Oxidative lipidomics of hyperoxic acute lung injury:
Mass spectrometric characterization of cardiolipin and phosphatidylserine peroxidation.

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Abstract: Reactive oxygen species have been shown to play a significant role in hyperoxia-induced acute lung injury, in part, by inducing apoptosis of pulmonary endothelium. However, signaling roles of phospholipid oxidation products in pulmonary endothelial apoptosis have not been studied. Using an oxidative lipidomics approach, we identified individual molecular species of phospholipids involved in the apoptosis-associated peroxidation process in a hyperoxic lung. C57BL mice were sacrificed 72 hrs after exposure to hyperoxia (100% oxygen). We found that hyperoxia-induced apoptosis (documented by activation of caspases 3 and 7 and histochemical Tunel staining of pulmonary endothelium) was accompanied by non-random oxidation of pulmonary lipids. Two anionic phospholipids – mitochondria-specific cardiolipin (CL) and extramitochondrial phosphatidylserine (PS) – were the two major oxidized phospholipids in hyperoxic lung. Using electrospray ionization mass spectrometry, we identified several oxygenation products in CL and PS. Quantitative assessments revealed a significant decrease of CL and PS molecular species containing C_{18:2}, C_{20:4}, and C_{22:5} and C_{22:6} fatty acids. Similarly, exposure of mouse pulmonary endothelial cells (MLEC) to hyperoxia (95% oxygen; 72 hr) resulted in activation of caspases 3 and 7 and significantly decreased content of CL molecular species containing C_{18:2} and C_{20:4} as well as PS molecular species containing C_{22:5} and C_{22:6}. Oxygenated molecular species were found in the same two anionic phospholipids – CL and PS - in MLEC exposed to hyperoxia. Treatment of MLEC with a mitochondria-targeted radical scavenger, a conjugate of hemi-gramicidin S (GS) with nitroxide, XJB-5-131, resulted in significantly lower oxidation of both CL and PS and a decrease in hyperoxia induced changes in caspase 3/7 activation. We speculate that cyt c driven oxidation of CL and PS is associated with the signaling role of these oxygenated species Participating in the execution of apoptosis and clearance of pulmonary endothelial cells, thus contributing to hyperoxic lung injury.

Keywords: Hyperoxia, lung, endothelium, cardiolipin, phosphatidylserine, cardiolipin hydroperoxides, phosphatidylserine hydroperoxides, apoptosis.
Introduction

Hyperoxia-induced acute lung injury is characterized by an influx of inflammatory cells, increased pulmonary permeability, and endothelial and epithelial cell death (41, 43, 59). Reactive oxygen species (ROS) have been postulated to have a significant role in hyperoxic acute lung injury, in part, by inducing cell death of pulmonary endothelium involving both extrinsic and intrinsic pathways (71). The mechanisms and link between two major factors contributing to genesis and maintenance of hyperoxic (acute) lung injury - an imbalance between production and elimination of partially reduced oxygen and nitrogen species and endothelial dysfunction due to endothelial cell apoptosis – are not well characterized.

Oxygenated fatty acids are well known signaling molecules that participate in regulation and coordination of cellular and body metabolism (38, 55). Their important role in cell proliferation, i.e. modulation of apoptosis, angiogenesis, inflammation, and immune surveillance, has been well documented (44, 57). The involvement of another oxygenated product of polyunsaturated docosahexaenoic acid, resolvin E1, in the pathogenesis of lung inflammatory injury has also been suggested (2, 23, 24), even though polyunsaturated phospholipids are the major oxidation targets in sn-2 position (32). Several decades ago, an activation of lipid peroxidation in the lung under hyperoxic conditions was evidenced by the accumulation of one of the secondary products of lipid peroxidation, malonyldialdehyde (28) as well as thiobarbituric acid material in cultured endothelial cells exposed to hyperoxia (21). Moreover, an elevation of oxygenated products formed from arachidonic acid - isoprostanes and isofuranes - has been documented in hyperoxic mouse lung (17). However, the participation and signaling roles of oxidatively modified phospholipids in pulmonary endothelial apoptosis in the hyperoxic acute lung injury have not yet been established.
Recently, the accumulation of oxidation products in two anionic phospholipids - a mitochondria-specific cardiolipin (CL), and extramitochondrial phosphatidylserine (PS) - has been associated with apoptosis, particularly with a release of pro-apoptotic factors from mitochondria into the cytosol and an externalization of PS on the cell surface of apoptotic cells, respectively (35, 36). Moreover, these two anionic phospholipids have been identified as oxidation substrates of cytochrome c (cyt c) catalyzed reactions \textit{in vitro} (36, 62) and \textit{in vivo} (62). Furthermore, oxidized phospholipids have been demonstrated to act as signals in monocyte activation, programmed cell death and phagocytotic clearance of apoptotic cells (14, 22, 42, 68, 69).

In the present study, we employed oxidative lipidomics to identify individual molecular species of phospholipids involved in the apoptosis-associated peroxidation process in hyperoxic lung and pulmonary endothelial cells. We found that hyperoxia-induced apoptosis was accompanied by non-random oxidation of pulmonary lipids. CL and PS were the two major oxidized phospholipids in both hyperoxic lung and mouse lung endothelial cells (MLEC). ESI-MS analysis revealed the formation of several oxygenation products in CL and PS. Treatment of MLEC with a mitochondria-targeted scavenger of electrons and radicals, GS-nitroxide (XJB-5-131) - a conjugate of hemi-gramicidin S (GS) representing a pentapeptide Leu-D-Phe-Pro-Val-Orn, with a stable nitroxide radical, 4-amino-Tempo - suppressed oxidation of both CL and PS and reduced hyperoxia induced apoptosis. Based on previous findings and the results of this work, we speculate that cyt c driven oxidation of CL and PS is associated with the signaling roles of these oxygenated phospholipid species in the execution of apoptosis and the clearance of pulmonary endothelial cells, thus contributing to hyperoxic lung injury.
Material and Methods

**Hyperoxia in vivo.** Wild type C57Bl6 male mice were placed in a plexiglass chamber with food and water *ad libitum* and exposed to 100% oxygen for up to 72 hrs with oxygen flowing at a rate of 15 L/min. Oxygen saturation in the chamber was measured periodically with an oxygen analyzer from Vascular Technology, Inc. All procedures were pre-approved and performed according to the protocols established by the Institutional Animal Care and Use Committee of the University of Pittsburgh. The choice of 72 hrs of exposure to hyperoxia was based on the published data that this is immediately before the lethal endpoint of hyperoxic lung injury with concomitant increase in neutrophil recruitment to the lungs (46).

**Isolation of murine pulmonary endothelial cells (MLEC).** Mice were sacrificed by CO2 inhalation and the chest was opened. Lungs were flushed with HBSS containing 10 U/mL heparin, removed, finely minced and digested in type I collagenase. The mixture was filtered, centrifuged and resuspended, and then incubated with magnetic beads coated with antibody (rat anti-mouse) to PECAM-1 (BD Pharmingen). Magnetic beads were removed gently via series of rinses (trypsin/EDTA) and cells isolated for subculture (60). At approximately passage 2, cells were incubated with fluorescently-labeled diacetylated LDL (diI-LDL) and sorted to homogeneity by FACS. The enriched PECAM and diI-LDL population was sub-cultured on a collagen/gelatin matrix in 2% O2, 5% CO2, 93% nitrogen in a Coy Hypoxic Glove Box/Chamber in Opti-MEM (Gibco), 10% FBS, 2 mM glutamine, 0.2% retinal derived growth factor (Vec Technologies), 10 U/mL heparin, 0.1 mM non-essential amino acid supplement (Gibco) and 55 µM β-mercaptoethanol. MLEC maintain an endothelial cell morphology and phenotype (PECAM positive; uptake of diI-LDL) for longer periods (e.g. more subcultures) when grown in 2% oxygen rather than room air.
**Exposure of MLECs to oxygen.** Cultured MLEC were exposed to 95% O₂ and 5% CO₂ for 72 hrs in a 37 °C humidified BillupsRothenberg modular incubator and contrasted their response to MLEC cultured at 2% O₂, 5% CO₂, and 93% N₂ in a Coy Hypoxic Glove Box/Chamber. At the end of exposure, caspase 3 and 7 activity was measured and lipids were extracted for analysis. A subgroup of MLEC was grown in room air and in early passage was placed in hyperoxia and caspase 3/7 activity measured after 72 hrs.

**Caspase 3/7 activity** in lung homogenates as well as in MLEC was measured using a luminescence Caspase-Glo™ assay kit obtained from Promega (Madison, WI). Luminescence was determined at zero time and following 1 hr incubation at 25 °C using a ML1000 luminescence plate reader (Dynatech). Caspase-3 activity was expressed as the luminescence produced within 1 h incubation per µg of protein.

**Effect of GS-nitroxide-XJB-131 on hyperoxia exposed MLEC.** MLEC were exposed to 95% O₂ (and 5% CO₂) for 72 hrs with or without addition of mitochondria-targeted electron scavenger, XJB-131 (20 uM), and oxidized phospholipids (see below) and apoptosis (as determined by caspase 3/7 activity) were analyzed.

**Immunohistochemical assessment of apoptosis.** Mice were anesthetized after 72 hrs of hyperoxia (or control), the chest was opened and the vascular space was flushed with PBS. Lungs were inflated with 2% paraformaldehyde and frozen sections were used for immunofluorescent detection of apoptosis using an Alexa 488-labeled Nick End labeling kit for assessing apoptosis from Promega (Madison, WI). All nuclei, including apoptotic nuclei, were labelled with DAPI such that healthy nuclei appear blue and apoptotic nuclei are labelled blue and green. To define the endothelial cell population, sections were also labelled with rat anti-mouse antibody to CD31 (an endothelial cell specific marker) (BD Pharmingen) and goat anti-rat
Cy3 conjugated secondary antibody (Jackson Immunoresearch). Images are confocal single plane images from an Olympus Fluoview 1000 scanning confocal microscope (Olympus America).

_Lipid extraction and 2D-HPTLC analysis._ Total lipids were extracted from lung homogenates and MLEC by Folch procedure (18). Lipid extracts were separated and analyzed by 2D-HPTLC (53). To prevent oxidative modification of phospholipids during separation plates were treated with methanol containing 1 mM EDTA, 100 µM DTPA prior to application and separation of phospholipids by 2D-HPTLC. Total lipids (60 nmol) were applied onto plates under flow of N₂ and the plates were first developed with a solvent system consisting of chloroform: methanol: 28% ammonium hydroxide (65:25:5 v/v). After the plates were dried with a forced N₂ blower to remove the solvent, they were developed in the second dimension with a solvent system consisting of chloroform:acetone:methanol:glacial acetic acid:water (50:20:10:10:5 v/v). The phospholipids were visualized by exposure to iodine vapors and identified by comparison with authentic phospholipid standards. For electrospray ionization mass spectrometry (ESI-MS) and analysis of phospholipid hydroperoxides (PL-OOH) by fluorescence HPLC using Amplex Red, the phospholipid spots on the silica plates were visualized by spraying the plates with deionized water. Subsequently, the spots were scraped from the silica plates and phospholipids were extracted in chloroform:methanol:water (10:5:1 v/v). Lipid phosphorus was determined by a micro-method (7).

_Quantitation of lipid hydroperoxides._ Lipid hydroperoxides were determined by fluorescence HPLC of resorufin formed in peroxidase-catalyzed reduction of specific PL-OOH with Amplex Red after hydrolysis by porcine pancreatic phospholipase A₂ (1 U/µl) in 25 mM phosphate buffer containing 1.0 mM Ca, 0.5 mM EGTA and 0.5 mM SDS (pH 8.0 at RT for 30 min). For the
peroxidase reaction, 50 µM Amplex Red and microperoxidase-11 (1.0 µg/µL) were added to the hydrolyzed lipids, and the samples were incubated at 4 °C for 40 min. The reaction was started by addition of microperoxidase-11 and terminated by a stop reagent (100 µL of a solution of 10 mM HCl and 4 mM BHT in ethanol). The samples were centrifuged at 10,000 g for 5 min and the supernatant was used for HPLC analysis. Aliquots (5 µL) were injected into a C-18 reverse phase column (Eclipse XDB-C18, 5 µm, 150 x 4.6 mm) and eluted using a mobile phase composed of 25 mM KH$_2$PO$_4$ (pH 7.0)/methanol (60:40 v/v) at a flow rate of 1 mL/min. The resorufin fluorescence was measured at 590 nm after excitation at 560 nm. Shimadzu LC-100AT vp HPLC system equipped with a fluorescence detector (model RF-10Ax) and an autosampler (model SIL-10AD vp) was used (64, 67).

*Electrospray ionization tandem mass spectrometry.* ESI-MS analysis was performed by direct infusion into linear ion-trap mass spectrometer LXQ™ with the Xcalibur operating system (Thermo Fisher Scientific, San Jose, CA) as previously described (64, 67). Samples collected after 2D-HPTLC separation were evaporated under N$_2$, re-suspended in chloroform:methanol 1:1 v/v (20 pmol/µL) and directly utilized for acquisition of negative-ion or positive-ion ESI mass spectra at a flow rate of 5 µL/min. The ESI probe was operated at a voltage differential of 3.5-5.0 kV in the negative or positive ion mode. Capillary temperature was maintained at 70 or 150°C. Using full range zoom (200-2000 m/z) in positive and negative ion mode, the profile spectra were acquired. Tandem mass spectrometry (MS/MS analysis) of individual phospholipid species was employed to determine the fatty acid composition. The MS/MS spectra were acquired using isolation width 1.0 m/z. Singly-charged ions were used for structural identification of CL as described by Hsu and Turk (25). The scan time setting of ion trap for full MS (range 1400-1600 m/z) was set at 50 microscans with maximum injection time 1000 msec. MS$^n$ analysis was performed using isolation width of 1 m/z, 5 microscans with a maximum injection time of 1000 msec. Two ion activation techniques were used for MS analysis: collision-
induced dissociation (CID, Q=0.25, low mass cut off at 28% of the precursor m/z) and pulsed-Q dissociation technique (PQD, with Q=0.7, and no low mass cut off for analysis of low molecular weight fragment ions). Based on the MS fragmentation data, the chemical structures of lipid molecular species were drawn using ChemDraw and confirmed by comparing with the fragmentation patterns presented in the Lipid Map Data Base (www.lipidmaps.org). Additionally, to quantitatively assess different molecular species of CL and PS, LC/ESI-MS was performed using a Dionex Ultimate™ 3000 HPLC coupled on-line to ESI and a linear ion trap mass spectrometer (LXQ Thermo-Fisher). The lipids were separated on a normal phase column (Luna 3 µm Silica 100A, 150x2 mm, (Phenomenex, Torrance CA)) with flow rate 0.2 mL/min using gradient solvents containing 5 mM CH₃COONH₄ (A – n-hexane: 2-propanol: water, 43:57:1 (v/v/v) and B - n-hexane: 2-propanol: water, 43:57:10 (v/v/v). Analysis of phospholipid oxidized molecular species (hydroperoxy- and hydroxy-) was performed as described (67). To account for isotopic interferences, we performed isotopic corrections by entering the chemical composition of each species into the Qual browser of Xcalibur (operating system) and using the simulation of the isotopic distribution to make adjustments for the major peaks. To minimize isotopic interferences between isolated masses M+2, the MS/MS spectra were acquired using an isolation width of 1.0 m/z. For identification and characterization of phospholipid molecular species, the spectra of CL and PS were acquired in negative mode and the scan data type was set to profile. For quantitative assessments of CL and PS molecular species in control and hypoxic lung, we used LC/MS. Because of the large size of LC/MS files obtained in the profile mode, the scan data type was set to centroid.

**Statistics.** The results are presented as mean ± S.D. values from at least three experiments, and statistical analyses were performed by either paired/unpaired Student's t-test or one-way ANOVA. The statistical significance of differences was set at p< 0.05.
Results

Effect of hyperoxia on phospholipid composition in mouse lung

First, we studied the effects of hyperoxia on the composition of the major phospholipids in the mouse lung. Lipids from the lungs of normal mice and mice exposed to hyperoxia (100% oxygen, 72 hrs) were isolated and separated by 2D-HPTLC (Fig. 1A insert). To quantitate phospholipids, the silica spots were scraped off the plates and lipid phosphorus was determined. The major classes of mouse lung phospholipids - in the order of their abundance - were: phosphatidylcholine (PC) > phosphatidylethanolamine (PE) > phosphatidylserine (PS) = phosphatidylinositol (PI) > CL > sphingomyelin (Sph) (Fig. 1A). No significant changes in the composition of the phospholipids were detected after exposure to hyperoxia (Fig.1A).

Effect of hyperoxia on the accumulation of hydroperoxy phospholipids in the mouse lung

The amounts of PL-OOH in major phospholipids were detected after their separation by 2D-HPTLC using the HPLC Amplex Red protocol (64, 67). Only two anionic phospholipids - CL and PS – underwent robust oxidation in the hyperoxic mouse lung (Fig. 1B). The CL hydroperoxide (CL-OOH) content was as high as 33.8 ± 8.0 pmol/nmol of CL for hyperoxia vs 4.5 ± 0.3 pmol/nmol of CL in the lung of control mice. The amount of accumulated PS hydroperoxide (PS-OOH) in the hyperoxic lung was 7.6 ± 1.7 pmol/nmol of PS vs 2.6 ± 0.7 in the control lung. Hyperoxia-induced increments of CL-OOH and PS-OOH constituted 29.3 pmol/nmol CL and 5.0 pmol/nmol PS, respectively. No significant oxidation in the major phospholipid classes such as PC, PE and PI was determined in the hyperoxic lung.

ESI-MS analysis of CL and PS molecular species of lung phospholipids

Molecular species of CL and PS were characterized by ESI-MS using the negative mode. Direct infusion experiments as well as LC/ESI-MS were employed in MS and MS^n analysis for identification of the molecular species containing polyunsaturated fatty acid residues that are
highly susceptible to oxidation (64). Typical full LC/ESI-MS spectra of lung CL and PS are presented in Fig 2A and 4A, respectively. CL molecular species were represented by four clusters (Fig. 2A). The major cluster contained molecular ions with \( m/z \) 1447.8, 1449.8. Molecular clusters at \( m/z \) 1421.8, 1473.8, and 1497.9 were also detectable in the full MS spectrum, but at relatively lower abundances. Analysis of PS revealed three major molecular clusters with \( m/z \) 788.5, 810.5 and 834.5. To identify molecular species of phospholipids we performed MS\(^2\) experiments using the PQD technique. Fragmentation (MS\(^3\)) analysis showed that both CL and PS contained linoleic (C\(_{18:2}\)), arachidonic (C\(_{20:4}\)) docosapentaenoic (C\(_{22:5}\)) and docosahexaenoic acid (C\(_{22:6}\)) (Table 1, 2). A quantitative assessment of these phospholipids revealed that CL was enriched with molecular species containing C\(_{18:2}\) (Table 1). PS was mainly represented by molecular species containing C\(_{20:4}\) and C\(_{22:6}\) (Table 2).

**Identification and quantitative analysis of oxidized molecular species of CL in hyperoxic mouse lung.**

Mouse lung CL was enriched with molecular species containing C\(_{18:2}\) and less unsaturated acyl groups (Table 1). For comparison, the sum of these species was 444.8 pmol/nmol CL vs. 227.2 pmol/nmol CL for molecular species containing at least one acyl group with 4, 5 and 6 double bonds – a 2-fold difference. Oxidation should cause disappearance of “oxidizable” species and appearance of their oxygenated products. Quantitative assessments revealed a significant reduction of CL molecular ions with \( m/z \) 1495.8, 1497.8 and 1499.8, i.e. less abundant but more unsaturated species (Fig. 2B,C). The amount of these molecular species was decreased 2.3-, 2.7- and 1.8-fold, respectively, after hyperoxia vs. the control mice. Essentially no quantitative changes were found in the clusters of less polyunsaturated CL molecules with \( m/z \) 1471.8-1475.8. While the relative intensity of most abundant molecular ion with \( m/z \) 1447.8, containing four C\(_{18:2}\) acyl residues, was slightly decreased in the hyperoxic lung compared to the control lung, quantitative assessments revealed no significant differences between the two types of
samples. In line with this, we found that CL oxidation involved less abundant molecular species containing – along with C\textsubscript{18:2} - C\textsubscript{20:4}, C\textsubscript{22:5} and C\textsubscript{22:6} fatty acid residues. As expected, the disappearance of “oxidizable” CLs was accompanied by the emergence of their oxygenated counterparts as illustrated by a typical fragmentation pattern of the CL molecular ion with m/z 1499.8 (Fig. 3Aa). Molecular ions of C\textsubscript{18:2} (m/z 279.3), C\textsubscript{18:1} (m/z 281.3), C\textsubscript{20:4} (m/z 303.3), C\textsubscript{22:6} (m/z 327.3) C\textsubscript{22:5} (m/z 339.3) along with other ions typical for CL fragmentation were detected in the MS\textsuperscript{2} spectrum (Fig. 3Ab). A molecular ion with m/z 1499.8 was represented by three highly unsaturated molecular species: C\textsubscript{18:1}/C\textsubscript{20:4}/C\textsubscript{20:4}/C\textsubscript{18:1}, C\textsubscript{18:0}/C\textsubscript{18:2}/C\textsubscript{18:2}/C\textsubscript{22:6}, C\textsubscript{18:1}/C\textsubscript{18:2}/C\textsubscript{18:2}/C\textsubscript{22:5}. Oxidized molecular species of CL with m/z 1528.1, 1530.1, 1532.1 were present in the spectra of from the hyperoxic lung. The MS\textsuperscript{2} analysis showed that these oxidized molecular species originated from the molecular species with m/z 1495.8, 1497.8 and 1499.8, respectively, after addition of two oxygen atoms. Using an MS\textsuperscript{2} analysis, we identified the peroxidized CLs as mono-hydroperoxy (C\textsubscript{18:1}/C\textsubscript{18:1}/C\textsubscript{18:2}-OOH/C\textsubscript{22:6}) and di-hydroxy (C\textsubscript{18:1}/C\textsubscript{20:4}-OH/C\textsubscript{20:4}-OH/C\textsubscript{18:1}/ and C\textsubscript{18:0}/C\textsubscript{18:2}-OH/C\textsubscript{18:2}/C\textsubscript{22:6}-OH) (Fig. 3Ba). The molecular ions of C\textsubscript{18:2}-OH (m/z 295.3), C\textsubscript{18:2}-OOH (m/z 311.3), C\textsubscript{20:4}-OH (m/z 319.3), and C\textsubscript{22:6}-OH (m/z 343.3) were identified after fragmentation of the parent ion with m/z 1532.1 (Fig. 3Bb). Thus, CL oxidation involves less abundant but highly unsaturated molecular species of CL which underwent oxidative modification under hyperoxic conditions. In some of them, however, oxygenation occurred not as expected in the most polyunsaturated acyl groups (C\textsubscript{20:4} and C\textsubscript{22:6}) but in the C\textsubscript{18:2} acyl moieties.

Identification and quantitative analysis of oxidized molecular species of PS in the hyperoxic mouse lung.

A comparative LC/ESI-MS analysis of PS isolated from normal and hyperoxic lungs detected the decrease in intensity of two molecular ions with m/z 810.5 (C\textsubscript{18:0}/C\textsubscript{20:4}) and 838.5 (C\textsubscript{18:0}/C\textsubscript{22:4}) (Fig. 4B). Accordingly, the quantitative assessment of PS from the hyperoxic lung revealed that the amounts of PS-C\textsubscript{18:0}/C\textsubscript{20:4}, and PS-C\textsubscript{18:0}/C\textsubscript{22:4} were reduced as well and were 135.0 ± 3.6 and
50.9 ± 2.4 pmol/nmol PS vs. 162.2 ± 7.7 and 69.0 ± 4.1 in control lung, respectively (Fig. 4C). It is likely that these two molecular species were oxidized in the mouse lung under hyperoxic conditions. To characterize the oxidation products, PS was isolated from hyperoxic lung, separated by 2D-HPLC and utilized for direct infusion ESI-MS experiments. The results of a fragmentation experiment (MS² spectra) using non-oxidized PS with m/z 810.5 and oxidized PS with m/z 842.5 are presented in Fig 5 Aa and Fig.5Bb, respectively. Daughter ions at m/z 283.3 and 303.3 corresponding to C₁₈:₀ and C₂₀:₄ along with other ions typical for PS fragmentation were detectable in the MS² spectrum of the molecular ion with m/z 810.5 (Fig. 5Aa). MS² fragmentation of the molecular ion with m/z 842.5 showed two overlapping molecular species. One of them is non-oxidized PS containing C₁₈:₁ (m/z 281.3) and C₂₂:₁ (m/z 337.3) fatty acids (Fig. 5Ab). Another one likely corresponds to a monohydroperoxy C₁₈:₀/C₂₀:₄-OOH or dihydroxy C₁₈:₀/C₂₀:₄-2OH molecular species originating from a molecular species with m/z 810.5 containing C₂₀:₄ in the sn-2 position. The molecular ions of C₁₈:₀ (m/z 283.3) and C₂₀:₄-OOH (m/z 335.3) fatty acids were detected in the MS² spectrum (Fig. 5Ab,c). In addition, we performed the MS² analysis of non-oxidized PS with m/z 838.5 (Fig. 5Ba) and an oxidized PS molecular species with m/z 870.5 (Fig. 5Bb). Daughter ions with m/z 283.3 and m/z 331.3 were detected in the MS² spectrum of the molecular ion with m/z 838.5 and corresponded to C₁₈:₀ and C₂₂:₄ fatty acids. The fragmentation pattern of molecular ion with m/z 870.5 revealed the presence of a molecular ion with m/z 363.3 along with an ion with m/z 283.3 corresponding to C₂₂:₄ containing two oxygens and C₁₈:₀ fatty acids, respectively (Fig. 5Bb,c). Thus, the detailed analysis of oxidized PS molecular species with m/z 842.5 and 870.5 confirmed that they originated from a molecular species of PS containing fatty acids with four double bonds (m/z 810.5 and 838.5) after addition of two oxygens and contain hydroperoxy-C₂₀:₄ (C₂₀:₄-OOH) and hydroperoxy-C₂₂:₄ (C₂₂:₄-OOH) or di-hydroxy-C₂₀:₄ (C₂₀:₄-2OH) and dihydroxy-C₂₂:₄ (C₂₂:₄-2OH) fatty acids, respectively. Accumulation of a hydroxy- and hydroperoxy-molecular species of PS originating from PS-C₁₈:₀/C₂₂:₆ was also observed (data not shown). Notably, hyperoxia-induced oxidation of PS
occurred only in acyl groups with four and six double bonds – in contrast to CL peroxidation where C\textsubscript{18:2} fatty acid residues were involved in the oxidative peroxidation along with acyl moieties containing four and six double bonds. No oxidation products in the other major phospholipid classes such as PC, PE and PI were detected by ESI-MS, in spite of the fact that these phospholipids contain highly unsaturated fatty acid residues (67).

**Hyperoxia-induced apoptosis in the lung**

Recently, we demonstrated that the selective oxidation of CL and PS is important for the execution of apoptotic cell death and the clearance of apoptotic cells by macrophages (33, 36). We reasoned that the enhanced oxidation of CL and PS in the hyperoxic lung might be associated with the activation of apoptosis. Measurements of caspase 3/7 activity in mouse lung 72 hrs after hyperoxia revealed a 1.4-fold higher activity vs. controls (p<0.05) (Fig. 6A). Furthermore, histochemical staining for TUNEL activity, 72 hrs after hyperoxia, revealed increased numbers of apoptotic (green) cells (indicated by yellow arrows) many of which were derived from CD31 positive (red) endothelial cells (Fig. 6B).

**Identification and quantitative analysis of phospholipid molecular species in MLECs exposed to hyperoxia.**

Assuming that the endothelium is a critical target of hyperoxic lung injury (16, 76), we performed oxidative lipidomics studies using MLEC isolated from mouse lung. We detected no significant changes in the phospholipid composition in MLEC exposed to hyperoxia (Fig. 7A). Similar to our results on phospholipid oxidation in the lung, hyperoxia caused the oxidation of two phospholipids, CL and PS (Fig. 7B). Hyperoxia-induced oxidation of CL in MLEC was very robust. In MLEC exposed to hyperoxia, the content of CL-OOH was estimated as 91.8 ± 0.9 pmol/nmol of CL vs. 5.3 ± 0.6 in control non-exposed MLEC. The hyperoxia-induced accumulation of PS-OOH in MLECs (35.5 ± 0.3 vs. 13.7 ± 4.6 pmol PS-OOH per nmol of PS)
was also significantly higher in MLEC than in hyperoxic lung. The hyperoxia-induced increases in the contents of CL-OOH and PS-OOH were 86.5 pmol/nmol CL and 21.8 pmol/nmol PS, respectively. No significant oxidation in the other major phospholipid classes such as PC, PE and PI was found.

Similar to the lung, molecular species of CL containing C_{18:2} were dominant in MLECs with two additional molecular species (compared to the lung) with \( m/z \) 1453.8 and 1455.8 and corresponding to \( C_{18:2}/C_{18:2}/C_{18:1}/C_{18:1} \) and \( C_{18:2}/C_{18:2}/C_{18:1}/C_{18:1} \), respectively (Table 1). We found no differences in the pattern of PS molecular species between the whole lung and MLEC. The same molecular species of PS were detected on MS spectra obtained from both whole lung and MLEC. However, PS in MLEC was significantly enriched with molecular species at \( m/z \) 788.5, and 836.5 corresponding to PS-\( C_{18:0}/C_{18:1} \) and PS-\( C_{18:0}/C_{22:5} \), respectively. In addition, the amounts of two molecular species of PS-\( C_{18:0}/C_{20:4} \) (\( m/z \) 810.5) and PS-\( C_{18:1}/C_{22:6} \) (\( m/z \) 832.5) were significantly lower in MLEC as compared to the lung. Three molecular species containing unsaturated fatty acids \( C_{18:0}/C_{20:4}, C_{18:0}/C_{22:6}, C_{18:0}/C_{22:6} \) along with highly saturated \( C_{18:0}/C_{18:0} \) were most abundant in MLEC (Table 2).

**Identification and quantitative analysis of oxidized molecular species of phospholipids in MLECs**

Similarly to the lung, oxidation of only two phospholipids - CL and PS - was detected in MLECs after exposure to hyperoxia (Fig. 8). Decreased intensity of PS molecular ions corresponding to molecular species with \( C_{22:6}, C_{22:5}, C_{22:4} \) in the \( sn-2 \) position was detected in LC/MS spectra of PS of hyperoxic cells. Specifically, the contents of PS-\( C_{18:0}/C_{22:6} \) (\( m/z \) 834.5), PS-\( C_{22:5}/C_{18:2} \) (\( m/z \) 836.5), and PS-\( C_{18:0}/C_{22:4} \) (\( m/z \) 838.5) in hyperoxia-exposed cells were 81.4 ± 4.3, 136.3 ± 2.8, and 69.3 ± 6.8 pmol/nmol PS vs. 131.8 ± 11.0, 166.7 ± 8.9 and 97.7 ± 5.1 in control cells, respectively (Fig. 8B). Quantitative MS analysis of CL in MLEC exposed to hyperoxia revealed significantly lower amounts of molecular species with \( m/z \) 1473.8, 1475.8, 1495.8, 1497.8 and
1499.8 (Fig. 8A). In addition, a significant decrease of the amounts of CL molecular species with m/z 1423.8 (C_{16:1}/C_{18:2}/C_{18:2}/C_{18:2}) 1425.8 (C_{16:1}/C_{18:2}/C_{18:1}/C_{18:1} or C_{16:0}/C_{18:2}/C_{18:2}/C_{18:1}) and 1447.8 (C_{18:2}/C_{18:2}/C_{18:2}/C_{18:2}), was also observed in MLEC after exposure to hyperoxia. Notably, a 2.5-fold activation of caspase 3/7 occurred in MLECs exposed to hyperoxia for 72 hrs (Fig. 9). We noted a similar 2-fold activation of caspase 3/7 in MLEC that were grown in room air and then placed in hyperoxia for 72 hrs (data not shown).

Mitochondria-targeted nitroxide (XJB-5-131) suppresses hyperoxia induced CL and PS oxidation in MLEC.

As a mitochondria-specific phospholipid, CL peroxidation occurs in this organelle likely due to the deregulated electron transport and production of reactive oxygen species (45, 52). Recently, we have developed mitochondria-targeted electron scavengers – conjugates of hemigramicidin S with nitroxide radicals (GS-nitroxides) - capable of preventing reactive oxygen species production and CL peroxidation in mitochondria (29, 74). We reasoned that GS-nitroxides may be effective in suppressing hyperoxia-induced phospholipid peroxidation in MLEC. Indeed, we found that GS-nitroxide (XJB-5-131) was able to protect CL (Fig. 10A) in mitochondria; moreover, PS peroxidation was also inhibited in hyperoxic MLEC (Fig. 10B). In addition, XJB-5-131 (10-40 μM) decreased hyperoxia-induced apoptosis in MLEC (as quantified by caspase 3/7 activation) in a concentration dependent manner (Fig. 9).
Discussion

Hyperoxic lung injury has generally been assumed to be a “free radical disease” resulting from an imbalance in production and elimination of partially reduced oxygen and nitrogen species (50, 56). The importance of these free radical pathways in mediating hyperoxic lung injury is supported by: a) observations that tolerance (secondary to LPS, cytokines or hyperoxia itself) is associated with induction of various antioxidants (15, 19, 26, 58); b) administration of antioxidants ameliorates such injury (13, 47, 61, 73); and c) overexpression of various forms of superoxide dismutase (SOD) protects transgenic mice against hyperoxia (1, 72, 75). The major sources of oxygen radicals, particularly superoxide anion-radical, are the increased electron leakage from mitochondrial respiration (20, 54) and the activation of NAD(P)H oxidases, including NOX family NADPH oxidases (11, 56). Both mechanisms have been confirmed in pulmonary capillary endothelial cells in situ (8, 56). Additionally, superoxide can be produced via one electron oxidation of oxy-ferro-Hb to its ferric form (10), particularly after Hb release form erythrocytes during hyperoxia induced hemolysis (12, 31).

Hyperoxia has been also known to activate cell death mechanisms in pulmonary endothelial cells (5, 48, 59), whereby both necrotic and apoptotic pathways are considered as important components of acute lung injury (5, 48, 59). Direct evidence for apoptotic vs. necrotic cell death pathways are equivocal: several studies demonstrated increased biomarkers of apoptosis (40, 48, 49, 71), whereas other workers failed to document apoptosis under hyperoxic conditions (5). Nonetheless, in the current study we used immunohistochemical (Tunel) techniques to reveal hyperoxia induced apoptosis of endothelial (CD31) origin and overall, extrinsic (Fas-mediated) and intrinsic (mitochondria-mediated) apoptosis is recognized as a prominent part of hyperoxic acute lung injury (40, 49, 70, 71). The role of mitochondria-dependent cell death signaling in the pathogenesis of hyperoxia-induced lung injury has been intensively discussed recently (9, 48, 59, 71). One of the major hallmarks of apoptosis is caspase cascade activation (13, 37, 77).
Both initiator and effector caspases are activated during hyperoxia through an angiopoietin 2 (Ang-2) dependent pathway (59). Accordingly, we found 1.4-fold increases in caspases 3 and 7 activity in the hyperoxic lung and prolonged exposure to hyperoxia resulted in endothelial cell apoptosis (Fig. 6) adding to insight into injury and death of endothelial cells in the lung (4, 48). In this regard, it is noteworthy that elements of hyperoxia and apoptosis could be revealed in 2-fold activation of caspases 3 and 7 that occurred in MLEC exposed to hyperoxia.

Execution of the mitochondrial stage of apoptosis has long been associated with the oxidation of CL (27, 36, 51). While the total content of CL was not altered by hyperoxia, significant changes were detected within individual species of CL - decreased amounts of several oxidizable molecular species of CL were found after hyperoxia. Our previous studies established that CL represents a selective target of oxidative attack, whereby cyt c forms a complex with CL, and catalyzes the oxidation, thus facilitating the release of pro-apoptotic factors, including cyt c, into the cytosol (36). Here, we demonstrate that hyperoxia-induced apoptosis is accompanied by CL oxidation in MLEC as well as in the lung. The remarkable feature of CL peroxidation during hyperoxia-induced apoptosis is its non-stochastic nature as evidenced by our oxidative lipidomics MS-analyses and identification of the hydroxy- and hydroperoxy-species of CL formed. The specific character of the peroxidation process has two manifestations: i) only CL but not other markedly more abundant phospholipids present in mitochondria – PC, PE – were involved in the process, and ii) not all but selected molecular species of oxidizable CL – C_{18:1}/C_{18:1}/C_{18:2}/C_{22:6}, C_{18:1}/C_{20:4}/C_{20:4}/C_{18:1}/ and C_{18:0}/C_{18:2}/C_{18:2}/C_{22:6} – were oxygenated, while others with highly oxidizable acyl groups remained non-oxidized. Moreover, in some batches of CL, with molecular species containing both C_{18:2} and C_{22:6} unsaturated, oxygenated products were detected in less polyunsaturated C_{18:2} acyl groups but not in C_{22:6} acyl moieties. While the mechanisms of selectivity towards CL are likely associated with the catalytic role of cyt c (3, 36) and its high affinity binding to CL, the specificity towards individual molecular species of CL
remains to be elucidated. Assuming that high affinity binding with cyt c and catalytic competence of the latter towards CL are the major prerequisites of hyperoxia-induced CL peroxidation, it is conceivable that the selectivity is due to the spatial separation, i.e. inaccessibility of specific CL molecular species confined to the inner leaflet of the mitochondrial inner membrane to cyt c from the intermembrane space.

Cyt c released from mitochondria into the cytosol can bind with PS and cause its peroxidation via the same peroxidase mechanisms to yield hydroxy- and hydroperoxy-species of PS that have been detected in the hyperoxic lung as well as in MLEC. Similarly to CL, total content of PS in the mouse lung was not changed after exposure to hyperoxia. However a significant decrease in the amounts of several oxidizable molecular species of PS was detected within individual species of PS. The signaling role of oxidized PS is realized through its participation in facilitating PS externalization and the appearance of PS and its oxidation products on the surface of apoptotic cells (65, 68). Both PS and PS oxidation products are recognition signals for several receptors (22) and subsequent clearance by macrophages (6, 34).

Recently, using pulmonary endothelial cells and LPS as an \textit{in vitro} model of acute lung injury, we demonstrated that LPS-induced apoptosis in sheep pulmonary endothelial cells was accompanied by accumulation of CL and PS oxidation products (63). Moreover, similar CL and PS oxidation products were detected in model systems in the cyt c driven reaction in the presence of H$_2$O$_2$ (63, 66). We speculate that the cyt c driven oxidation of CL and PS is associated with the execution of apoptosis in pulmonary endothelial cells, thus contributing to hyperoxic acute lung injury.

The pulmonary endothelium is the locus of early structural and functional changes in hyperoxic lung injury and apoptosis of pulmonary endothelium is a contributing factor to the genesis and
maintenance of hyperoxic lung injury. Nonetheless, the early molecular pathways that account for hyperoxic-induced pulmonary endothelial cell apoptosis are unknown and therapies for acute lung injury remain entirely palliative. A rational approach may be the delivery of agents selectively to the pulmonary endothelium and, more precisely, to mitochondria, to inhibit apoptosis before the point-of-no-return – release of pro-apoptotic factors from mitochondria and caspase activation. Inhibition of PS oxidation in cells treated with GS-nitroxide could be due to the drug’s ability to prevent release of cyt c from mitochondria. Our previous work demonstrated that oxidation of CL is essential for the mitochondrial membrane permeabilization and release of cyt c into the cytosol (36). The released cyt c can interact with PS localized in the inner leaflet of plasma membrane and form cyt c/PS peroxidase complex with PS oxidation catalytic competence. Accordingly, GS-nitroxide-dependent prevention of CL oxidation and cyt c release from mitochondria can be responsible for the inhibition of PS oxidation. We suggest that prevention of CL oxidation may be a preferred target for anti-apoptotic strategies to protect pulmonary endothelial cells during acute lung injury. Homologues of hemi-gramicidin S attached to cyclic nitroxides, GS-Tempo conjugates, showed a high level of effectiveness in the accumulation in mitochondria (30, 74). Indeed, we found that the electron scavenging activity of GS-nitroxide conjugates was associated with the inhibition of CL and PS oxidation in MLEC exposed to hyperoxia. Importantly, this effect of XJB-5-131 was associated with a decreased hyperoxia-induced apoptosis in pulmonary endothelium (as quantified by caspase 3/7 activation). Recently, we noted that XJB-131 could inhibit oxidant mediated apoptosis in intact organs of experimental animals, in vivo (39), and thus future studies will be focused on developing additional mitochondria-targeted strategies to protect the lung against hyperoxia-induced injury.

Supported by NIH HL70755, HL094488 and HL65697.
References


Table 1. Identification and content of major CL molecular species in mouse lung and mouse lung endothelial cells

<table>
<thead>
<tr>
<th>m/z</th>
<th>CN:DB</th>
<th>Molecular species</th>
<th>Whole mouse lung pmol/nmol total CL</th>
<th>MLEC pmol/nmol total CL</th>
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<tr>
<td>1421.8</td>
<td>70:7</td>
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<td>C_{16:0}/C_{18:2}/C_{18:2}/C_{18:1}</td>
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<td>72:8</td>
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<td>1449.8</td>
<td>72:7</td>
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<td>140.6 ± 4.1</td>
<td>99.4 ± 7.0*</td>
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<td>49.2 ± 4.3</td>
<td>6.9 ± 0.7*</td>
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<td>64.8 ± 4.8</td>
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All data are Mean ± SEM, for Whole lung n=3, for MLEC n=4; *p<0.01 vs. whole lung. CN- carbon number, DB- double bonds.
**Table 2.** Identification and content of major PS molecular species in mouse lung and mouse lung endothelial cells

<table>
<thead>
<tr>
<th>m/z</th>
<th>CN:DB</th>
<th>Molecular species</th>
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<th>MLEC pmol/nmol total PS</th>
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<td>40.8 ± 4.7</td>
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<td>175.9 ± 6.8*</td>
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<td>810.5</td>
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<td>C_{18:0}/C_{20:4}</td>
<td>158.7 ± 5.8</td>
<td>111.7 ± 10.0*</td>
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<td>812.5</td>
<td>38:3</td>
<td>C_{18:0}/C_{20:3}</td>
<td>60.0 ± 2.1</td>
<td>77.4 ± 7.9</td>
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<tr>
<td>832.5</td>
<td>40:7</td>
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<td>13.9 ± 2.0</td>
<td>3.6 ± 2.0*</td>
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<td>834.5</td>
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<td>166.7 ± 8.9*</td>
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<td>838.5</td>
<td>40:4</td>
<td>C_{18:0}/C_{22:4}</td>
<td>65.8 ± 3.8</td>
<td>97.7 ± 5.1*</td>
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All data are Mean ± SEM, for whole lung n=3, for MLEC n=4; *p<0.01 vs. whole lung, CN - carbon number, DB - double bonds.
Figure legends

**Figure 1.** Phospholipid composition and accumulation of phospholipid hydroperoxides in lung of mouse exposed to hyperoxia.

A. Phospholipid composition of control and hyperoxic lung. Mice were exposed to hyperoxia (100% of oxygen) for 72 hrs and sacrificed thereafter. Lipids were extracted, separated by 2D-HPTLC. Spots of phospholipids were scraped and lipid phosphorus was determined. Insert: Typical 2D-HPTLC chromatogram of total lipids extracted from control mouse lung. NL, neutral lipids, PC, phosphatidylcholine, PE, phosphatidylethanolamine, PS, phosphatidylserine, PI, phosphatidylinositol, CL, cardiolipin, Sph, sphingomyelin. Open bars or number 1 – control; closed bars or number 2 – hyperoxia. Data are mean ± SD, n=3 (for control); n=4 (for hyperoxia).

B. Accumulation of PL-OOH in lung of mice exposed to hyperoxia (100% of oxygen for 72 hrs). Lipids were extracted and separated by 2D-HPTLC. PL-OOH were detected using Amplex Red protocol. PC, phosphatidylcholine, PE, phosphatidylethanolamine, PS, phosphatidylserine, PI, phosphatidylinositol, CL, cardiolipin,

Open bars – control; closed bars – hyperoxia. Data are mean ± SD, *p< 0.05 vs. control, n=3 (for control); n=4 (for hyperoxia).

**Figure 2.** Oxidation of CL in lung of mice exposed to hyperoxia.

A. Typical full negative LC/ESI-MS spectrum of CL isolated from control mouse lung. The major cluster contains molecular ions with m/z 1447.8, 1449.8 along with clusters at m/z 1421.8, 1473.8, and 1497.9 were detectable in the spectrum.
B. Typical full negative LC/ESI-MS spectrum of CL isolated from hyperoxic mouse lung. MS analysis revealed significant reduction of CL molecular ions with \( m/z \) 1495.8, 1497.8 and 1499.8.

C. Quantitative assessment of CL molecular species in control lung and lung isolated from mice exposed to hyperoxia. Open bars – control; closed bars – hyperoxia. Data are mean ± SD, *\( p < 0.05 \) vs. control, \( n=3 \) (for control); \( n=4 \) (for hyperoxia).

**Figure 3.** ESI-MS\(^2\) analysis of CL isolated from control and hyperoxic mouse lungs.

A. a) MS\(^2\) fragmentation of CL species with \( m/z \) 1499.8 from control lung.

Ions \([a]^{-}\) and \([b]^{-}\) with \( m/z \) 721.8 corresponding to \( \text{C}_{18:1}/\text{C}_{20:4}\)-PA as well as characteristic daughter \([a+136]^{-}\) and \([b+136]^{-}\) (\( m/z \) 857.0); ions \([a-(\text{C}_{18:1})]^{-}\) and \([b-(\text{C}_{18:1})]^{-}\) (\( m/z \) 440.6); \([a-(\text{C}_{20:4})]^{-}\) and \([b-(\text{C}_{20:4})]^{-}\) (\( m/z \) 419.5) were formed during fragmentation of the molecular ion with \( m/z \) 1499.5 corresponding to molecular species of CL \( \text{C}_{18:1}/\text{C}_{20:4}/\text{C}_{20:4}/\text{C}_{18:1} \). Fragmentation of molecular species of CL \( \text{C}_{18:1}/\text{C}_{18:2}/\text{C}_{18:2}/\text{C}_{22:5} \) revealed the presence of \([a]^{-}\) and \([b]^{-}\) ions with \( m/z \) 697.7 and 746.8; mainly corresponding to \( \text{C}_{18:1}/\text{C}_{18:2}\)-PA and \( \text{C}_{18:2}/\text{C}_{22:5}\)-PA. In addition ions with \( m/z \) 753.6 (\([a+56]^{-}\)); 833.6 (\([a+136]^{-}\)); 802.6 (\([b+56]^{-}\)); 882.6 (\([b+136]^{-}\)) were detected on MS\(^2\) spectrum. Moreover, \([a]^{-}\) and \([b]^{-}\) ions with \( m/z \) 699.5 and 744.6 corresponding to \( \text{C}_{18:0}/\text{C}_{18:2}\)-PA and \( \text{C}_{18:2}/\text{C}_{22:6}\)-PA. In addition ions with \( m/z \) 755.6 (\([a+56]^{-}\)); 835.6 (\([a+136]^{-}\)); 800.4 (\([b+56]^{-}\)); 880.7 (\([b+136]^{-}\)) were formed after fragmentation of molecular ion corresponding to molecular species of CL \( \text{C}_{18:0}/\text{C}_{18:2}/\text{C}_{18:2}/\text{C}_{22:6} \).

b) Part of MS\(^2\) spectrum of molecular ion with \( m/z \) 1499.8 shown in the range of \( m/z \) 270 – 350.

Molecular ions of \( \text{C}_{18:1} \) (\( m/z \) 281.3), \( \text{C}_{18:2} \) (\( m/z \) 279.3), \( \text{C}_{22:5} \) (\( m/z \) 329.3) and \( \text{C}_{22:6} \) (\( m/z \) 327.3) were presented in MS\(^2\) spectrum as well. Thus, MS\(^2\) analysis of the singly-charged ion (\( m/z \) 1499.8) revealed the co-presence of molecular species of CL \( \text{C}_{18:1}/\text{C}_{20:4}/\text{C}_{20:4}/\text{C}_{18:1} \) along with CLs \( \text{C}_{18:1}/\text{C}_{18:2}/\text{C}_{22:5}, \text{C}_{18:0}/\text{C}_{18:2}/\text{C}_{18:2}/\text{C}_{22:6} \).
B. a) MS\(^2\) fragmentation of CL species with \(m/z\) 1532.1 from hyperoxic lung.

Fragments \([a+136+16 \text{ (one oxygen)}]\) and \([b+136+16 \text{ (one oxygen)}]\) of oxidized molecular species of CL \((C_{18:1}/C_{20:4}^{+}\text{OH}/C_{20:4}^{+}\text{OH}/C_{18:1})\) with \(m/z\) 873.5 corresponding to \([C_{18:1}/C_{20:4}^{+}\text{OH}-\text{PA} +136]\) were detected on MS\(^2\) spectrum. In addition, fragmentation of CL molecular species \(C_{18:1}/C_{18:2}^{+}\text{OOH}/C_{18:2}/C_{22:5}\) resulted in appearance of fragments \([a+32 \text{ (two oxygen)}]\) with \(m/z\) 729.6 corresponding to \(C_{18:1}/C_{18:2}^{+}\text{OOH}-\text{PA}\). Moreover, fragments with \(m/z\) 760.8 \([a+16 \text{ (one oxygen)}]\), corresponding to \(C_{18:0}/C_{18:2}^{+}\text{OH}-\text{PA}\), \(m/z\) 896.6 \([a+136+16 \text{ (one oxygen)}]\), corresponding to \(C_{18:0}/C_{18:2}^{+}\text{OH}-\text{PA} +136\), \(m/z\) 771.6 \([b+56+16 \text{ (one oxygen)}]\), corresponding to \(C_{18:2}/C_{22:6}^{+}\text{OH}-\text{PA}\) and, 851.5 \([b+136+16 \text{ (one oxygen)}]\), corresponding to \(C_{18:2}/C_{22:6}^{+}\text{OH}-\text{PA}\) were detected in the MS\(^2\) spectrum and originated from a molecular species of CL , \(C_{18:0}/C_{18:2}^{+}\text{OH}/C_{18:2}/C_{22:6}^{+}\text{OH}\).

b) Part of MS\(^2\) spectrum of molecular ion with \(m/z\) 1532.1 shown in the range of \(m/z\) 270 – 370. Molecular ions of oxygenated fatty acids \(C_{18:2}^{+}\text{OH} \ (m/z\) 295.3), \(C_{18:2}^{+}\text{OOH} \ (m/z\) 311.3), \(C_{20:4}^{+}\text{OH} \ (m/z\) 319.3) and \(C_{22:6}^{+}\text{OH} \ (m/z\) 343.3) alone with non oxidized fatty acids \(C_{18:0} \ (m/z\) 283.3), \(C_{18:1} \ (m/z\) 281.3), \(C_{18:2} \ (m/z\) 279.3), \(C_{22:5} \ (m/z\) 329.3) and \(C_{22:6} \ (m/z\) 327.3) were presented in the MS\(^2\) spectrum of CL molecular ion with \(m/z\) 1532.1.

**Figure 4. Oxidation of PS in lung of mice exposed to hyperoxia.**

A. Typical full negative LC/ESI-MS spectrum of PS isolated from control mouse lung. The major molecular ions of PS with \(m/z\) 788.5, 810.5 and 834.5 were detectable in spectrum.

B. Typical full negative LC/ESI-MS spectrum of PS isolated from hyperoxic mouse lung. The decrease of intensity of two molecular ions with \(m/z\) 810.5 \((C_{18:0}/C_{20:4})\) and 838.5 \((C_{18:0}/C_{22:4})\) in hyperoxic lung was detected.
C. Quantitative assessment of PS molecular species in normal and hyperoxic lung. Open bars – control; closed bars – hyperoxia. Data are mean ± SD, *p< 0.05 vs. control; n=3 (for control); n=4 (for hyperoxia).

**Figure 5.** ESI-MS² analysis of PS isolated from control and hyperoxic mouse lungs.

A. a) MS² fragmentation of PS species with m/z 810.5 from control lung. b) MS² fragmentation of oxidized PS molecular species with m/z 842.5 from hyperoxic lung. c) Part of MS² spectrum of molecular ion with m/z 842.5 shown in the range of m/z 270 – 350.

The ion with m/z 153.0 corresponded to glycerophosphate without molecule of water, a common ion formed during phospholipid fragmentation. Molecular ions with m/z 283.3 and m/z 303.3 corresponding to C₁₈:0 and C₂₀:₄ fatty acids, respectively, were also formed during fragmentation of parent ion with m/z 810.5. Molecular ions with m/z 335.3 corresponding to C₂₀:₄ fatty acid with two oxygen added was formed during fragmentation of parent ion with m/z 842.5. The loss of serine group of PS yielded the fragments with m/z 723.6 and 751.6. Product ions with m/z 419.3 originated from fragments with m/z 723.6 and 755.5 after loss of arachidonic acid C₂₀:₄ and oxidized arachidonic acid C₂₀:₄-OOH, respectively. Thus, the molecular ion at m/z 810.5 corresponds to molecular species of PS C₁₈:₀/C₂₀:₄ whereas the molecular ion with m/z 842.5 corresponds to molecular species of oxidized PS C₁₈:₀/C₂₀:₄-OOH.

B. a) MS² fragmentation of PS species with m/z 838.5 from control lung. b) MS² fragmentation of oxidized PS molecular species with m/z 870.6 from hyperoxic lung. c) Part of MS² spectrum of molecular ion with m/z 870.5 shown in the range of m/z 270 – 370. The ions corresponded to glycerophosphate without molecules of water (m/z 153.0) were detected on MS² spectra. Fragmentation of parent ion with m/z 838.5 results in formation of molecular ions with m/z 283.3 and m/z 331.3 corresponding to C₁₈:₀ and C₂₂:₄ fatty acids, respectively. MS² analysis of molecular ion with m/z 870.5 revealed the presence of molecular ions with m/z 363.3
corresponding to C\textsubscript{22:4} after addition of two oxygens. The molecular ion with m/z 365.3 was formed during fragmentation process of non-oxidized molecular species of PS with m/z 870.5 (PS\textsubscript{C\textsubscript{18:1}/C\textsubscript{24:1}}) and corresponds to nervonic acid (C\textsubscript{24:1}). The loss of serine group of PS yielded the fragments with m/z 755.7 and 783.6. Product ion with m/z 419.3 originated from fragments with m/z 751.6 and 783.6 after loss of C\textsubscript{22:4} fatty acid and C\textsubscript{20:4}-OOH, respectively. Therefore, molecular ions at m/z 838.5 and m/z 870.5 correspond to molecular species of PS C\textsubscript{18:0}/C\textsubscript{22:4} and product of its oxidation C\textsubscript{18:0}/C\textsubscript{22:4}-OOH.

**Figure 6.** Exposure of mice to hyperoxia results in activation of caspase 3/7 activity in lung.

**A.** Assessment of caspase 3/7 activity in mouse lung using Caspase-Glo assay. Open bars – control; closed bars – hyperoxia. Data are mean ± SD, *p*< 0.05 vs. control; n=3 (for control); n=4 (for hyperoxia).

**B.** Exposure of mice to hyperoxia for 72 hrs results in apoptosis of endothelial cell origin. Lungs were inflated with 2% paraformaldehyde and frozen sections were used for immunofluorescent detection of apoptosis using an Alexa 488-labeled (green) Nick End labeling kit. There were very few apoptotic cells in lungs of animals breathing room air as evident at low (10X) and higher (40X) magnification (upper panels). In contrast, numerous apoptotic cells were apparent (10 and 40X) in the hyperoxic group and many of these were associated with CD31 positive (e.g. endothelium) cells as was evident in the insert at 100X (bottom right panel). Boxed areas at low magnification indicate the subsequent area at high magnification.

**Figure 7.** Phospholipid composition and accumulation of phospholipid hydroperoxides in MLEC\textsubscript{s} exposed to hyperoxia.

**A.** Phospholipid composition of control and hyperoxic MLEC. MLEC were exposed to hyperoxia (95% O\textsubscript{2} and 5% CO\textsubscript{2} for 72 hrs in a 37°C). Lipids were extracted, separated by 2D-HPTLC, spots of phospholipids were scraped and lipid phosphorus was determined.
B. Accumulation of PL-OOH in MLEC exposed to hyperoxia.

Lipids were extracted and separated by 2D-HPTLC. Phospholipid hydroperoxides were detected using Amplex Red protocol. Open bars – control; closed bars – hyperoxia. Data are mean ± SD, *p< 0.05 vs. control; n=3.

Figure 8. Oxidation of CL and PS in MLEC exposed to hyperoxia.

Quantitative assessments of CL (A) and PS (B) molecular species in MLECs. Open bars – control; closed bars – hyperoxia. Data are mean ± SD, *p< 0.05 vs. control, n=3

Figure 9. Activation of caspase 3/7 in MLEC exposed to hyperoxia.

MLEC (15,000 per well) were treated with XJB-5-131 (10-40 uM, for 4 hrs). After that, the drug was removed and cells were exposed to hyperoxia (95% O₂ and 5% CO₂ for 72 hrs). At the end of the exposure caspase 3/7 activity was measured. * - significantly different from control (p<0.05); # - significantly different from hyperoxia alone (p<0.05) Open bars – control; closed bars – hyperoxia. Data are mean ± SD, n=3.

Figure 10. Mitochondria-targeted small-molecule, XJB-5-131, protect both CL (A) and PS (B) against oxidation induced by hyperoxia in MLEC (95% oxygen, 72hrs). Open bars – control; closed bars – hyperoxia. Data are mean ± SD, *p< 0.05 vs. control, n=3.
Figure 1
Figure 4

A

B

C

PS molecular species, pmol/nmol PS

Control

Hyperoxia

m/z

810.5 838.5

*
Figure 5
Figure 6

(A) Caspase 3/7 activity, Luminescence AU/mg protein

(B) Control

Hyperoxia, 72hrs
Figure 7

A

PL content, % of total

B

PL-OOH, pmol/nmol PL

Control

Hyperoxia

*
Figure 8
Figure 9

Caspase 3/7 activity, Luminescence AU

Control (no Hyperoxia) vs Hyperoxia

XJB-5-131

0 µM 10 µM 20 µM 40 µM
Figure 10

A

Oxidized CL molecular species, pmol/nmol CL

1423.8 1473.8 1475.8 m/z

[Graph showing data for Oxidized CL]

B

Oxidized PS molecular species, pmol/nmol PS

834.5 836.5 383.5 m/z

[Graph showing data for Oxidized PS]

Legend:
- □ Hyperoxia
- □ Hyperoxia+JXB-131

* Indicates statistical significance.