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Cigarette Smoke-Induced Alterations in Frontal White Matter Lipid Profiles Demonstrated by MALDI-Imaging Mass Spectrometry: Relevance to Alzheimer's Disease

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Abstract

Background—Meta-analysis has shown that smokers have significantly increased risks for Alzheimer's disease (AD), and neuroimaging studies showed that smoking alters white matter (WM) structural integrity.

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SUPPLEMENTARY MATERIAL

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Objective—Herein, we characterize the effects of cigarette smoke (CS) exposures and withdrawal on WM myelin lipid composition using matrix assisted laser desorption and ionization-imaging mass spectrometry (MALDI-IMS).

Methods—Young adult male A/J mice were exposed to air (8 weeks; A8), CS (4 or 8 weeks; CS4, CS8), or CS8 followed by 2 weeks recovery (CS8 + R). Frontal lobe WM was examined for indices of lipid and protein oxidation and lipid profile alterations by MALDI-IMS. Lipid ions were identified by MS/MS with the LIPID MAPS prediction tools database. Inter-group comparisons were made using principal component analysis and R-generated heatmap.

Results—CS increased lipid and protein adducts such that higher levels were present in CS8 compared with CS4 samples. CS8 + R reversed CS8 effects and normalized the levels of oxidative stress. MALDI-IMS demonstrated striking CS-associated alterations in WM lipid profiles characterized by either reductions or increases in phospholipids (phosphatidylinositol, phosphatidylserine, phosphatidylcholine, or phosphatidylethanolamine) and sphingolipids (sulfatides), and partial reversal of CS's inhibitory effects with recovery. The heatmap hierarchical dendrogram and PCA distinguished CS exposure, duration, and withdrawal effects on WM lipid profiles.

Conclusion—CS-mediated WM degeneration is associated with lipid peroxidation, protein oxidative injury, and alterations in myelin lipid composition, including shifts in phospholipids and sphingolipids needed for membrane integrity, plasticity, and intracellular signaling. Future goals are to delineate WM abnormalities in AD using MALDI-IMS, and couple the findings with MRI-mass spectroscopy to improve *in vivo* diagnostics and early detection of brain biochemical responses to treatment.

Keywords

Alzheimer's disease; cigarette smoke; imaging mass spectrometry; MALDI; mouse model; neurodegeneration; tobacco; white matter

INTRODUCTION

Alzheimer's disease (AD) is largely sporadic in occurrence, indicating that factors other than genetics strongly influence its onset and progression. Non-genetic risk factors include environmental and lifestyle-related exposures that can be modified. Several studies have linked chronic cigarette smoking to cognitive impairment [1–3] and brain atrophy [2, 4–12] involving white matter (WM) [3, 11, 13]. In addition, meta-analyses showed that smoking is associated with atrophy of brain regions targeted by AD [14], and that cigarette smoking increases risk for developing AD [15]. Although these studies are provocative, research is needed to demonstrate mechanisms of tobacco smoke-induced neurodegeneration.

The present study characterizes the effects of cigarette smoke (CS) exposures on neurodegeneration, focusing on WM myelin lipids. The rationale is that although most AD research is concerned with gray matter structure degeneration, there is considerable evidence that WM degeneration occurs early and is important. WM pathology in AD was first recognized by Brun and England [16–19], and later shown to be present at early, pre-clinical stages of disease [20]. WM degeneration in AD is associated with partial loss of myelin

sheaths, axons, and oligodendroglial cells [19]. Furthermore, it has been suggested that myelin breakdown is a key component of the disease process in AD due to increased susceptibility of oligodendrocytes to free radical and other metabolic damage [21]. In addition, WM in AD includes significant reductions in oligodendrocytes accompanied by increased populations of astrocytes [22], decreased myelin density [23], and decreased expression of myelin basic protein [24].

Altogether, the efforts applied to studying WM degeneration in AD pale in comparison with the research devoted to gray matter pathology, including mechanisms of amyloid- β and phospho-tau associated lesions. One likely explanation is that tissue-based approaches needed to advance research on WM degeneration have only recently become accessible, whereas growth in neuroimaging technology has outpaced correlative histopathological research. Consequently, it has been difficult to validate interpretations made by magnetic resonance imaging, positron emission tomography, diffusion tensor imaging, and magnetic resonance spectroscopy [25–28].

This study demonstrates the potential co-factor role of CS exposures in the pathogenesis of WM degeneration using matrix-assisted laser desorption and ionization-imaging mass spectrometry (MALDI-IMS) as a tool for characterizing WM biochemical pathology. Herein, we assessed the degrees to which CS exposures and short-term withdrawal alter brain lipid profiles using MALDI-IMS. The experimental A/J mouse model simulated effects of sidestream smoke, i.e., secondhand CS exposures.

MATERIALS AND METHODS

Experimental models

The experimental models were generated at the University of Southern California. Young adult (8 weeks old) A/J male mice ($n = 5/\text{group}$) were exposed to cigarette smoke (CS) or air as follows: 8 weeks of room air only (A8); 4 weeks CS (CS4); 8 weeks CS (CS8); and 8 weeks CS followed by 2 weeks recovery (CS8 + R) [29, 30]. CS was generated from research grade Kentucky 3R4F cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY) using an industry standard Teague Enterprises, TE-10 Smoking Machine (Davis, CA). The cigarettes contained 11 mg of total particulate matter and 0.73 mg of nicotine. Sidestream and mainstream smoke were mixed in a ratio of 89% to 11%, which is similar to environmental tobacco smoke exposures. Six cigarettes were puffed simultaneously, one time per min for 9 puffs. The cigarettes were burned for 6 h/day, 5 days/week, and for 4 or 8 weeks duration. Mice were adapted to CS by ramping up concentration and exposure period in the first week. The chamber atmosphere was monitored for total suspended particles, which usually ranged from 80–100 mg/m³ when 6 cigarettes were lit at one time. Under these conditions, the chamber atmosphere had 21% oxygen and approximately 3 ppm of carbon monoxide. CO levels were measured with a TSI Q-Trak air quality monitor. Previous studies demonstrated carboxy-hemoglobin levels to be less than 8% immediately following CS exposures. Before use, the cigarettes were kept for 48 h in a standardized atmosphere humidified with 70% glycerol-30% water. Throughout the experiment, mice were housed under humane conditions and kept on a 12-h light/dark cycle with free access to food. Upon sacrifice, freshly harvested brains were snap-frozen and

stored at -80°C . Frozen frontal WM was divided for protein and lipid extraction, or cryo-sectioning. All experiments were performed in accordance with protocols approved by the University of Southern California's Institutional Animal Care and Use Committee, and conformed to guidelines established by the National Institutes of Health.

Competitive enzyme-linked immunosorbant assays (ELISAs)

Frontal lobe homogenates were prepared and protein concentration was measured as previously described [31]. Competitive ELISAs were used to measure Keratin-18 (USCN Life Science Inc, Hubei, PRC), protein carbonyl, 4-hydroxy-2-nonenal (HNE), and isoprostane 8-iso-PGF 2α (Cell Biolabs Inc, San Diego, CA) according to the manufacturers' protocols. Results were normalized to sample protein concentration.

Matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS)

Frontal lobe WM cryo-sections (10 μm thick) were thaw-mounted onto indium tin oxide (ITO)-coated slides (Delta Technologies, Loveland, CO) and prepared for lipid analysis by sublimation coating with $200 \pm 13 \text{ mg/cm}^2$ 2,5-dehydroxybenzoic acid (DHB; Sigma-Aldrich Co, St. Louis, MO) as a thin uniform layer of matrix [32, 33]. Sublimation is a dry method that does not require a matrix solvent, and ideally suited for lipid analytes which could become delocalized. Matrix sublimation was achieved using a commercial apparatus (Chemglass Life Sciences, Vineland, NJ) in which $300 \pm 5 \text{ mg}$ of DHB crystals were evenly dispersed on the bottom of a lower flask that was tightly sealed to an upper flask that contained the MALDI target slide attached to its bottom surface with conductive copper tape. Under vacuum pressure (0.05 Torr) and with chilling of the upper flask, heat applied to the lower flask causes the DHB to transition from solid to gas phase, and subsequently condensate onto the surface of the cooled slide [33]. Imaging was performed with a reflectron geometry MALDI-time-of-flight (TOF)/TOF mass spectrometer (Ultraflextreme, Bruker Daltonics, Bremen, Germany). Analyses were performed by focusing a Smartbeam II Nd:YAG laser onto 100 μm^2 areas, with imaging data acquired in the negative ion mode as described in the literature [33] to better profile WM lipids, particularly phosphatidylinositol (PI), sulfatide (ST), and ganglioside species [34].

Data analysis and statistics

Competitive ELISA data depicted in graphs reflect group means + S.E.M. Those data were analyzed by one-way analysis of variance (ANOVA) with the Tukey *post-hoc* test using GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA). MALDI data were processed using FlexAnalysis v3.4 (Bruker Daltonics, Billerica, MA) and visualized with FlexImaging software v4.0 (Bruker Daltonics, Billerica, MA). Results were normalized to total ion count and analyzed statistically using ClinProTools v3.0 (Bruker Daltonics, Billerica, MA). Lipids were identified by comparing the precursor and product ion m/z values with those catalogued in the LIPID MAPS prediction tool database (<http://www.lipidmaps.org/tools/index.html>). Their identities were confirmed by tandem mass spectrometry (MS/MS) in the LIFT-TOF/TOF mode. The heatmap was constructed using Version 3.2.2 of R software [35]. Extensive testing was used to verify high quality, consistency and reproducibility of data. Several transformations were applied to the row values. To scale the data, row means were subtracted from each cell. The resulting values

were further divided by the standard deviation in order to obtain a z-score of each individual cell, yielding a mean of 0 and a standard deviation (S.D.) of 1. The data were plotted using a cosmetically modified heatmap library function in R with a 6-color palette. We also applied hierarchical clustering algorithm using Euclidean distance function on the overall table to display a dendrogram of lipid ions.

RESULTS

CS exposures cause WM lipotoxicity

We used competitive ELISAs to measure immunoreactivity to Keratin-18, protein carbonyls, HNE, and 8-iso-PGF2 α . One-way ANOVA tests demonstrated significant inter-group differences for HNE (Fig. 1A) and 8-iso-PGF2 α (Fig. 1B), and protein carbonyl (Fig. 1C), but not Keratin-18 (Fig. 1D). *Post-hoc* Tukey repeated measures tests demonstrated that frontal lobe WM levels of HNE and protein carbonyl were similar in the A8 and CS4 groups, but significantly elevated in CS8 relative to the other three groups. In contrast, 8-iso-PGF2 α was significantly elevated in CS4 relative to the other three groups. The higher mean levels of HNE and protein carbonyl in CS8 versus CS4 indicate that WM oxidative stress-related injury and degeneration increase with CS exposure duration. However, the finding that CS8+R, HNE, 8-iso-PGF2 α , and protein carbonyl levels were either normalized and similar to A8, or significantly reduced relative to the other groups suggests that some adverse effects of CS exposure on WM could be reversed by withdrawal.

CS alteration of WM lipid ion profiles

Frontal lobe WM lipid extracts were subjected to MALDI in negative ion mode. The Peak Statistic report identified different lipid ions with mass/charge ratio (m/z) values between 705.52 and 1097.13 (Supplementary Table 1). Initial inter-group comparisons were made using the intensity/area versus m/z profiles (Fig. 2). Higher peak and area under curve reflect greater abundance of a specific lipid ion. CS exposures differentially altered lipid ion abundance as was manifested by selective increases or decreases in their intensities relative to control; 2) CS4 and CS8 effects were directionally similar but the effects were sometimes more pronounced with longer exposure duration; and 3) short-term recovery partly reversed the effects of CS4 and/or CS8.

Heatmap depiction of differential CS dose/duration and withdrawal effects on frontal WM lipid ion profiles

The heatmap generated with hierarchical clustering helped illustrate overall effects of CS exposures on frontal lobe expression of lipids in WM (Fig. 3). The heatmap revealed three dominant hierarchical clusters and one or two minor clusters. The upper segment (a) was characterized by high levels of lipid ion expression in A8 control frontal WM and sharply lower levels in the CS4 and CS8 samples. Approximately three sub-group responses were detected within the (a) cluster. The uppermost a1 subgroup was characterized by similarly sharply reduced lipid ion levels in the CS4 and CS8 samples and partial normalization of levels in the CS8 + R brains. The a2 and a3 subgroups had reduced lipid ion levels in the CS4 samples and further reductions in the CS8 brains. For a2, the lipid ion levels were similarly suppressed in the CS8 and CS8 + R brains, whereas for a3, CS8 + R samples

exhibited partial reversal of the CS inhibitor effects on lipid ion expression. The (b) cluster was associated with very low lipid ion levels in A8 control brains, moderately increased lipid ion expression in the CS4 samples, further increases in the CS8 samples, and partial normalization of responses in the CS8 + R samples. The (c) cluster was characterized by similarly low levels of lipid ion expression in the A8 and CS4 samples, but with two different responses in CS8 and CS8 + R. In the c1 subgroup, lipid ion expression was sharply increased in CS8 relative to A8 and CS4, but variably reduced in the CS8 + R samples. The c2 subgroup exhibited similarly low levels of lipid ions in the CS8 samples as compared with A8 and CS4 brains, but CS8 + R treatment sharply increased lipid ion expression to the highest levels.

Structural assignment of lipid ions

Lipid ions were identified using MALDI-LIFT-TOF/TOF based on their fragmentation patterns and structural characteristics found in LIPID MAPS. Structural assignments required detection of the product ions belonging to both the head group and fatty acid chains. We selected 16 lipid ions for characterization and analysis of CS effects. The lipids were identified as phospholipids or sphingolipids (sulfatides) (Supplementary Table 2). Sub-structural assignments for three phospholipids (m/z 's 713.4, 751.5, and 958.7) and two sulfatides (890.9 and 904.9) could not be made using the LIPID MAPS database as the library is not yet complete and Fourier transformation, which is needed to maximize resolving power of very closely related ions was not available on our instrument. Among the phospholipids that could be assigned, one was phosphatidylcholine, PC(30 : 5) which had an $m/z = 729 : 4$; another was phosphatidylethanolamine PE(38 : 4), which had an m/z of 767.4; five others were phosphatidylinositols PI(27 : 2), PI(36 : 4), PI(38 : 3), PI(38 : 4), and PI(38 : 5) with m/z 's of 735.6, 857.7, 886.8, 885.9, and 883.8, respectively; and the last two were phosphatidylserines, PS(36 : 1) and PS(40 : 6) with m/z 's of 788.9 and 834.7. Three phospholipids could not be assigned due to limitations of the LIPID MAPS database. The sulfatides that could be assigned had m/z 's of 888.9 and 906.9, and were identified as ST(24 : 1) and ST(24 : 0)(OH), respectively. Details about how the structural assignments were made are provided in the Supplementary Material including Supplementary Table 1 and Supplementary Fig. 1.

Effects of CS exposures on lipid ion intensities in frontal lobe

MALDI-IMS analysis demonstrated that CS exposures differentially altered frontal WM phospholipid and sulfatide expression relative to control (Table 1). The main effects of CS were to decrease WM levels of phospholipids with m/z 's of 713.4, 751.5, and 958.7, PC(30 : 5), PE(38 : 4), and PI(27 : 2), and increase PI(36 : 4), PI(38 : 3), PI(38 : 4), PI(38 : 5), and PS(40 : 6). In addition, sulfatide m/z 890.9 and ST(24 : 1) were increased by CS, whereas sulfatide m/z 904.9 and ST(24 : 0)(OH) were reduced. In contrast, there were no detectable CS effects on PS(36 : 1). In general, the directional responses for CS4 and CS8 were similar, and CS8 produced bigger effects than CS4 with respect to five of the lipid ions. Otherwise, the maximum responses occurred after just 4 weeks of CS exposure. CS8 + R reduced the CS inhibitory effects for 7 of the lipid ions, but did not blunt CS-associated increases in lipid ion expression. In other words, CS-induced increases in specific phospholipids or sulfatides

were not abrogated by CS-withdrawal, whereas the inhibition of their expression was frequently reduced by recovery.

Principle component analysis (PCA)

PCA of the phospholipid and sulfatide ion profiles generated four distinct clusters: The A8 (control) cluster was separate from the three CS clusters. CS4 and CS8 overlapped extensively and for the most part, the signals were distantly clustered from those of A8 (Fig. 4). However, despite their overlapping profiles, the CS4 and CS8 groups could be delineated to some degree with respect to small sub-populations of lipid ions. In this regard, a portion of the CS4 cluster was positioned close to the A8 controls, whereas the remainder overlapped with the CS8 signals. The CS8 + R signals were largely clustered close to A8 controls rather than the CS8 and CS4 signals, suggesting that the recovery abrogated many effects of the CS exposures. On the other hand, a subset of the CS8 + R lipid ion signals overlapped with CS8, indicating that not all of the CS effects could be reversed by halting the exposures.

DISCUSSION

The main findings were that CS exposures had neurotoxic and degenerative effects on WM, as manifested by increased levels of lipid and protein adducts and strikingly altered lipid profiles affecting both phospholipids and sphingolipids. The effects of CS worsened with duration of exposure, but were partly reversed by short-term withdrawal. Furthermore, the abnormalities correspond with recent findings of CS-associated impairments in insulin and IGF signaling and altered expression of myelin-associated genes in frontal lobes of the CS-exposed mice [36, 37]. The use of MALDI-IMS to detect biochemical indices of WM degeneration holds promise for future investigations, including studies of human brains with AD.

Tobacco smoke contains hundreds of toxins, including volatile, non-volatile, and tobacco-specific nitrosamines. The most potent and abundant tobacco-specific nitrosamines in CS are 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN) [38–40]. A single cigarette may contain between 1 µg and 9 µg of tobacco-specific nitrosamines, and up to 8 µg of the other classes of nitrosamines, releasing up to 2 µg of nitrosamine products into air [41]. Therefore, nitrosamine exposures via second-hand smoke are significant. Although the vast majority of research on nitrosamine-induced diseases pertains to carcinogenesis, emerging evidence supports the concept that low-level nitrosamine exposures also threaten health by causing progressive degenerative diseases linked to tissue injury, inflammation, impairments in insulin/IGF signaling through cell survival and metabolic pathways, oxidative and nitrosative stress, and dysregulated lipid metabolism [42–44]. In this regard, we have shown that NNK and other nitrosamines cause neurodegeneration [31, 44] including in WM [45–47] with abnormalities similar to those described herein and in recent CS exposure related publications [36, 37].

The present work circles back to the main clinical and epidemiological concerns as to whether chronic CS exposures themselves cause neurodegeneration, focusing on WM, i.e., myelin lipids. The rationale was that WM atrophy and degeneration are early, major

manifestations of neurodegeneration in AD [16–20]. Histopathologically, WM degeneration in AD is associated with partial loss of myelin sheaths, axons, and oligodendroglial cells [19], possibly due to myelin breakdown. Mechanistically, a key component of this disease process in AD may be increased susceptibility of oligodendrocytes to free radical and other metabolic damage [48]. Correspondingly, WM degeneration in AD includes reduced oligodendrocyte and increased astrocyte populations [22], decreased myelin density [23], and decreased expression of myelin basic protein [24].

We examined indices of stress in WM by using competitive ELISAs to measure Isoprostane 8-iso-PGF2 α , 4-HNE, protein carbonyls, and Keratin 18. Isoprostane 8-iso-PGF2 α is formed by free radical-catalyzed peroxidation of arachidonic acid and marks lipid peroxidation [49–52]. HNE is a dominant aldehydic product of lipid peroxidation of membrane polyunsaturated fatty acids that exerts cytotoxic effects, including via post-translational modification and impairment of enzymes [53]. Protein carbonylation is a marker of protein oxidation that reflects stress [54]. Keratin 18 marks cellular injury [55–57] and death following toxin exposure [58]. Our studies showed that CS exposures increased lipid adducts and protein carbonylation in frontal lobe WM (Fig. 1). The greater responses in CS8 versus CS4 indicate that lipotoxicity is driven by exposure duration, providing a mechanism for WM degeneration among smokers and individuals exposed to second-hand smoke. The reduced indices of lipid and protein adduct formation following recovery is reassuring and they suggest that CS-mediated WM degeneration is at least partly reversible. The caveat is that the phenomenon occurred in young mice (<20 weeks old), and may not apply in older individuals or following chronic CS exposures, i.e., longer than 12 weeks.

Dysregulated lipid metabolism is a critical abnormality linked to lipotoxicity, which worsens inflammation, cell death, and impairments in insulin/IGF signaling, all of which occur in AD [59–63]. To better characterize CS-mediated WM atrophy and degeneration, we used MALDI-IMS and MALDI lipid spot assays to examine CS exposure effects on myelin lipid profiles. Using these approaches, we observed reductions or increases in the levels of multiple phospholipids and sulfatides in the CS4 and/or CS8 groups, and partial normalization of responses in the CS8 + R group (Fig. 2, Table 1). In addition, most of the responses were directionally similar for CS4 and CS8, and for many of the lipid ions, the responses were more pronounced and striking in the CS8 versus the CS4 group. Altogether, the data suggest that longer durations of CS exposures lead to greater alterations in myelin lipid profiles, and that cessation of CS exposure permits reversal or recovery of the CS effects. These effects are well-illustrated by both the heatmap and PCA figures (Figs. 3 and 4, respectively). It is noteworthy that reductions in brain sphingolipids (sulfatides) and corresponding increases in ceramides are features of AD neurodegeneration [60, 62–69].

Membrane phospholipids are integral components of plasma membranes and play critical roles in regulating receptor functions and microdomains (lipid rafts), and reduced phospholipid levels have been linked to insulin resistance [70, 71]. Therefore, CS-mediated reductions in frontal lobe WM phospholipids, including phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol could be responsible for some of the related impairments insulin/IGF signaling, including disruption of downstream metabolic signaling as occur in AD [72–75]. Membrane phospholipid content is regulated by

phospholipase hydrolysis. Correspondingly, both CS [76] and tobacco-specific nitrosamine [77] exposures decrease lung phospholipids (phosphatidylcholine, phosphatidylglycerol, and phosphatidylserine) by enhancing phospholipase A2 activity [29, 77]. Additional likely downstream mediators of injury include increased activation of phospholipase A2 neuro-inflammatory pathways, release of pro-oxidative arachidonic acid, and formation of 4-HNE-protein adducts in the brain [78, 79]. Altogether, these findings could suggest that CS exposures partly mediate their adverse effects on insulin/IGF signaling by inhibiting phospholipid synthesis or maintenance, possibly through increased activation of phospholipases.

In addition to phospholipids, CS exposures reduced expression of sulfatides. Sulfatides are glycosphingolipids synthesized by oligodendrocytes and localized on the extracellular leaflet of myelin plasma membranes [80]. Sulfatides are synthesized via sulfonation of galactocerebroside, which is formed from ceramide and galactose. Sulfatide can be degraded back to ceramide and sulfate via galactosylceramidase and sulfatidase [80, 81]. Sulfatide and galactocerebroside comprise nearly 30% of myelin lipids and are markers of oligodendrocytes. Sulfatides play key roles in protein trafficking, neuronal plasticity, memory, adhesion, myelin maintenance, glial-axonal signaling, insulin secretion, and oligodendrocyte survival [82]. Reductions in membrane sulfatide content disrupt myelin sheath structure and function and impair neuronal conductivity. In this regard, mice rendered sulfatide deficient exhibit demyelination and loss of axonal function in the CNS [83]. Increased degradation of sulfatide back to ceramide also can be problematic due to ceramide accumulation and attendant activation of inflammatory cytokines, increased generation of reactive oxygen species, and apoptosis [84]. Other studies have already linked increased ceramide levels in brain to cognitive impairment and neurodegeneration mediated by oxidative stress, neuro-inflammation, insulin resistance, and deficits in oligodendrocyte myelin-associated gene expression [85, 86]. These pathogenic processes are evident in AD [87], possibly due to combined effects of sulfatide deficiency [61] and ceramide accumulation [87]. Further studies are needed to determine if CS exposures reduce brain sulfatide levels by decreasing ceramide galactosyltransferase and galactocerebroside sulfotransferase, or increasing galactosylceramidase and sulfatidase gene expression or enzyme activity.

On the other hand, we also observed that CS exposures increased the levels of several PI, PS, and ST species, and that these effects were not muted or abrogated by CS withdrawal. Phosphatidylinositols are acidic glycerophospholipids that when phosphorylated, play key roles in lipid signaling, intra-cellular messaging, and membrane trafficking. Phosphatidylserines are acidic glycerophospholipids synthesized from phosphatidylcholine or phosphatidylethanolamine via exchange of the head group with serine. Although these responses could potentially serve as seemingly irreversible indices of brain CS exposure, further studies are needed to characterize their mechanisms and pathophysiological significance.

In conclusion, this study demonstrates that CS exposures cause lipid peroxidation, protein oxidation, and broad, striking alterations in phospholipid and sulfatide profiles in WM. The groups were distinguishable based on clustered responses as illustrated with both heatmap

and PCA diagrams. Importantly, many of the pathophysiological responses to CS exposure were reversed or markedly reduced by a brief period of recovery. These studies are novel in that they illustrate in situ biochemical abnormalities in WM caused by CS exposure, and hint at mechanisms of CS-mediated neurodegeneration. Furthermore, extension of this research to *in vivo* imaging via magnetic resonance spectroscopy could help establish new biomarkers for reversible stages of WM degeneration in human diseases such as AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

CS effects on stress indices in frontal lobe WM. Competitive ELISAs were used to measure (A) 4-hydroxy-2-nonenal (HNE), (B) 8-iso-PGF-2 α , (C) protein carbonyl, and (D) cytokeatin 18, with results normalized to sample protein content. Inter-group comparisons were made by one-way ANOVA. Significant F -values and p -values are included in the panels. Results of *post-hoc* Tukey multiple comparison tests are shown (* $P < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

Fig. 2. Relative lipid ion intensity profiles in frontal WM extracts. Lipid ion m/z and intensity were detected by MALDI-TOF. The spectra show relative intensities of lipid ions between m/z 700–1000 Da in A8 controls compared with the CS4, CS8, and CS8 + R groups.

Fig. 3. Heatmap illustrating IMS hierarchical clustering of different lipid ion species. Results shown with the 6-color palette correspond to z-scores, which were scaled to have a mean of 0 and S.D. of 1. A hierarchical clustering algorithm was applied using the Euclidean distance function on the overall table to display a dendrogram of lipid ions. A8 = control room air exposed $\times$ 8 weeks; CS4 = cigarette smoke exposed $\times$ 4 weeks; CS8 = cigarette smoke exposed $\times$ 8 weeks; CS8 + R = cigarette smoke exposed $\times$ 8 weeks followed by 2 weeks exposure to room air. Clustered responses, a1, a2, b, c, and d are described under results.

Fig. 4. Principal component analysis (PCA) of IMS data acquired in the negative-ionization mode. Based on spectral similarities and WM lipid profiles, three distinct groupings were identified: the A8 control (red), CS4 (green), and CS8 (blue). CS8 + R (yellow) overlapped with A8 as well as CS4 and/or CS8, corresponding with its partial reversal or blunting of CS effects.

Table 1

MALDI-IMS-Cigarette smoke effects on frontal white matter lipid ion expression

m/z	Lipid assignment	CS4	CS8	CS8 + R
713.4	Phospholipid	↓	↓↓	↓
751.5	Phospholipid	↓↓	↓↓	↓
958.8	Phospholipid	↓	↓↓	↓
729.4	PC(30 : 5)	↓	↓↓	↓
767.4	PE(38 : 4)	↓↓	↓↓	↓
735.6	PI(27 : 2)	↓	↓↓	↓
857.7	PI(36 : 4)	↑	↑	↑
886.7	PI(38 : 3)	↑	↑	↑
885.7	PI(38 : 4)	↑	↑↑	↑↑
883.8	PI(38 : 5)	↑	↑	↑
788.9	PS(36 : 1)	↔	↔	↔
834.8	PS(40 : 6)	↑	↑	↑
890.8	Sulfatide	↑	↑	↑
904.8	Sulfatide	↓↓	↓↓	↓
906.8	ST(24 : 0)(OH)	↓	↓	↓
888.8	ST(24 : 1)	↑	↑	↑

Adult A/J mice were exposed to air (control), cigarette smoke (CS) for 4 or 8 weeks, or CS-8 weeks followed by 2 weeks exposure to air (recovery; R). Tandem mass spectrometry (MS/MS) with MALDI LIFT-TOF/TOF was used to fragment phospholipids in the negative ion mode. Lipid species assignment was achieved by searching the LIPID MAPS database. Ions designated as phospholipid or sulfatide could not be further characterized due to limitations of the LIPID MAPS database. Directional arrows correspond to the mass spectra peak intensities (abundances) of phosphatidylethanolamine (PE), phosphatidylserine (PS), or phosphatidylinositol (PI) ions in the experimental groups relative to control (↓ or ↑ = Moderately reduced or increased; ↓↓ or ↑↑ sharply reduced or increased; ↔).