LIPIDOMICS: STUDY OF TOTAL PHOSPHOLIPIDS IN IMMORTALIZED LIVER CELLS EXPOSED TO DIFFERENT FATTY ACID SUBSTRATES

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ABSTRACT

Accumulation of fatty acids into non-adipose tissues can lead to cell dysfunction and cell death. Lipotoxicity from accumulation of long chain fatty acids is specific to, or made more severe by saturated fatty acids. Using H4IIE rat hepatoma cells as a model system of fatty liver disease, changes to the cellular phospholipid pool following exposure to saturated or unsaturated fatty acids were studied.

INTRODUCTION

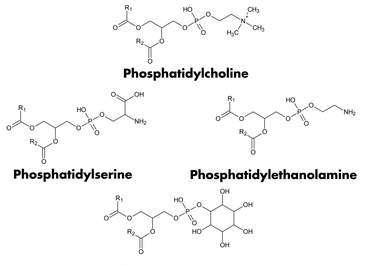
Metabolic syndrome is a collection of diseases including hyperlipidaemia, obesity, and diabetes mellitus. These diseases share a number of interdependent components: insulin resistance, impaired fatty acid metabolism, and altered lipid profiles. In the liver, these metabolic processes result in a progression of diseases known collectively as nonalcoholic fatty liver disease.[1] Nonalcoholic fatty liver disease (NAFLD) is a chronic disease syndrome initially characterized by the accumulation of fatty acids in the liver (steatosis) with progression, in some individuals, to nonalcoholic steatohepatitis (NASH) and end-stage liver disease.

It has been proposed that the trigger for progression into more advanced stages of NAFLD involves an acute insult, or "second hit", superimposed on hepatic steatosis.[2] The identity of this acute insult, including the cellular intermediates, and signaling pathways used to transmit its signal have not been established. We have recently demonstrated that hepatic steatosis characterized by increased saturated fatty acids leads to increased caspase-3 activity, endoplasmic reticulum stress and liver injury in rats.[3] In addition, we have demonstrated that the saturated fatty acids, stearate and palmitate, potently induce endoplasmic reticulum stress and apoptosis in H4IIE liver cells, independently of ceramide accumulation.[4] Thus, the presence of increased saturated fatty acid delivery to or accumulation in the liver may constitute an intrinsic second hit in the steatotic liver. The present study sought to characterize the cellular lipid environment in H4IIE liver cells following incubation with either palmitate or oleate.

A number of analytical techniques have been used to study the lipid fraction of cells and biological membranes. In particular, analysis of the non volatile membrane components such as phospholipids has relied on ESI-MS. Gross and other researchers have used infusion and nanoelectrospray based analytical strategies based on lipid class pre-fractionation and tandem mass spectrometry techniques with triple quadrupole mass spectrometers.[5-8] Although rich in information content, these strategies do not allow for the discovery of new molecular species.

Separation of these lipids is complicated by the variable phospholipid head group and the distribution of fatty acyl chain length and degree of unsaturation. Most HPLC separations of lipids utilize normal phase separations that are plagued by poor reproducibility and poor peak capacity. For this reason, a reversed phase HPLC method using water/acetonitrile and a C_8 column packed with highly efficient sub 2 µm particles coupled with ESI TOF mass spectrometry was developed. In addition to the accurate mass measurements that are used to indicate phospholipid class, fatty acyl chain total carbon number, and degree of unsaturation for each component; the novel MS^E technique was used to determine the identity of the individual fatty acids.[9] MS^E delivers parallel precursor and product ion analysis with exact mass in a single run. This is particularly useful in UPLC separations where qualitative and quantitative data is needed.

Major Phospholipid Classes



METHODS

Cell Culture and Sample Preparation

H4IIE cells were cultured in DMEM and 10% FBS. At 90% confluence, the media was supplemented with either 500 µM palmitic or oleic acid, bound to albumin, for 6 hours. A separate culture was used as a control Total cellular lipids were extracted using chloroform:methanol and di-luted 3:1 prior to analysis.

Liquid Chromatography/Mass Spectrometry

Waters ACQUITY UPLC

Mobile Phases
- 50 mM Aqueous Ammonium Acetate pH 5.0
- Acetonitrile

Column ACQUITY BEH C8 2.1x100 mm 1.7 µm d_p
- Flow Rate 550 µL/min
- Temperature 50 °C

Gradient:
- Hold 35% B 0.1 minute
- Ramp to 65% B in 1 minute
- Ramp to 95% B in 13 minutes
- Hold 2 minutes

Waters Q-Tof Premier
- ESI Negative Mode
- Capillary= 3.5 kV
- Cone Voltage= 40 V
- Collision Energy = 4 eV
- LockSpray using Leucine Enkephalin
- W Mode resolution (fwhm) = 15,000 @ *m/z* 554

MS^E Experiments
- Collision energy ramp from 20 to 40 eV during high energy accumulation.

RESULTS

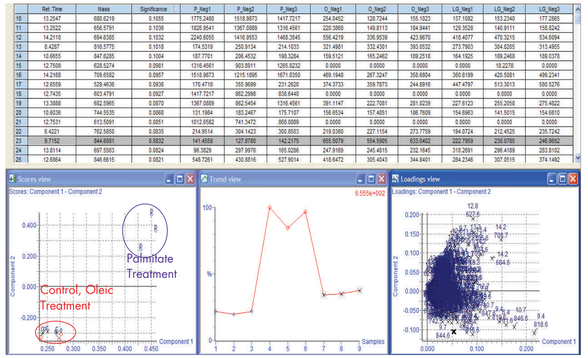


Figure 1. MarkerLynx report for negative Ion LC/MS analysis of total phospholipids isolated from H4IIE cells. Top pane: Table of components and normalized peak areas. Bottom left pane: Scores plot showing class separation. Bottom middle pane: Trend plot for component at *m/z* 844.6081@9.71min. Bottom right pane: Loadings plot data labeled with *m/z* retention time pair.

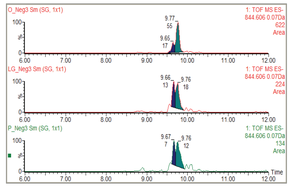


Figure 2. Narrow XIC for component *m/z* 844.608 @9.71 min. Top trace: Oleic acid treatment, Middle trace: Control treatment, Bottom trace: Palmitic acid treatment.

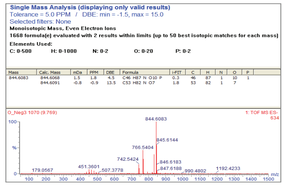


Figure 3. Elemental composition report for component *m/z* 844.6083 @ 9.71 min.

Phospholipid	Ret. Time	Mass	Palmitic Acid	Oleic Acid	Average Peak Area Control	Palmitic/Control	Oleic/Control	Palmitic/Oleic	Formula	ppm error
PI C36:ω 4	6.5671	858.523	103.62	6.41	10.80	959		1616	$C_{44}H_{81}O_{13}P$	2.8
PI C34:ω 0	7.3831	835.536	153.67	12.50	49.80	309		1229	$C_{42}H_{83}O_{13}P$	3.2
PS C34:ω 0	7.4679	760.515	100.03	24.67	15.28	655		405	$C_{42}H_{75}NO_{10}P$	2.5
PE C36:ω 6	7.5368	736.5	52.30	6.75	21.18	247		775	$C_{41}H_{71}NO_8P$	4.1
PS C40:ω 4	7.582	838.564	110.01	12.87	36.45	302		855	$C_{46}H_{81}NO_{10}P$	5.7
PI C36:ω 2	7.7355	861.549	51.69	172.84	79.57				$C_{45}H_{83}O_{13}P$	2.7
PS C36:ω 2	7.7738	786.527	24.64	59.12	15.70		376	42	$C_{44}H_{79}NO_{10}P$	3.9
PI C38:ω 4	7.818	885.551	395.23	343.93	243.25	162			$C_{47}H_{85}O_{13}P$	3.3
PS C36:ω 0	8.0265	790.562	544.69	229.31	318.94	171	72	238	$C_{44}H_{81}NO_{10}P$	1.3
PS C35:ω 0	8.0278	776.547	114.18	51.75	59.30	193		221	$C_{43}H_{79}NO_{10}P$	1.3
PS C42:ω 3	8.1788	864.578	93.67	69.88	82.73				$C_{48}H_{81}NO_{10}P$	0.3
PS C36:ω 0	8.2756	790.562	274.09	17.83	76.21	360	23	1537	$C_{44}H_{81}NO_{10}P$	-0.1
PE C34:ω 2	8.3459	714.51	65.95	61.01	68.92				$C_{39}H_{73}NO_8P$	-1.5
PE C36:ω 4	8.3648	738.508	137.42	108.39	123.26				$C_{41}H_{73}NO_8P$	-1.9
PS C37:ω 1	8.4191	802.562	48.51	81.33	60.64		134	60	$C_{45}H_{84}NO_{10}P$	1.9
PS C38:ω 1	8.4287	816.578	213.18	348.99	297.37	72		61	$C_{46}H_{86}NO_{10}P$	-0.2
PS C40:ω 3	8.431	840.578	200.34	64.88	67.07	299		309	$C_{48}H_{86}NO_{10}P$	-0.1
PS C39:ω 3	8.4311	826.562	39.32	14.85	14.18	277		265	$C_{47}H_{84}NO_{10}P$	-1.9
PS C38:ω 1	8.6363	816.574	59.05	54.29	59.34				$C_{46}H_{86}NO_{10}P$	-2.4
PI C38:ω 3	8.6593	887.566	75.18	154.84	95.78		162	49	$C_{47}H_{89}O_{13}P$	3.3
PS C37:ω 0	8.7082	804.578	114.33	67.35	100.22			170	$C_{45}H_{86}NO_{10}P$	0.6
PS C36:ω 0	8.7613	790.549	9.01	40.66	18.45				$C_{44}H_{84}NO_{10}P$	1.4
PE C38:ω 3	8.7788	764.524	76.68	129.49	89.00		145	59	$C_{43}H_{79}NO_8P$	1.8
PS C40:ω 2	8.8204	842.59	99.29	63.02	57.75	172			$C_{48}H_{88}NO_{10}P$	4.4
PS C35:ω 1	8.9861	778.561	113.29	0.00	8.67	1307			$C_{43}H_{81}NO_{10}P$	1.8
PS C36:ω 1	8.9962	792.577	468.38	12.49	55.20	849	23	3750	$C_{44}H_{83}NO_{10}P$	3.8
PS C39:ω 1	9.0435	830.59	38.31	75.47	81.20	47		51	$C_{47}H_{86}NO_{10}P$	3.7
PE C34:ω 1	9.3262	716.522	106.95	82.80	87.12				$C_{39}H_{75}NO_8P$	0.1
PS C37:ω 0	9.3789	804.578	157.24	122.86	129.69	121		128	$C_{45}H_{86}NO_{10}P$	-3
PS C38:ω 0	9.3798	818.593	528.37	460.42	454.46	116		115	$C_{46}H_{88}NO_{10}P$	1.1
PE C40:ω 5	9.4424	790.54	38.49	71.17	45.77		155		$C_{45}H_{76}NO_8P$	2.1
PS C44:ω 5	9.4955	892.61	58.05	34.60	40.33	144	86	168	$C_{50}H_{84}NO_{10}P$	-0.3
PS C40:ω 1	9.5613	844.612	76.00	68.61	57.24				$C_{48}H_{90}NO_{10}P$	3.8
PE C36:ω 2	9.6235	742.54	72.54	248.79	100.82	72	247	29	$C_{41}H_{77}NO_8P$	2.3
PS C40:ω 1	9.7152	844.608	137.18	614.38	233.25	59	263	22	$C_{48}H_{90}NO_{10}P$	3.3
PS C39:ω 1	9.7188	830.592	47.03	218.82	62.61	75	349	21	$C_{47}H_{88}NO_{10}P$	5.1
PE C38:ω 4	9.7203	766.54	300.09	258.48	199.41	150			$C_{43}H_{79}NO_8P$	4.3
PS C42:ω 3	9.7621	868.608	39.07	28.38	32.72				$C_{50}H_{88}NO_{10}P$	4.5
PE C40:ω 5	9.7667	792.555	71.14	24.01	42.74	166		296	$C_{45}H_{78}NO_8P$	5
PS C42:ω 2	10.164	870.629	57.23	23.94	34.82	164		239	$C_{50}H_{90}NO_{10}P$	4.7
PS C41:ω 1	10.271	858.625	19.24	33.80	33.41			57	$C_{49}H_{88}NO_{10}P$	3.7
PE C38:ω 3	10.555	768.556	61.39	64.50	42.47	145	152		$C_{43}H_{81}NO_8P$	2
PE C36:ω 1	10.604	744.554	163.39	166.97	150.29				$C_{41}H_{79}NO_8P$	5.9
PS C40:ω 0	10.658	846.623	353.63	303.46	316.18	112		117	$C_{48}H_{92}NO_{10}P$	0
PS C39:ω 0	10.665	832.609	140.42	114.10	123.72			123	$C_{47}H_{90}NO_{10}P$	4.4
PS C42:ω 1	10.875	872.639	82.05	134.20	76.91		174	61	$C_{50}H_{92}NO_{10}P$	-0.8
									StdDev (ppm)	2.3

Table 1. Identification of phospholipids observed in negative ion ESI/LC/MS analysis of H4IIE cells. Phospholipids are identified by class, acyl chain carbon number and degree of unsaturation. Significant peak area differences (95% confidence level (Students *t* > 2.78)) are expressed as a ratio of mean peak areas* 100.

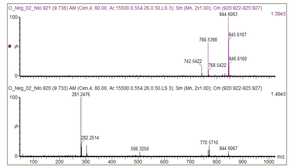


Figure 4. Top panel: TOF MS spectrum of component *m/z* 844.6083 @ 9.71 min. Bottom panel: TOF MS^E spectrum.

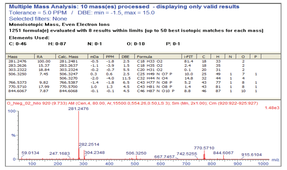


Figure 5. Elemental composition report for fragment ions from component *m/z* 844.6083 @ 9.71 min generated by MS^E. The compound is a PS containing oleate (C18:ω 1) and behenate (C22:ω 0) acyl chains.

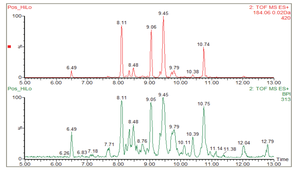


Figure 6. Top panel: XIC of *m/z* 184 from LC/MS^E analysis of phospholipids. The *m/z* 184 ion is specific to phosphatidylcholine and sphingomyelin. Bottom panel: ESI +ve LC/MS BPI chromatogram.

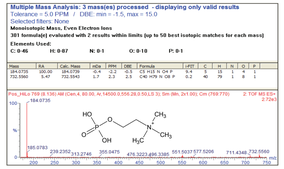


Figure 7. Elemental composition report for MS^E spectrum at 8.11 minutes. The phospholipid is PC C32:ω 1 (*m/z* 732.5560).

Data Visulization with SIMCA-P

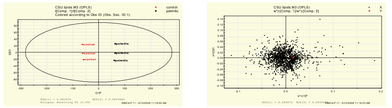


Figure 8. SIMCA-P orthogonal Partial Least Squares (o-PLS) plots for control and Palmitic acid treatment groups. Right panel: Scores plot. Left Panel: Loadings plot.

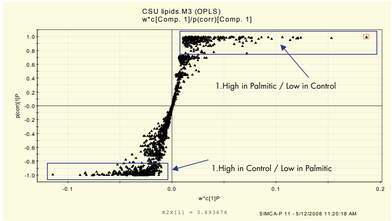


Figure 9. O-PLS 'S' Plot for Palmitic acid treatment vs. Control group. This plot highlights the components responsible for the group separation.

CONCLUSIONS

- **Clear separation of Palmitic acid treatment group from Control and Oleic acid treatment groups**
- **Identification of phospholipids that cause class separation**
- **Use of MS^E to determine phospholipid acyl chain composition**
- **Use of positive ion MS^E to identify and characterize PC and SM phospholipids**

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