinical translation. In the next section, we will briefly review these strategies.

Targeting oxidative stress and lipid oxidation via antioxidants

With strong evidence of increased lipid peroxidation following TBI and a correlation with neurological outcome, antioxidant therapy targeting oxidation of lipids was an obvious therapeutic strategy following brain trauma. These therapies include nonspecific antioxidants such as vitamin C and vitamin E that function through free radical scavenging mechanisms, as well as antioxidant enzymes including superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX). Despite the success of antioxidant therapy targeting lipid oxidation in experimental models of TBI, protective effects of antioxidant therapy largely have not translated in the large multicenter randomized controlled trials in TBI patients [69]. Three major reasons for this failure include: *1*) Relatively slow reaction rate of antioxidants with oxidants thus requiring non-physiological or unachievable in vivo concentrations for their antioxidant activity [70]. In this regard, vitamin E is an exception

that is a potent antioxidant at relatively low concentrations. However, the reaction rates for effective scavenging by Vitamin E are limited to hydroperoxyl radicals [71, 72]. 2) Insufficient understanding of the role of oxidative stress in the pathophysiology of disease processes due to lack of proper oxidative stress markers [73]. 3) Insufficient attention to the enzymatic reactions in which free radical intermediates can be formed. In this case, random chemical reactions and respective rate constants although important do not define the overall protective potency. Therefore, unless the mechanisms and sources of lipid peroxidation are better understood, blanket use of free radical scavengers and antioxidants may not be fully successful. Better understanding of mechanisms of lipid peroxidation, identification, and quantification of oxidized lipid species are needed. With the advent of oxidative lipidomics that includes a combination of various separation techniques and mass spectrometry over the last 10 years, our understanding of targets of lipid peroxidation and of the essential role oxidized lipids play in signaling during brain injury and repair have improved. In the next section, we will focus on the different signaling roles PLs play in neuronal health and disease.

Brain phospholipids and their signaling in TBI

Lipids are of critical importance to brain structure and function, comprising approximately 60% of brain weight. The brain lipidome is rich and complex consisting of 5,713 putative species of which 2,330 have been identified [74, 75]. PLs contribute substantially to the diversity and abundance of brain lipids, comprising 35–50% of total lipid weight [76, 77] and 60% of the number of lipids [75]. Polyunsaturated fatty acids (PUFAs) contain one or more methylene-interrupted dienes and are enriched at the sn2 position of PLs. Similar to diacylated PLs, the tetra-acylated PL cardiolipin (CL) in the brain is also enriched with PUFAs. PUFAs are prone to non-enzymatic free radical and enzymatic oxidation due to the ease of abstraction of hydrogens from the methylene bridges. Therefore, PUFA-containing PLs form one the most important groups of oxidizable molecules in the brain's cellular milieu. Despite the diversity of the PL head group and acyl chain composition, enzymatic mechanisms enable selective oxidation in a time-dependent manner. In the following section, we will review the mechanisms of PL oxidation and the associated cellular signaling that have been described after TBI.

Signaling by oxidized free fatty acids

Release of free fatty acids (FFA) from PLs is one of the earliest events following mechanical insult. Elevations of FFA in the injured brain are reported as early as 5 minutes after experimental TBI and remain up to 24 hr after injury [78]. In humans with severe TBI, polyunsaturated FFA have been shown to remain elevated up to a week after injury in CSF [79, 80]. This increase in FFA is due to calcium-dependent activation of phospholipase A2 (PLA2) resulting in the hydrolysis of esterified PUFAs from the sn2 position of PLs [81]. Concurrent with the increase in FFA, expression of lipid peroxidation enzymes including LOX [48] and COX [82] also increase after injury. This results in massive enzymatic FFA oxidation, which peaks at 1 hr after injury [51]. Various oxidized FFA products such as eicosanoids, docosanoids, and octadecanoids are elevated after TBI. More specifically, well-characterized lipid mediators [82] including hydroxyoctadecadienoic acids (HODEs),

hydroxyeicosatetraenoic acids (HETEs), DiHETEs, prostaglandins, lipoxins, neuroprotectins, and resolvins [83] were among the 244 oxidized FFA observed following TBI [51]. Oxidized FFA are dynamic lipid mediators that regulate both the promotion and resolution of inflammation [84]. Production of pro-inflammatory oxidized FFA occurs by 1 hr after impact, whereas production of anti-inflammatory oxidized FFA predominates by 24 hr following injury. The early increase in oxidized FFA correlates with other early pro-inflammatory events such as microglia activation [85], blood brain barrier (BBB) damage [86], and neutrophil infiltration [87]. The later increase in the anti-inflammatory and resolving signals correlates with other events in the resolution of inflammation (**Fig 1**) [88]. The PLA2-mediated hydrolysis of fatty acids from PLs generates an additional lipid product, lysophospholipids (lyso-PL), with roles in various signaling pathways. Among the various lyso-PLs, lysophosphatidylcholine has known involvement in disruption of the BBB and induction of T-cell and neutrophil response [89]. Additionally, lyso-phosphatidic acid is involved in the initiation of neuropathic pain [90] via G protein-coupled receptor (GPCR)-mediated signaling [91]. As an alternative mechanism of the production of PUFA lipid mediators, oxidation of different PLs, particularly CLs, and their subsequent hydrolysis by Calcium independent phospholipases A2 leading to the release of oxygenated PUFA has been documented [47].

Signaling by oxidized phosphatidylethanolamine

Direct enzymatic oxidation of PLs, resulting in the initiation of cell death pathways in the injured brain, is also triggered by TBI [33]. As an early response, oxidation of CLs by cyt c takes place in mitochondria and leads to the execution of apoptosis [67]. Another death pathway realized in TBI is ferroptosis - the recently described, iron-dependent form of cell death [92]. Ferroptosis is triggered by doubly- and triply-oxygenated phosphatidylethanolamine (PE), especially those containing omega-6 fatty acids such as arachidonic acid (AA) and adrenic acid (AdA) [65]. Production of these oxidized PE is accelerated by the formation of a transient protein complex through the binding of phosphatidylethanolamine-binding protein 1 (PEBP1) and 15-lipoxygenase (15LO) [48]. This complex allosterically alters the substrate specificity of 15LO from FFA to AA-PE. Under normal conditions, hydroperoxy-PLs are reduced to less active hydroxy-PLs by the action of glutathione peroxidase 4 (GPX4) in the presence of glutathione (GSH) [93]. The accumulation of ferroptotic signal molecules can occur as a result of overproduction by the 15LO/PEBP1 complex or by inefficient clearance by the GPX4/GSH system. Unlike apoptosis, there are no specific morphological features of ferroptotically dying cells revealed by conventional (fluorescence) or electron microscopy. However, a recent study identified the complex of 15-LO with PEBP1 as a required stage of ferroptosis leading to the production of pro-ferroptotic hydroperoxy-arachidonoyl-phosphatidylethanolamines [48]. These complexes can be visualized by high-resolution fluorescence microscopy using dual object recognition protocol[94] [48]. Application of this technique showed remarkably increased contents of these complexes in the ferroptotically dying neurons after TBI. This is illustrated in the new **Fig 2** where the 15-LO-2/PEBP1 complexes are comparatively shown for the normal vs injured brain. These studies suggest that ferroptotic cell death is initiated early (1 hr) after the impact progressing up to 24 hr post-injury (**Fig 3**) [48]. Though the

exact role and mechanisms of ferroptotic cell death in disease pathology is yet to be explored, the number of neurological diseases where ferroptosis has been shown to play an important pathogenic role is growing [95]. Therapies targeting ferroptosis, such as Ferrostatin-1 or inhibitors of LOX, have been shown to reduce neuronal death and infarct size in animal models of intracerebral hemorrhage [96]. Similarly another ferroptosis inhibitor, Liproxstatin-1 given after ischemia reperfusion injury was shown to improve motor function in rats [97].

Signaling by oxidized cardiolipin

As indicated above, oxidation of CL and apoptosis occurs early during TBI. CL is typically confined to the inner membrane of mitochondria but can be externalized to the mitochondrial outer membrane upon the changes in mitochondrial physiology. As this process requires at least three translocations of CL across the inner and outer mitochondrial membranes, several mechanisms may be implicated in the transmembrane migration of CLs including: Ca^{2+} -dependent phase transition [98], Ca^{2+} -dependent migration by phospholipid scramblase 3 [99], and nicotinamide diphosphate kinase D (NDPKD)-catalyzed translocation [100]. At this new location, CL can form a complex with cyt c, the electron shuttle between complex III and IV of the electron transport chain. CL binding destabilizes the structure of cyt c [101] resulting in its conversion to a peroxidase [37]. CL is oxidized by this CL/cyt c peroxidase using H_2O_2 , generating more than 150 unique oxidized CL products in the injured brain [67]. Oxidation of CL results in cytosolic release of pro-apoptotic cyt c to initiate apoptosis after TBI (**Fig 4**) [37]. The temporal profile of CL oxidation in the injured brain indicates the initiation of apoptosis 3 hr following TBI [33]. Various cell types in brain exhibit varying levels of sensitivity towards apoptosis. For example, neurons are sensitive to apoptotic cell death while microglia are resistant [21]. In TBI, the apoptosis regulation is also mediated by various other factors such as, B-cell lymphoma-2 (Bcl-2) family of proteins [102] and mitogen-activated protein kinase (MAPK) signal-transduction pathways [103]. Studies suggest that TBI sets into motion both apoptotic as well regulated necrotic cell death pathways such ferroptosis [8, 104]. The exact regulating factors determining initiation of apoptosis versus ferroptosis after TBI is not known. It is possible that mitochondrial injury beyond the repair capacity might predispose the neurons to trigger apoptotic cell death pathway via oxidation of CLs.

In addition to the role of CL oxidation in apoptosis, oxidized CL species can be further hydrolyzed by the calcium-independent phospholipase A2 (iPLA2- γ) producing oxidized FFA and monolyso-CL (MLCL). Like oxidized FFA produced via the classical lipid mediator pathway described above, mitochondrial-based lipid mediator production contributes to inflammatory signaling by oxidized FFA [47]. MLCL is also implicated in the activation of apoptosis. Increased MLCL aids in outer mitochondrial membrane (OMM) localization of truncated BH3 interacting domain protein (tBID), leading to OMM permeabilization and release of apoptogenic factors [105, 106]. Interestingly, cells devoid of iPLA2- γ were resistant to *in vitro* TBI using a mechanical stretch model of injury, supporting a role for MLCL in the activation of apoptosis (unpublished results). Furthermore, increased MLCL content due to mutation in CL remodeling enzyme tafazzin results in cognitive defects, such as lower visual spatial skills and mathematical performance

in patients with Barth syndrome [107–109]. Barth syndrome is an X-linked inherited disease that is caused solely by the impairment of tafazzin to reacylate CL leading to dilated cardiomyopathy, skeletal myopathy and neutropenia [110].

While the oxidation of CL in the OMM induces apoptosis and inflammation, externalized CL is involved in several other key signaling events. Externalized CL can interact with the key autophagy protein, microtubule-associated-protein-1-light chain-3 (LC3), to initiate autophagosome formation and thus mitophagy [99]. As mitochondrial dysfunction plays a crucial role in secondary injury following TBI, removal of damaged mitochondria via mitophagy is important in preventing mitochondrial-derived oxidative and inflammatory signaling [111]. In TBI, inhibition of mitophagy has been shown to increase inflammation, while activation of mitophagy reduces inflammation [112]. Mitochondria with externalized CL which fail to undergo mitophagy can be released to the systemic circulation. Once outside the cell, externalized CL is recognized by CD36 scavenger receptors on phagocytes and can modulate inflammatory response by TLR4-mediated pathways [113].

Signaling by oxidized phosphatidylserine

As previously discussed, CL oxidation by the CL/cyt c peroxidase results in the release of cyt c into the cytoplasm where it is involved in the initiation of apoptosis. cyt c displays high affinity towards anionic phospholipids, and thus once in the cytoplasm it can bind to anionic phosphatidylserine (PS) in the plasma membrane. This cyt c-PS complex again enables the peroxidase activity of cyt c with resulting oxidation of PS [114]. Along with caspase-mediated changes in phospholipid flippase and scramblase activity, oxidized PS can function as a non-enzymatic scramblase leading to PS externalization [115–118] which serves as an “eat me” signal in efferocytosis. Oxidized PS is an even more potent “eat me” signal than non-oxidized PS for the recruitment of phagocytes to the vicinity of apoptotic cells (**Fig 5**). Studies indicate that oxidized PS is a ligand for the scavenger receptor CD36 and MFG-E8 in mediating macrophage response [119, 120]. In line with its role in the clearance of apoptotic cells, PS oxidation occurs only by 24 hr. Efferocytosis serves a critical role in modulating the inflammatory response and promoting injury resolution [67, 68]. Importantly another lipid mediator implicated in resolution of inflammation, resolvins, are also pronounced during this time, indicating synergetic effects of oxidized lipid mediators in the resolution of inflammation. Aside from its role in efferocytosis and inflammation resolution, oxidized PS may also directly bind to protein kinase C inhibitor therefore preventing the inactivation of blood coagulation factor [121]. To date, no studies have directly connected PS oxidation and coagulation status in TBI, but it is possible that oxidized PS may prevent injury propagation through an unexplored role in promotion of coagulation.

Targeting phospholipid oxidation signaling in TBI

The oxidized PL signaling pathways present both potential targets and challenges when considering the design of effective neuroprotective therapies. However, opportunity does exist for the design of small molecule inhibitors to target common pathways involved in the synthesis and signaling of oxidized PL in TBI. Common and non-specific drug targets in preventing lipid peroxidation have been reviewed previously [30]. With new insight into

targets and time course of lipid peroxidation gained through mass spectrometry-based lipidomics, here we will focus on new possibilities of drug targeting aimed at signaling by oxidized PL (**Table 2**).

Inflammation mediated by FFA oxidation is a major driving force in TBI pathogenesis and thus a viable option for secondary injury prevention [122]. Targeting this process can include both PLA2 and lipid oxidation enzymes. Despite the success of enzyme inhibition or genetic knockdown in animal models, nearly all clinical trials targeting FFA oxidation or inflammation demonstrated limited or no success [123]. It is now recognized neuro-inflammation is necessary for tissue regeneration and repair. Early inflammation is required for removal of dead cells generated during the impact but chronic inflammation is deleterious. Identification of a switch between beneficial and harmful inflammation will be critical in designing therapies targeting FFA oxidation or inflammation [124].

Interfering with CL oxidation and thereby preventing apoptosis is another avenue for development of neuroprotective therapies in TBI. One option is to prevent the peroxidase activity of cyt C/CL complex by irreversibly blocking the accessibility to the oxidizing heme in cyt c. Proof-of-concept studies have demonstrated that mitochondrial-targeted imidazole-substituted derivatives of stearic acid and oleic acid (TPP-ISA and TPP-IOA) inhibit cyt C/CL peroxidase activity while reducing caspase activation and cell death following γ -irradiation injury [130]. Alternatively, ongoing post-injury generation of H_2O_2 necessary for cyt C/CL activity can be inhibited through treatment with mitochondria-targeted nitroxide-based electron scavengers (i.e. XJB-5-131, JP4-039). XJB-5-131 has been shown to reduce lesion volume, neuronal cell death, and improve functional outcome following experimental TBI [67]. Reducing the abundance of oxidizable CL species using mitochondrial-targeted TPP-conjugated oleic acid is also effective in prevention of apoptotic cell death [67]. Finally, iPLA2 γ , which hydrolyzes oxidized acyl chains including those esterified to CL, can be specifically and potently inhibited by (R)-bromoenol lactone [(R)-BEL], leading to significant reduction in oxidized FFA following γ -irradiation [47]. The potential neuroprotective role of (R)-BEL is worth exploring in TBI. Collectively, TPP-ISA, TPP-IOA, XJB-5-131, (R)-BEL, and other mechanistically similar approaches may provide protection after TBI by limiting generation and subsequent release of CL-generated lipid mediators to their distal sites of action.

Due to the highly regulated nature of ferroptosis, this cell death pathway may offer a better option with multiple avenues for therapeutic targeting. The targets for prevention of ferroptosis include: 1) augmenting the defense system which remove oxidized PE products, 2) preventing the formation of 15LOX/PEBP1 complex, thereby reducing the rate of PE oxidation, and 3) directly preventing PE oxidation with 15LOX inhibition. The defense system which removes oxidized PE, namely GSH and GPX4, can be augmented with N-acetylcysteine (NAC) [126] and ebselen [127] supplementation. NAC is a precursor for GSH production and showed protective effects in adult TBI patients [131, 132]; however, the protective effects have not borne out in pediatric TBI [133]. Early administration of ebselen, a GPX mimetic that reduces hydroperoxy-PL, showed significant improvement in stroke patients [134, 135]. Furthermore, ebselen treatment ameliorated neurological injury in a rat model of TBI [136]. 15LOX inhibitors such as baicalein can improve functional outcome

after TBI [128, 129], while various 15LOX inhibitors were shown to be protective in stroke models [137, 138]. Other small molecule inhibitors such as Liproxstatin and Ferrostatin-1 also reduce oxidized PE accumulation and decrease ferroptosis. As previously discussed, accumulation of oxidized AA- or AdA-containing PE results in ferroptosis, therefore inhibition of acyl-coA synthetase long-chain family member 4 (ACSL4) and thus the formation of AA- and AdA-esterified PE may also protect against TBI [139].

Future perspectives.

The current understanding of oxidized PL signaling has identified promising treatment targets for mitigation of TBI, however, several outstanding questions remain unanswered regarding PL oxidation in TBI. Brain regions perform specific neuronal functions [140] and display differential proteomes [141] and metabolomes [142]. A recent metabolomics analysis of different brain regions showed significant PL species variation across the brain [142]. In line with the functional and metabolomic differences, the susceptibility of different brain regions to injury significantly differs [143–145]. Together, these findings strongly suggest that different brain regions may exhibit differential pathogenesis and lipid signaling. Though information on the temporal course of lipid oxidation after TBI can be studied using liquid chromatography mass spectrometry (LC-MS) analysis of brain homogenates, spatial information about the lipid mediators is lost with this methodology. Unlike LC-MS methods in which each lipid species or classes are separated prior to mass analysis, imaging methods can acquire information on complex mixture of lipids with a wide range of concentrations; however, the identification of low-abundance, signaling PL is difficult with imaging mass spectrometry techniques. Using a combination of various tissue treatments to remove abundant lipids and enhance ionization, we have utilized imaging mass spectrometry to identify the specific loss of PUFA-containing CL species from the contusional cortex, hippocampi (CA1 and CA3), and thalamus after experimental TBI [146, 147]. Although the low abundance of oxidized lipids precluded their detection, the specific loss of PUFA-containing CL indicated that oxidation may explain the decrease in tissue CL after TBI. Recently, oxidatively truncated CL species were imaged using Desorption Electrospray Ionization Mass Spectrometry (DESI-MS) in thyroid oncocytic tumors [148]. The latest advances in imaging mass spectrometry methods including gas cluster ion beam-secondary ion mass spectrometry (GCIB-SIMS), sub-micrometer focused ionization beams, and enhanced and high-resolution mass detection techniques could enable cellular-level imaging of PL in the near future.

In addition to the importance of brain region-specific oxidized PL signaling, the implications of subcellular location of PLs cannot be ignored. The cellular distribution of PLs is not uniform, with the enrichment of several PL classes in specific subcellular compartments. For example, CL is restricted to the mitochondria [149], PE is enriched in mitochondria as well as endoplasmic reticulum, and PS is confined to the plasma membrane [150]. Identification of oxidized PL does not only provide information related to the mechanism and time course of injury, it can also provide insight to the cellular organelle involved in the injury mechanism. Understanding of oxidized PL signaling may aid in the development of organelle-targeting drugs to increase the treatment specificity and efficacy. While CL oxidation is clearly linked to the mitochondria, our understanding of the subcellular location

of all other PL oxidation pathways is limited. Combining the strength of subcellular organelle lipidomics [151, 152] and oxidative lipidomics can offer a solution to identify the exact location of oxidized PL production.

Conclusions

The pathophysiology of TBI is a complex and dynamic process which involves signaling through multiple pathways including inflammation and cell death. While oxidative stress and lipid peroxidation have long been implicated in TBI pathogenesis, our understanding of the mechanisms of lipid peroxidation and their role in the regulation of disease pathogenesis remain limited. With the advent of oxidative lipidomics, we have begun to unravel the complexity and diversity of oxidized lipid mediators and the time course of their production after TBI. While free radical-based non-enzymatic reactions cannot be ignored, a substantial portion of lipid peroxidation following TBI is generated through tightly controlled, enzymatically regulated mechanisms that may be a leading cause of secondary injury. Such enzymatic lipid peroxidation produces distinct mediators during different stages of inflammation and activation of specific cell death pathways. This temporal differentiation of signaling events offer many advantages as certain phase of otherwise deleterious processes are essential in the prognosis of the TBI. In addition to providing new targets for therapeutic intervention, our current knowledge of PL oxidation in TBI provides a possible explanation as to why previous clinical trials targeting lipid oxidation were unsuccessful and what changes in treatment strategy are needed to improve outcomes after TBI. Despite the improved understanding of lipid signaling pathways in the context of TBI, further study is needed. Future research should aim to identify exact targets for neuro-therapeutic intervention, spatial and temporal signaling, and association of (oxidized) lipids with cell death and repair pathways.

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Fig 2. Ferroptosis in injured Brain.

Ferroptosis in injured (Control cortical impact) rat brain is shown using various indicators of ferroptosis. (A) Co-localization of PEBP1 and 15LO2 in brain tissue. Stitched image showing high resolution large area confocal scanning of 3×5 image fields. Left: the overlaid emissions for the immunolocalization of PEBP1 (red), 15LO2 (green), and nuclei (blue). Right: co-localization analysis for 15LO2 and PEBP1, with the number of spots having both proteins appearing yellow. Scale bar, 200 μm . (B) Number of co-localized 15LO2 and PEBP1 in brain tissue 4 hr after injury. (C) Changes in GPX4 activity in rat brain cortex at 4 hr after injury. (D) Volcano plot demonstrates changes in the content of PEox at 1 hr post injury. The figure has been reproduced from Wenzel et. al., [48] with permission from Elsevier.

Fig 3. Production and signaling of phosphatidylethanolamine (PE) oxidation after traumatic brain injury (TBI).

After TBI, the expression of 15-LOX and its formation of a complex with PE binding protein 1 (PEBP1) increase. This complex oxidizes arachidonyl (AA)-PE to 15-hydroperoxyeicosatetraenoic acid-PE (15-HpETE-PE). The glutathione (GSH) and glutathione peroxidase 4 (GPX4) system responsible for the reduction of 15-HpETE-PE to 15-hydroxyeicosatetraenoic acid-PE (15-HETE-PE) is ineffective post-TBI, resulting in 15-HpETE-PE accumulation and ferroptosis. Structure of AA-PE and 15-HpETE-PE are shown in inset.

Fig 4. Production and signaling of oxidized cardiolipin (CL-ox) after traumatic brain injury (TBI).

After TBI, cardiolipin (CL) translocate from the inner leaflet to the outer leaflet of the inner mitochondrial membrane (IMM). Once in the outer leaflet, CL binds cytochrome (cyt) c, an electron shuttle of the mitochondrial electron transport chain, to form a complex with peroxidase activity. Using hydrogen peroxide (H_2O_2) as an oxidizing equivalent, this enzyme oxidizes CL. This CL-specific oxidation leads to the release of cyt c from the intermembrane space (IMS) into the cytosol triggering apoptosis. Oxidized CL can be hydrolyzed by calcium-independent phospholipase A_2 (iPLA $_2\gamma$) to generate monolyso-CL and oxidized free fatty acids (FFA-ox) involved in inflammatory signaling. Inset showing the structure of cardiolipin and oxidized cardiolipin.

The cytochrome (cyt) c released from mitochondria into the cytosol during apoptosis can bind to phosphatidylserine (PS) in the inner leaflet of the plasma membrane to form a peroxidase. This PS-specific oxidation leads to oxidation of PS. Externalization of PS and PS-ox to the outer leaflet of the plasma membrane results in efferocytosis by serving as “eat me” signals for phagocytic cells. Structures of PS and PS-ox also shown in the figure.

Table 1.

Oxidative stress markers commonly used in the studies of TBI

oxidative stress markers	Analysis Method	Reference
<i>Lipid peroxidation markers</i>		
Isoprostanes	Immuno assay/LC-MS	[19, 31, 39, 43]
Oxidized phospholipids	LC-MS	[33, 47, 48]
Oxidized free fatty acids	LC-MS	[49–51]
4-Hydroxynanoenal	Immuno assay/LC-MS	[52]
Malondialdehyde	Immuno assay/LC-MS	[41, 42]
<i>Protein oxidation Markers</i>		
Protein carbonyls	Immuno assay/LC-MS	[53, 54]
Protein adducts	Immuno histochemistry	[53, 55]
<i>DNA oxidation products</i>		
8-hydroxy-2'-deoxyguanosine (8-OHdG)	LC-MS	[56, 57]
<i>Enzymes related to oxidative stress</i>		
NADPH oxidase	Spectroscopy	[58]
Xanthine oxidase	Spectroscopy	[59]
Nitric oxide synthase	Immuno assay	
Catalase	Spectroscopy	[60]
Glutathione peroxidase	Spectroscopy	[60, 61]
<i>Antioxidants</i>		
Ascorbate	Spectroscopy	[31]
Glutathione	Spectroscopy	[62]
Vitamin E	HPLC	[46]

Table 2.

List of potential Drug/small molecule to inhibit phospholipid oxidation pathways.

Drug/Small molecule	Pathway	Model	Reference
Imidazole-substituted derivatives of stearic acid and oleic acid	cyt C/CL peroxidase activity	Mouse whole body irradiation	[125]
nitroxide-based electron scavengers (i.e. XJB-5-131, JP4-039)	cyt C/CL peroxidase activity	Rat TBI, global cerebral ischemia/reperfusion	[9, 67]
(R)-bromoenol lactone [(R)-BEL]	Hydrolysis of Clox	Mouse whole body irradiation	[47]
N-acetylcysteine	GSH precursor, Ferroptosis inhibitor	Human TBI	[126]
ebesen	GPx mimetic, ferroptosis inhibitor	Human stroke	[127]
Baicalein	15LOX, ferroptosis inhibitor	Rat TBI, Cerebral Ischemia	[128, 129]