Label free Differential Lipid Analysis for Rat Brain Samples Affected fy Stroke using a Linear Ion Trap Orbitrap Mass Spectrometer

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Overview

Purpose: Comparing lipid profiles in normal and stroke affected rat brain by ESI and MALDI

Methods: Lipid extraction from stroke affected brain and tissue slices from the corresponding rat brain used for this study

Results: The two different ionization methods; ESI and MALDI gave complementary data on the stroke and control rat brain samples.

Introduction

Apart from water, lipids are the most common biomolecules found in the brain (12 %) and make up 50 % of its dry weight. Furthermore, lipids are the major building blocks of biomembranes; play a key role in signal transduction, and are an important reservoir of energy in biological systems. Additionally, changes in the levels of lipids, in particular ceramides and glycerophospholipids, have been observed in apoptosis or cell death. Lipids represent a large and very diverse group of biomolecules that have one or both of the following properties: soluble in organic solvents and the presence of long hydrocarbon chains. Lipids can be organized into eight categories: fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides. Glycerophospholipids are widely abundant in nature, especially in biological membranes, and are divided into classes based upon their head group as follows: glycerophosphocholines (PCs), glycerophosphoethanolamines (PEs), glycerophosposerines (PSs), glycerophosphoglycerols (PGs), glycerolphosphoinositols (PIs), and glycerophosphates (PAs). Sphingolipids contain a sphingoid base backbone and include sphingomyelins (SMs), sulfatides (STs), ceramides, cerebrosides, and gangliosides.

In this study, LC ESI MS and MALDI-MS will be used to analysis the lipidome for an animal model of stroke with particular focus on glycerophospholipids and sphingolipids.

Methods

Animal Work: All the animal work in this study abides by the Guide for the Care and Use of Laboratory Animals (NIH). Adult male Sprague-Dawley rats (250-300 g) were used. Animals were anesthetized with chloral hydrate (400 mg/kg i.p.) and the right middle cerebral artery (MCA) was ligated and the common carotids (CCAs) were bilaterally clamped for 60-min to generate focal ischemia (stroke) in the cerebral cortex. Twenty-four hours after ligation, the animal were perfused with PBS. The brains were removed and the cerebellum and anterior 4 mm were cut away using a brain block. The remaining tissue was immediately frozen in isopentane on dry ice and stored at -80 °C.

Lipid Extraction: For extraction studies, brains were sectioned into right and left hemispheres in order to compare stroke and non-stroke hemispheres. Lipids were extracted according to Folch extraction[1] using 0.88% KCl or distilled water as the Folch upper phase. Each lipid extracts (for 1 g brain, 20 mL Chloroform/Methanol 2:1 v/v) was sonicated, vortexed, and extracted for two and half hours. KCl 0.88% or distilled water was added at a ratio of 1mL for 5mL of Chloroform/methanol 2:1 v/v. After vortexing for 1 min, the mixture was centrifuged 10 min at 4000 rpm. Gangliosides were enriched in the upper phase (aqueous phase). The organic phase containing all other lipids was evaporated to dryness using nitrogen and conserved at -20 °C.

LC-ESI MS/MS: ESI experiments were performed on an LTQ Orbitrap Velos instrument (Thermo Fisher Scientific, San Jose, USA) operating in positive ion mode. The heated electrospray probe (HESI-II) was used. The instrument was operated in full scan mode from *m/z* 400-1000 at 60K and 100K resolution. For the LC portion, we used a Hypersil GOLD™ (50x2.1, 1.9um) column (Thermo Fisher Scientific) with water and methanol for solvents A and B respectively. The raw files were analyzed using label free differential analysis software package SIEVE 1.2 (Thermo Fisher Scientific). The resulting peak lists were detected using the following settings: noise level = 350,000; min time span = 3.0; *m/z* tolerance=0.0020.

MALDI Imaging: Frozen brain tissue was cut into thin sections (18 µm thickness) in a cryostat. The tissue samples were attached to the cryostat sample stages using ice slush made from distilled water. The ice slush only came in contact with the tissue blocks at the surface opposing the sample stages, and was frozen into a thin layer of ice within 5 seconds. Water was used to attach the tissue to the sample holder.

The tissue sections were placed directly onto the MALDI sample targets. A digital light microscope picture was taken of the tissue section before and after matrix addition. The matrix used in this study was **2,6-dihydroxyacetphenone (DHA).** It was prepared as a saturated solution in 50% ethanol with 125 mM ammonium sulfate and 0.05% heptafluorobutyric acid (HFBA). DHA has previously been demonstrated as an excellent MALDI matrix for lipids but had a limitation due to its sublimation in vaccum.[2] Recently, it has been shown that by adding ammonium sulfate and HFBA to DHA matrix solutions its lifetime in vacuum can be extended to allow for imaging experiments to be conducted.[3] The matrix solution was sprayed on the tissue sections with an artistic airbrush (Aztek® A470/80 Airbrush System, The Testors Corporation, Rockford, IL).

A Thermo Scientific LTQ XL with a MALDI source (nitrogen laser at 337 nm) was used to acquire MALDI imaging data. The data was recorded in positive ion mode with a *m/z* range of 700-850 with normal mass range and scan rate. The imaging parameters were 1 microscan/step with 10 laser shots per step at a laser energy of 10 µJ and a raster step size of 80 µm. The two dimensional ion density maps (MALDI images) were generated using the ImageQuest software (Thermo Fisher Scientific).

ESI – LC-MS/MS: Lipid Analysis

FIGURE 1. Example of SIEVE differential analysis comparing lipids from stroke brain and control; the RIC on top represents *m/z* 724.52757; (PE36p:4+H) showing a small increase in the stroke area. The bottom PCA analysis shows the distribution of the control samples in relation to the stroke lipids.

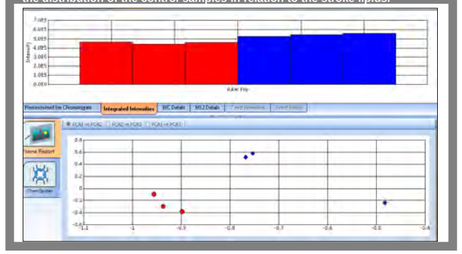


Figure 1 is an example of SIEVE differential analysis on lipids extracted from rat brain affected by stroke and the control lipids in this example were extracted from the area of the brain not affected by stroke. For the data analysis in the examples shown in Figures 2 and 3; an increase/decrease of areas >3X were considered to by up/down regulated.

FIGURE 2. SIEVE label-free differential analysis on control brain and brain associated with stroke for lipids within 3 ppm mass accuracy

Assignment	Formula	*m/z(calc)*	*m/z(obs)*	Int. Change	Error (ppm)
PE 36p:4+H	C35H70NO8P+H	664.49116	664.49153	Control	0.57
PC 30:0+Na-N(CH3)3	C35H67O8P+Na	669.44658	669.44729	Control	1.06
PE30p:0+Na	C35H70NO7P+Na	670.47821	670.47877	Control	0.83
PA 30:0-H+Na+K	C33H65O8P-H+Na+K	681.38681	681.3864	Control	0.6
SM 16:0+K-N(CH3)3	C36H70NO6P+K	682.45724	682.4571	Control	0.2
PE30p:0+Na	C35H70NO8P+Na	686.47313	686.47372	Control	0.85
PA 32:0+K	C35H69O8P+K	687.43616	687.43661	Control	0.65
SM 18:0+Na-N(CH3)3	C38H74N2O6P+Na	694.5146	694.51418	Control	0.6
PA 30:0-H+2K	C33H65O8P-H+2K	697.36074	697.36069	Control	0.07
PA 34:1+Na	C37H71O8P+Na	697.47788	697.47755	Control	0.47
PA 35:4+H	C39H69O8P+H	697.48028	697.48155	Control	1.82
PC 32:0+Na-N(CH3)3	C37H71O8P+Na	697.47788	697.47755	Control	0.47
PE32p:0+Na	C37H74NO7P+Na	698.50951	698.5093	Control	0.3
PC 30:0+H	C38H76NO8P+H	706.53813	706.5379	Control	0.32
PE30p:0+H2O+K	C35H70NO7P-H+Na+K	708.4341	708.43449	Control	0.55
PE30p:0+H2Na	C35H70NO8P-H+2Na	708.45507	708.45593	Control	1.21
PA 32:0-H+Na+K	C35H69O8P-H+Na+K	709.41811	709.41881	Control	0.98
SM 18:0+K-N(CH3)3	C38H74N2O6P+K	710.48654	710.48837	Control	0.23
PC 32:1+K-N(CH3)3	C37H69O8P+K	711.43616	711.43686	Control	0.96
PA 34:1+K	C37H71O8P+K	713.45181	713.45148	Control	0.46
PC 32:0+K-N(CH3)3	C37H71O8P+K	713.45182	713.45148	Control	0.47
PC 34:1+H	C42H82NO8P+H	760.585	760.5849	Control	0.13
PE 36p:4+H	C41H74NO7P+H	724.5275	724.5274	No Change	0.13
PC 32:0+H	C40H80NO8P+H	734.5684	734.5683	No Change	1.49
PE34p:1+H	C39H76NO8P+H	718.53813	718.53898	Stroke	1.18
PA 38:6+H	C41H69O8P+H	721.48028	721.48155	Stroke	1.76
PC 34:2+Na-N(CH3)3	C39H71O8P+Na	721.47788	721.47755	Stroke	0.45
PC 34 + H	C42H84NO8P+H	762.57507	762.575	Stroke	0.09
PC 38:5+H	C46H82NO8P+H	808.58508	808.58532	Stroke	0.29

FIGURE 3. SIEVE label-free differential analysis on stroke side of the brain versus the non-stroke side of the same brain for lipids within 3 ppm.

Assignment	Formula	*m/z(calc)*	*m/z(obs)*	Int. Change	Error (ppm)
SM 16:0+K-N(CH3)3	C36H70NO6P+K	682.45724	682.4571	Control	0.21
SM 18:0+Na-N(CH3)3	C38H74NO6P+Na	694.5146	694.51518	Control	0.83
SM 18:0+K-N(CH3)3	C38H74NO6P+K	710.48654	710.48737	Control	1.64
PC 32:1+K-N(CH3)3	C37H69O8P+K	711.43617	711.43686	Control	0.96
PA 34:1+K	C37H71O8P+K	713.45181	713.45148	Control	0.46
PC 32:0+K-N(CH3)3	C37H71O8P+K	713.45182	713.45148	Control	0.47
PC 34:1+H	C42H82NO8P+H	760.585	760.5849	Control	0.13
SM 20:0+Na-N(CH3)3	C40H78NO6P+Na	722.5459	722.5456	No Change	0.41
PA 36:2+Na	C39H73O8P+Na	723.49353	723.49327	Stroke	0.35
PA 38:5+H	C41H71O8P+H	723.49593	723.49527	Stroke	0.91
PC 34:1+Na-N(CH3)3	C39H73O8P+Na	723.49353	723.49427	Stroke	1.02
PE 36p:4+H	C41H74NO7P+H	724.5275	724.5274	No Change	0.13
PC 32:0+H	C40H80NO8P+H	734.5694	734.5683	No Change	1.49
SM C18:0/d18:2+H	C41H81N2O6P+H	729.5905	729.59139	Stroke	1.21
SM 20:0+K-N(CH3)3	C40H78NO6P+K	738.51984	738.51854	Stroke	1.76
PA 36:2+K	C39H73O8P+K	739.46747	739.46725	Stroke	0.29
PC 34:1+K-N(CH3)3	C39H73O8P+K	739.46747	739.46725	Stroke	0.29
PC 30:0+K	C38H76NO8P+K	744.49402	744.49473	Stroke	0.95
PE36a:1+H	C41H80NO8P+H	746.56943	746.56836	Stroke	1.43
PE36a:0+H	C41H82NO8P+H	748.58508	748.58404	Stroke	1.39
PA 40:5+H	C43H75O8P+H	751.52723	751.52851	Stroke	1.7
PC 36:1+Na-N(CH3)3	C41H77O8P+Na	751.52483	751.52351	Stroke	1.75
PC 34 + H	C42H84NO8P+H	762.57507	762.575	Stroke	0.09
PA 38:5+K	C41H71O8P+K	761.45181	761.45178	Stroke	0.04
PC 36:4+K-N(CH3)3	C41H71O8P+K	761.45182	761.45178	Stroke	0.05
GalCer C18:0(OH)/d18:1+Na	C42H81NO9+Na	766.58036	766.58029	Stroke	0.09

MALDI – LTQ – XL: Tissue Imaging

FIGURE 4. Surface light microscope pictures of tissue section on MALDI target plate before matrix addition.

FIGURE 5. Surface light microscope pictures of tissue section on MALDI target plate before matrix addition

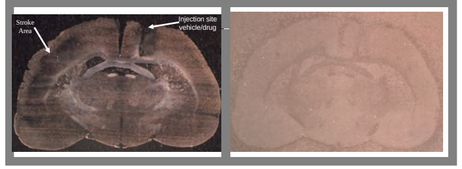


FIGURE 6. Cumulative MALDI mass spectrum acquired for the rat brain tissue in positive ion mode from the section

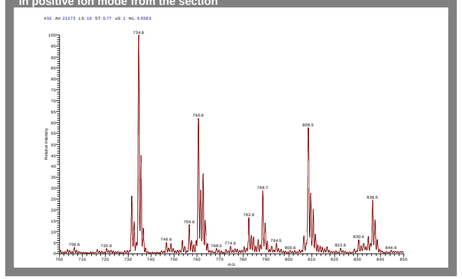


FIGURE 7. MALDI images of

(a)PC 32:0+H *m/z* = 734.6,

(b) PC 36:1+H *m/z* = 788.6,

(c) PC 34:1+H *m/z* = 760.6, and

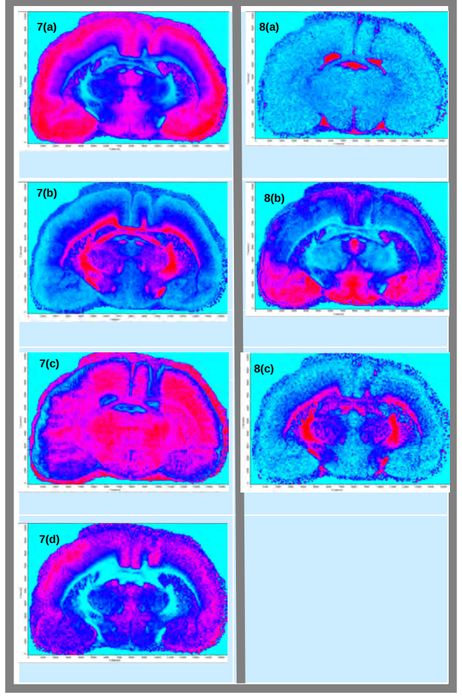
(d)PC 34:0+H *m/z* = 762.6 from the tissue section in Figure 4.

FIGURE 8. MALDI images of

(a)SM C16:0/d18:1+H *m/z* = 703.6,

(b) SM C18:0/d18:1+H *m/z* = 731.6,

(c) SM C24:1/d18:1+H *m/z* = 813.7 *from tissue section in Figure 4.*



Discussion

ESI-MS/MS: For ESI analysis, three experiments were conducted. In the first experiment (results shown in Figure 2), lipids extracted from a control brain from a healthy rat was compared with rat brain affected by stroke. Considering the top 2000 frames from a SIEVE experiment; it was shown that the more abundant lipid species were present in higher amounts in the control brain compared to the stroke affected brain. Similar to the MALDI results, the PC at *m/z* 734.573 did not show any change in the control and stroke brains.

In the second experiment, lipids extracted from the stroke brain were compared to the non-stroke side of the same brain (results shown in Figure 3). Some of the lipids; for example, PE 36p+H at *m/z* 724.527 (identified in the top 2000 SIEVE frames) showed a small increase in the cumulative runs from the stroke portion of the brain compared to the non-stroke side of the brain. In general, some of the abundant PC and PE species showed an increase in the non-stroke portion of the brain. Compared to the expt. in Figure 2, more lipid species identified in this case showed an increase in the stroke affected brain compared to the non-stroke portion of the same brain. Similar to the MALDI results, the PC species at *m/z* 760.6 shows a decrease in the stroke affected brain and 762.6 shows an increase in the stroke brain compared to the non-stroke brain in both Figures 2 and 3.

MALDI Imaging: Figure 6 shows the total MALDI mass spectrum in positive ion mode for the rat brain tissue section in Figure 4. Similar to previous results, this spectrum is dominated by mass peaks corresponding to PCs and SMs species.[4] Three major PC species observed in this spectrum are PC 32:0 (M+H, *m/z* = 734.6), PC 36:1 (M+H, *m/z* = 788.6), and PC 34:1 (M+H, *m/z* = 760.6). Previous work[4] has shown that these PCs have distinct regional distribution with PC 32:0 heavily concentrated in gray matter; PC 34:1 observed in abundantly in both white and gray matter, and PC 36:1 overwhelmingly present in white matter. Figure 7 (a)-(c) shows the MALDI images for these three PC species in the rat stroke brain section. PC 32:0 and PC 36:1 shown clear gray and white matter distribution and do not show any significant changes due to the stoke area in the left side of the image. However, PC 34:1 is abundantly present in both gray and white matter as expected but shows a decrease in the cerebral cortex on the left side of the image (Figure 7c) which corresponds to the stroke area. Figure 7d shows an image of PC 34:0 which is increased in relative abundance in the cerebral cortex on the left side of the image corresponding to the stroke area.

Figure 8 illustrates MALDI images for three SM species. SM C16:0/d18:1 (M+H, *m/z* = 703.6) is concentrated in the ventricles of the rat brain while SM C24:1/d18:1 (M+H, *m/z* = 813.7) is abundant in the ventricles and corpus colloseum. However neither species appears to be affected by the stroke area. SM C18:0/d18:1 (M+H, *m/z* = 731.6), one of the most abundant SM specie in mammalian brain, is widely distributed but the outer cerebral cortex region on the left side of the image (Figure 8b) is disrupted in the stroke region of the brain.

Conclusions

1. ESI-MS/MS results showed similar results to MALDI-MS for the PC species and the more abundant lipid species
2. ESI expts. conducted with stroke brain in comparison with control from a healthy rat brain showed more increased intensities of lipid species in control brain
3. ESI expts. conducted with stroke brain in comparison with non-stroke portion of the stroke affected brain showed a surge in the lipid species in the stroke
4. For MALDI analysis, the stroke affected areas showed disruption in lipid species compared to control portion of the brain
5. DHA proved to be a better matrix for evaluating lipid species on the MALDI LTQ compared to the matrices currently available.

References

1. Folch J., Lees M., Sloane Stanley G.H. *J. Biol. Chem.* (1957) 226: 497-509.
2. Jackson, S. N., Wang H-Y. J., and Woods, A. S. *J. Am. Soc. Mass Spectrom.* (2007) 18: 17-26.
3. Colsch, B and Woods, A. S. *Glycobiology* (2010) In Press.
4. Jackson, S. N., Wang H-Y. J., and Woods, A. S. *Anal. Chem.* (2005) 77: 4523-4527.