Targeted LC-MS/MS profiling for the identification of the key enzymes involved in lysophosphatidic acid metabolism

Emily Armitage[1]; Aurelien Tripp[2]; Nikos Koundouros[2,3]; David J Magee[2,4]; Alan Barnes[1]; Neil J Loftus[1]; George Poulogiannis[2]

[1] Shimadzu MS/BU, Manchester, United Kingdom; [2] Signalling and Cancer Metabolism Team, Division of Cancer Biology, The Institute of Cancer Research, London, United Kingdom; [3] Alan Meyer Cancer Center, Weill Cornell Medicine, New York, NY; [4] Pain Medicine Department, The Royal Marsden Hospital, London, United Kingdom

Overview

- Targeted LPA profiling by LC-MS/MS demonstrates the role of key metabolic enzymes in the production of different LPA species.
- Five different CRISPR-mediated knockouts were studied revealing cytosolic phospholipase A2 as a key player in LPA production.

1. Introduction

Lysophosphatidic acid (LPA) refers to a group of bioactive phospholipids with different lengths and saturation that play a key role in the activation of oncogenic signalling pathways. LPA-induced signalling is recognised as a central component of several hallmarks of cancer through its contribution in metabolic reprogramming, promoting tumour growth and metastasis and facilitating immune evasion. Setting up therefore a robust LC-MS/MS method to detect different LPA species and identifying the key metabolic enzymes that regulate their production would be of immense clinical benefit. In this study, we developed a targeted LC-MS/MS method to measure LPA species in cell and media extracts with the aim to gain a greater understanding of some of the key metabolic enzymes (PLA1A, cPLA2, PLD2, ENPP2 and FABP1) involved in LPA production and transport.

2. Methods

Isogenic MCF-10A cell lines with either control or CRISPR-mediated knockout of PLA1A, cPLA2, PLD2, ENPP2 and FABP1 were generated. Cells were cultured until 80% confluent and cell pellets and media underwent lipid extraction using the Folch chloroform/methanol method. Samples were evaporated to dryness and resuspended in 350µL cold methanol before being processed for LC-MS/MS analysis (LCMS-8060, Shimadzu Corporation), mobile phase: water, acetonitrile with 0.1% ammonium hydroxide, Acquity BEH column (C18, 1.7µm, 100x2.1mm). LPA species were targeted in an unscheduled MRM method. MRM transitions were derived theoretically, and collision energy was optimised on extracted test samples. Five biological replicates were analysed for each of the experimental samples and these were run in a randomised order with pooled QCs every 5 injections.

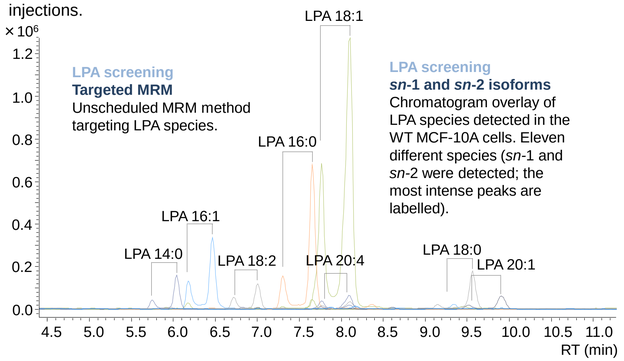

Figure 1. Mass chromatograms of LPA species detected in a wild type MCF-10A cell extract, measured by unscheduled MRM. 11 different LPAs were detected across all samples in both *sn*-1 and *sn*-2 isoforms.

2.1 Method development

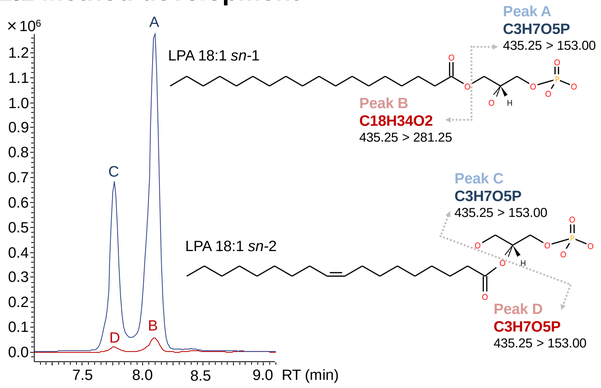

Figure 2. Theoretically derived MRM transitions for LPA species shown for LPA 18:1 as an example. Structures for the *sn*-1 and *sn*-2 isoforms are presented indicating the fragmentation leading to the two transitions for LPA 18:1. Each transition is observed for both isoform which are separated chromatographically.

Table 1. MRM transitions for LPA screening. Each LPA was targeted in an unscheduled MRM method enabling both *sn*-1 and *sn-2* isoforms to be detected. Two transitions were set, corresponding to the common dehydrated glycerol phosphate fragment (T1) and the specific fatty acid fragment (T2). Lipid Maps IDs are given for the *sn*-1 form of each LPA species; those marked with * are from Lipid Maps ® Lipid Standards, while the rest are from the Lipid Maps ® Structure Database (LMSD).

LPA	Formula	Lipid Maps ID	Precursor m/z	T1 m/z	CE	Dwell	T2 m/z	CE	Dwell
14:0	C17H35O7P	LMGP10050007*	381.20	153.00	10	22	227.20	10	25
16:0	C19H39O7P	LMGP10050006*	409.25	153.00	5	25	255.25	10	28
16:1	C19H37O7P	LMGP10050016	407.20	153.00	5	25	253.20	10	28
18:0	C21H43O7P	LMGP10050005*	437.25	153.00	5	25	283.25	10	28
18:1	C21H41O7P	LMGP10050008*	435.25	153.00	5	25	281.25	10	28
18:2	C21H39O7P	LMGP10050017	433.25	153.00	10	25	279.25	10	28
18:3	C21H37O7P	LMGP10050023	431.20	153.00	10	25	277.20	10	28
18:4	C21H35O7P	LMGP10050024	429.20	153.00	10	25	275.20	10	28
20:0	C23H47O7P	LMGP10050018	465.30	153.00	10	25	311.30	10	28
20:1	C23H45O7P	LMGP10050026	463.30	153.00	10	25	309.30	10	28
20:2	C23H43O7P	LMGP10050027	461.25	153.00	10	25	307.25	10	28
20:3	C23H41O7P	LMGP10050028	459.25	153.00	10	25	305.25	10	28
20:4	C23H39O7P	LMGP10050013*	457.25	153.00	10	25	303.25	10	28
20:5	C23H37O7P	LMGP10050033	455.20	153.00	10	25	301.20	10	28
22:0	C25H51O7P	LMGP10050031	493.35	153.00	10	25	339.35	10	28
22:1	C25H49O7P	LMGP10050029	491.30	153.00	10	25	337.30	10	28
22:2	C25H47O7P	LMGP10050030	489.30	153.00	10	25	335.30	10	28
22:4	C25H43O7P	LMGP10050020	485.25	153.00	10	25	331.25	10	28
22:6	C25H39O7P	LMGP10050019	481.25	153.00	10	25	327.25	10	28

3. Results

Cell and media extracts from control (CTRL) and CRISPR-mediated knockout (KO) of PLA1A, cPLA2, PLD2, ENPP2 or FABP1 were analysed using the LPA screening method. The datasets obtained from cells or media extractions were treated separately for statistical analysis. Each dataset was scale normalized using the Limma package in R (4.1.0) and analysed using the MetaboAnalyst software. cPLA2 KO led to the most significant changes in LPA abundance (peak area) when compared to the CTRL cells. Intracellular levels of *sn*-1 LPAs (18:0, 20:1 and 20:3) were significantly reduced following cPLA2 KO (Figure 3 & 4), whilst 9 different LPA species were significantly reduced and 13 were increased in the media (Figure 4). PLA1A KO led to a significant reduction in LPA 16:1 *sn*-1 in cell extracts (Figure 3). No significant differences were observed in the PLD2 model and only a few marginal changes were observed in the ENPP2 and FABP1 datasets.

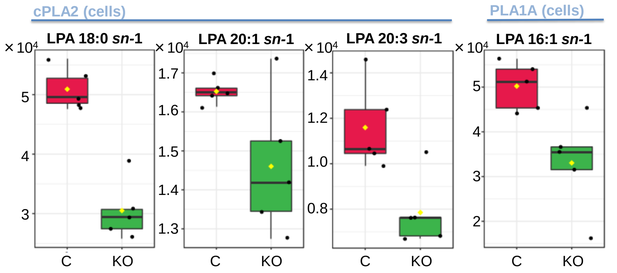

Figure 3. Boxplots generated using MetaboAnalyst presenting 3 LPAs that significantly differed in response (peak area) to CRISPR KO of cPLA2 and one LPA that significantly differed in response to CRISPR KO of PLA1A.

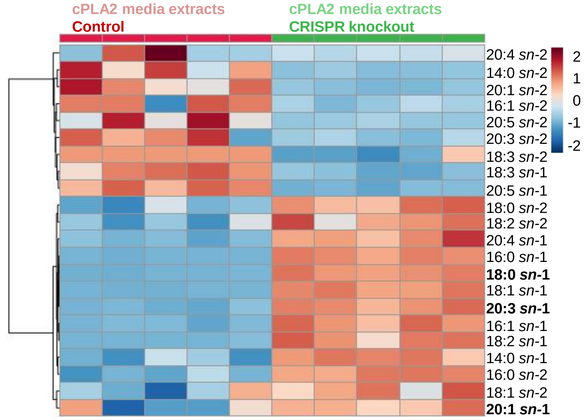

Figure 4. Heatmap generated using MetaboAnalyst highlighting the significantly different abundance of LPA species in the media extracts from cells with cPLA2 CRISPR KO versus wildtype CTRL. LPA species that were found to be significantly different in the corresponding cell extracts (Figure 3) are highlighted in bold.

4. Discussion

Several different enzymes are involved in regulating LPA production. Phospholipase D2 (PLD2) catalyzes the hydrolysis of phosphocholine to phosphatidic acid, from which LPA can be derived intracellularly through the cleavage of the 1-acyl by phospholipase A1 (PLA1, encoded by PLA1A gene) or the 2-acyl by cytosolic phospholipase A2 (cPLA2). Fatty acid binding protein 1 (FABP1) facilitates intracellular transport of LPA and its export where it joins other sources of LPA production extracellularly from ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2). Figure 5 shows a schematic of LPA production both intracellularly and extracellularly as well as the interplay between the 5 different enzymes studied herein. In line with cPLA2 being one of the key enzymes contributing to LPA production, its KO a significant decrease in *sn*-1 LPAs intracellularly and a significant increase extracellularly suggesting that there may be of a compensatory mechanism contributing to *sn*-1 production. PLA1A knockout resulted in only one significant difference (LPA 16:1 *sn*-1) intracellularly and few significant differences were detected in the remaining knockouts with no obvious trends observed.

KEY:
PA: phosphatidic acid
PC: phosphatidylcholine
PE: phosphatidylethanolamine
LPA: lysophosphatidic acid
LPC: lysophosphatidylcholine
PLA1/2: phospholipase A 1/2
PLD: phospholipase D
cPLA2: cytosolic phospholipase A2
FABP1: fatty acid binding protein 1
ENPP2: ectonucleotide pyrophosphatase/phosphodiesterase 2

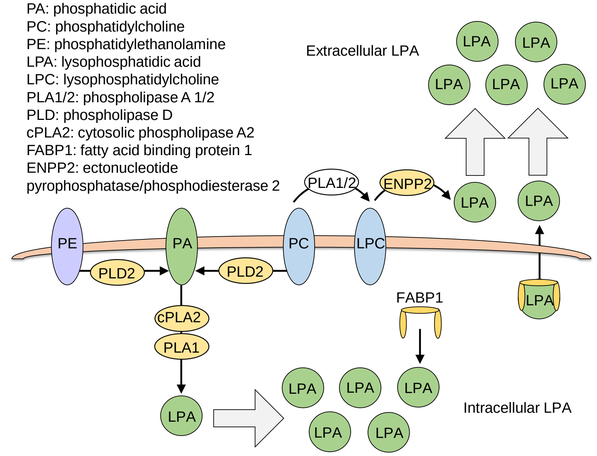

Figure 5. Intracellular and extracellular LPA production. LPA is produced intracellularly from PC and PE via PA, is transported facilitated by FABP1 and is produced extracellularly from PC via LPC. Enzymes highlighted in yellow are the ones studied through CRISPR KO of the encoding genes.

5. Conclusions

- An LPA screening method has been developed using LC-MS/MS (LCMS-8060, Shimadzu Corporation).
- cPLA2 is a key enzyme for the metabolism of LPAs but appears to have a distinct role for extracellular versus intracellular levels of LPA species.

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