Lipidomics identifies a requirement for peroxisomal function during influenza virus replication

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Abstract  Influenza virus acquires a host-derived lipid envelope during budding, yet a convergent view on the role of host lipid metabolism during infection is lacking. Using a mass spectrometry-based lipidomics approach, we provide a systems-scale perspective on membrane lipid dynamics of infected human lung epithelial cells and purified influenza virions. We reveal enrichment of the minor peroxisome-derived ether-linked phosphatidylcholines relative to bulk membrane glycerophospholipids (GPLs) via phospholipase activity, and (to some extent), are metabolized in peroxisomes. We find that the unique cholesterol compositions for virus production. Host cell lipid metabolism and plasma membrane microdomains are implicated in the biogenesis of virus envelopes. Several studies have dissected the lipid inventory of purified influenza virions (9, 10), whereas others have demonstrated the requirements for de novo fatty acid and sphingolipid biosynthesis and unique cholesterol compositions for virus production at budding sites (11–14).

In addition to the importance of host cell lipid metabolism for the biogenesis of influenza virus envelopes, recent findings suggest a major role for soluble lipid mediators in antiviral responses against influenza virus infection in vivo (15, 16). These soluble lipid mediators originate from membrane glycerophospholipids (GPLs) via phospholipase activity, and (to some extent), are metabolized in peroxisomes. For example, β-oxidation in the peroxisome is crucial for the retroconversion of DHA, the precursor of the lipid mediator protectin D1, which prevents nuclear export of influenza virus RNAs; protectin D1 production is directly inhibited by influenza virus (15). The role of peroxisomes during influenza virus replication is further evident by interaction between influenza virus nonstructural protein 1 (NS1) and multifunctional protein 2 (MFP2/HSD17B4), an antiviral protein essential for peroxisomal β-oxidation (17). Therefore, the collective literature indicates an apparent role for peroxisomes as the initial sites of antiviral signaling (18).

Influenza viruses hijack host cell machineries for efficient replication and acquire a host-derived lipid envelope during budding. Recent systems-scale studies have primarily addressed the individual roles of genes (1–5) and proteins (6–8) in this process, yet have failed to illustrate how they function together to generate macromolecular precursors for virus production. Host cell lipid metabolism and plasma membrane microdomains are implicated in the biogenesis of virus envelopes. Several studies have dissected the lipid inventory of purified influenza virions (9, 10), whereas others have demonstrated the requirements for de novo fatty acid and sphingolipid biosynthesis and unique cholesterol compositions for virus production at budding sites (11–14).

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METHODS

Virus strains, virus production, and purification

Virus stocks were prepared by passaging egg-grown virus strains once in MDCK cells. Virus strains were purified from A549 and MDCK cells as described in the supplementary data.

Lipid extraction of infected cells and purified viruses

A549, Chinese hamster ovary (CHO)-K1, and NRel-4 cells were seeded into 10 cm cell culture dishes 24 h prior to infection. Cells at 80–100% confluence were infected with a 5 ml inoculum of purified influenza virus A/PR/8/34 H1N1 at MOI 5. Virus-infected cells and mock-infected cells were collected at 12, 18, and 24 hours postinfection (hpi) (for A549 cells) or only at 18 hpi (CHO-K1 and NRel-4 cells). Lipid extraction was conducted according to a modified Bligh and Dyer protocol described in the supplementary data.

Quantitative analysis of lipids by HPLC MS/MS

Samples with spiked internal standards were analyzed by ESI-MS. Signal intensities for each lipid species were extracted according to their retention time, normalized to the representative spiked internal standards, and represented as a molar fraction of the total amount of measured lipids. Statistical significance was calculated using an unpaired Student’s $t$-test ($P < 0.05$; two-tailed) or a block-design three-way ANOVA corrected by a false discovery rate procedure (for more details, see the supplementary data).

D609 and GW7647 treatment of influenza virus-infected cells

D609 and GW7647 were purchased from Tocris Bioscience (Bristol, UK). A549 cells were infected with influenza virus A/PR/8/34 H1N1 (MOI <1), and serum- and antibiotics-free medium supplemented with D609 (10 µM and 100 µM) or GW7647 (1 µM, 2 µM, and 5 µM) were added at 12 hpi and 1 hpi, respectively.

RESULTS AND DISCUSSION

To systematically characterize the temporal changes of host cell membrane lipid composition during influenza virus infection, human lung epithelial (A549) cells were infected with purified influenza virus A/PR/8/34 H1N1 and total cellular lipids were extracted 12, 18, and 24 hpi. A high multiplicity of infection (MOI5) was used to ensure a synchronous, one-round of infection (19). A total of 175 lipid species, representing GPL and sphingolipids (SPLs), two major membrane lipid classes analyzed in this study, were measured using established methodology based on HPLC and ESI-MS and operated in multiple reaction monitoring mode (Fig. 1A, supplementary Table II) (20, 21). The levels of 90 lipid species (i.e., ~52% of all measured lipids) were significantly altered between H1N1-infected and mock-infected cells ($q < 0.006$; Fig. 1B, C) at either 18 hpi, 24 hpi, or both. Of these, 35 (Fig. 1B, red pie chart) and 15 (Fig. 1B, blue pie chart) lipid species had correlation coefficients of $>0.9$ or $<0.9$, respectively, with virus titer (Fig. 1B, supplementary Tables I and II).
Lipids implicated in influenza virus replication

Fig. 1. Influenza virus infection impacts peroxisomal and sphingolipid metabolism in the host. A: A549 cells were infected with purified influenza virus. 175 lipid species were analyzed by mass spectrometry at 12, 18, and 24 hpi. B: Distribution of Pearson correlation coefficients between 175 lipid species and virus titer (supplementary Tables I, II). Black, red (>0.9) and blue (<0.9) indicate 90 lipid species altered in influenza virus-infected cells (q < 0.006; supplementary Table II). C: Heatplot showing fold ratios (infected/mock) of 90 lipid species with altered levels upon infection (q < 0.006). Yellow and blue indicate elevated and decreased concentrations, respectively. Lipid species, which correlate with virus titer (B), are indicated by red (>0.9) and blue (<0.9) fonts. Representative structures to illustrate the differences between ester-linked (D), odd chain (E), and ether-linked (F) PC lipids. Please note that we were able to determine the total carbon fatty acyl composition but did not dissect the exact carbon composition of the two fatty acyl constituents in the measured GPL species. Hence, the two fatty acyl constituents shown in the structures can vary as long as they add up to the respective total carbon fatty acyl compositions. G: Fold ratios of changes in fatty acid chain length composition of Cer, HexCer, and SM lipid species. Results in panels A, B, C, and G are from three independent experiments with three replicates each (n = 9 for each condition). Error bars in G represent ± SDs.
influenza virus-infected cells (22). However, additional SM synthases (SMS1 and SMS2, which transfer the choline donor S-adenosylmethionine required for the de novo methylation pathway for aPC biosynthesis, was significantly downregulated in influenza virus-infected cells (22, 24)). These changes coincided with the increasing levels of another choline containing lipid, SM, suggesting an important correlation of influenza virus replication with choline lipid metabolism (Fig. 1B,C). Increase in SM and decrease in aPC species could possibly be explained by the activities of inter-related enzyme systems including sphingomyelin synthases (SMS1 and SMS2, which transfer the choline headgroup of PC onto a ceramide backbone to produce SM) and ethanolamine kinase 1, which is downregulated in influenza virus-infected cells (22). However, additional experiments such as quantitative proteomics and enzymatic assays would be required to draw such conclusions.

The results presented here provide a good starting point for generation of hypotheses and such future investigations. The upsurge in the proportion of long chain (>38 fatty acyl carbons, >C38) aPC species with polyunsaturated fatty acyls (Fig. 1D), odd chain aPC (Fig. 1E), and ePC (Fig. 1F) suggested altered peroxisomal lipid metabolism in infected cells. Consistent with impaired peroxisomal β-oxidation, we observed an enrichment in C26:0 but a decrease in C24:1 fatty acids in SPL species (Fig. 1G) (25–27); this provided further support for an important role of fatty acyl metabolism during influenza infection (15, 16).

We next tested whether alterations in host membrane lipid levels are detectable also in envelopes harvested from purified virus particles (Fig. 2A). Indeed, aPC, phosphatidylethanolamine, phosphatidylcholine (aPC) species, but increased SPL such as many SM and hexosylceramide (HexCer) species (Fig. 1B, C). These observed changes in lipid species were also reflected by the increase in total amounts of ether-linked PC (ePC), odd chain aPC and SM lipid classes and by the decrease in the total amount of Ganglioside GM3 lipid class across the three independent experiments (supplementary Figs. I, II).

The decrease in the proportion of ganglioside GM3 species likely reflected influenza virus neuraminidase activity (9). The reduced levels of aPC species in influenza virus-infected cells have been previously proposed to be related to impaired aPC biosynthesis as measured by metabolite rates of phospholipid precursors and by global gene and protein expression experiments (8, 22, 23). SREBP1, a major regulator of the one-carbon cycle producing the methyl donor S-adenosylmethionine required for the de novo methylation pathway for aPC biosynthesis, was significantly downregulated in influenza virus-infected cells (22, 24).

To account for the confounding effect of variations in peroxisomal activity among different host cell lines as well as differences in experimental approaches in the published literature, we decided to use the difference between the ePC/aPC ratios of virus particles and uninfected producer cells ($\Delta_{\text{virus/Cell}} = \text{ePC/aPC}_{\text{Virus}} - \text{ePC/aPC}_{\text{Cell}}$) as a molecular proxy of PC lipid class remodelling to compare a wide variety of different studies (Fig. 2C–F). Envelopes of influenza viruses (circles in Fig. 2C, F) had a significantly higher ePC/aPC ratio than their uninfected producer cells (bars in Fig. 2C, F), whereas other enveloped viruses (including human immunodeficiency virus, murine leukemia virus, vesicular stomatitis virus, dengue virus, and hepatitis C virus) showed equal or lower ePC/aPC ratios (diamonds in Fig. 2D, F, supplementary Table IV). Therefore, peroxisome-dependent remodelling of lipids within the abundant PC class, rather than overall changes to total PC concentration, is specific to influenza virus.

We next determined the lipid composition of two closely related H3N2 influenza virus strains differing in pathogenicity. The parent influenza A strain A/Aichi/2/68 H3N2 (P0) was adapted by ten passages in mice (P10), which ultimately showed higher virulence with enhanced replication fitness because of nonconservative point mutations in hemagglutinin (HA) (Gly218Glu) and NS1 (Asp125Gly) (28). NS1 regulates lipid metabolic genes in a severity-dependent manner (22) and interacts with MFP2/HSD17B4 (see above) (17). The latter mutation has been shown to produce high virus titres with enhanced interferon-β antagonism and to differentially regulate host gene expression (29, 30). We assumed a negligible effect of the mutation in HA (Gly218Glu) on host lipid metabolism because the mutation lies in a region involved in sialic acid linkage recognition, important for entry rather than influenza virus replication within the host cell (28). We showed that the more pathogenic H3N2 strain (P10) exhibited a ~25% higher ePC/aPC ratio in its envelope than the less pathogenic P0 strain (Fig. 2E, supplementary Fig. III, supplementaryTables IV, V), which was comparable with the variation in ePC/aPC ratios between different influenza viruses (Fig. 2G). Collectively, these results suggest strain-dependent differences with regard to PC class remodelling which might reflect influenza virus pathogenicity and underscore the conserved role of peroxisomes in influenza infection (Fig. 2F).

We next sought a systems-scale perspective on the broader impact of influenza virus-induced perturbations of lipid metabolism (Fig. 3). To do so, we used the aforementioned data from (i) A549 cell infection (host), (ii) purified H1N1 virus (virus), and (iii) H3N2 P10 virus (pathogenicity) for unsupervised cluster analysis (Fig. 3A, supplementary Table VI). We assigned 13 clusters (AU $p$-value <0.09, Fig. 3B) with unique patterns of lipid regulation (Fig. 3C). For instance, lipids with increased concentrations in virus-infected cells but decreased levels in virions are suggestive of intracellular requirements for virus replication as revealed for long chain fatty acid-containing ePC and two aPC species with long but saturated fatty acyls (cluster 6 in Fig. 3C, D). Unsaturated ester-linked PE and PC species were reduced both in virions as well as in infected cells (Fig. 3E), consistent with the downregulation of genes implicated in ester-linked GPL metabolism (22). On the contrary, lipids enriched in both infected cells and virions are indicative of a possible function in virus morphogenesis, as seen for saturated short-chain fatty acid-containing ePC species (cluster 10 in
Lipids implicated in influenza virus replication (Fig. 4A). This result strengthens the previously identified requirement of intact SM biosynthesis for influenza virus production (Fig. 1) (14), but more specifically, implies importance of the salvage pathway.

To further scrutinise the necessity of ether lipid metabolism during influenza replication, we infected wild-type CHO-K1 and ether lipid-deficient CHO cells (NRel-4) (32) with influenza virus H1N1 A/PR/8/34. NRel-4 cells exhibited lower expression levels of influenza virus proteins NS1 and M2, and a four- to five-fold decrease in virus production when compared with CHO-K1 cells (Fig. 4B). The lipid alterations induced by influenza virus in CHO cells were consistent with the changes in A549 cells (supplementary Fig. V) with the obvious exception of ePC, which cannot be generated in NRel-4 cells because of the reported impairment in their peroxisomal dihydroxyacetone phosphate acyltransferase (DHAPAT) activity. DHAPAT catalyses the first committed step in ether lipid biosynthesis, attaching a fatty acid to dihydroxyacetone phosphate (DHAP).

As acyl-DHAP can also be redirected into TAG biosynthesis (33), and because the two CHO cell variants were not isogenic, we therefore decided to further investigate
A

Pathogenicity
Lipid
Lipid
Lipid

Pathogenicity

Virus

Host

B

C

Recasting

Clustering

Hierarchical Clustering using Pearson correlation distances (uncentered) with average-linkage

D

Cluster 6
(intracellular)

Pathogenicity

Virus

Host

P10^0
H1N1^1
18hpi^2
24hpi^2

E

Cluster 7
(antiviral)

Pathogenicity

Virus

Host

P10^0
H1N1^1
18hpi^2
24hpi^2

F

Cluster 12
(Envlope)

Pathogenicity

Virus

Host

P10^0
H1N1^1
18hpi^2
24hpi^2

G

Cluster 13
(pathogenicity)

Pathogenicity

Virus

Host

P10^0
H1N1^1
18hpi^2
24hpi^2

H

Correlation with virus titre

Cluster

Relative Change

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
ether lipid involvement using two siRNA constructs against peroxisomal AGPS. AGPS is the immediate downstream enzyme of DHAPAT in ether lipid biosynthesis, which exchanges the fatty acid of acyl-DHAP with a fatty alcohol. Both probes led to the substantial reduction in enzyme levels (70%), as judged by Western blotting (Fig. 4C).

### Fig. 3
Life cycle-dependent clusters of lipids revealed by comparative analysis of host and viral lipid profiles. A: Hierarchical clustering was performed on 146 lipid species describing pathogenicity-related differences in lipid composition, influenza virus-enriched lipids, and alterations in host cell lipid metabolism. Values were rescaled to make different data sets comparable for cluster analysis (see Methods for details). B: Approximately unbiased (AU) p-values and standard errors (bootstrap resampling; n = 10,000) of tree splits (gray circles) and 13 assigned clusters (black circles). C: Dendrogram indicating increased (red) and decreased (blue) levels of individual lipid species (supplementary Table VI). D–G: Average changes in lipid species (gray lines) and average relative trends (black lines ± SDs) for intracellular (D), antiviral (E), envelope-enriched (F) and pathogenicity-dependent (G) clusters. Correlations of individual lipid species with virus titer is indicated by red (>0.9) and blue (<0.9) fonts, respectively; * altered in a pathogenicity-dependent fashion; † enriched in H1N1; ‡ alterations in infected cells as determined in Figs. 1 and 2. H: Correlation of the 13 clusters with virus titer. Robust correlations are colored in blue (negative) and red (positive).
supplementary Fig. VI A), to a moderate (<1.5-fold) but significant decrease in ether lipid levels (supplementary Fig. VI B), and, importantly, to a 60% reduction in infectious virus production (Fig. 4C). These results were comparable to siRNA constructs targeting Rab11a, which is required for assembly and budding (34), and demonstrate the functional importance of ether lipid biosynthesis in influenza virus production.

To examine the functional role of the peroxisome, we treated infected cells with a PPARα agonist (GW7647) that induces peroxisomal fatty acyl β-oxidation (35, 36). We hypothesized impaired peroxisomal β-oxidation in influenza virus-infected cells due to the following evidence: 1) catalase activity correlates with peroxisomal β-oxidation and was decreased (Fig. 4D) (37), 2) accumulation of SPL-containing long chain fatty acyls (C26:0) are a molecular marker for impaired peroxisomal β-oxidation (Fig. 1D), and 3) the decrease of C24:1 fatty acyls is linked to reduced acyl-CoA oxidase 1 activity, an enzyme essential for peroxisomal β-oxidation (Fig. 1D) (27). Accordingly, virus production was reduced upon GW7647 treatment of A549 cells without impacting cell viability (Fig. 4E). These findings were consistent with independent studies identifying MFP2/HSD17B4 (see above), acyl-CoA oxidase 1, and carnitine O-octanoyltransferase as antiviral mediators of influenza virus infection (5, 8, 17). They further establish the antiviral role of peroxisomal β-oxidation and PPARα activation and provide a functional explanation for the usage of PPARα agonists as an alternative treatment for influenza virus infection (38).

Finally, we explored the potential in vivo relevance of peroxisomal and SPL metabolism for influenza virus infection through an examination of the reported susceptibility factors in mice (39). We found 23 genes associated with lipid metabolism [5% of total genes measured (39)] that were enriched for peroxisomal and SPL metabolism (Fig. 4F). The platelet-activating factor (PAF) acetylhydrolase, PL2AG7, which exhibits the strongest association, has been independently identified as a host susceptibility factor for influenza infection (40). PL2AG7 hydrolyses PAF, an ePC and activator of platelets and inflammation, producing lyso-PAF, which can be converted to ePC by lyso-PC acyltransferase 2 (Fig. 4G). Consistent with elevated ePC levels in infected cells, influenza virus infection correlated with higher expression levels of PL2AG7 and induced lyso-PC acyltransferase 2 activity in mice (39, 41). These findings provide a link between the metabolic pathways of peroxisomes and lipid mediators involved in inflammation in vivo.

In summary, we present a detailed account of the temporal changes in host cell membrane lipids during influenza virus replication in relation to the composition of virus envelopes and virus pathogenicity. While our study does not directly expose the mechanistic actions of identified lipids in the influenza virus life cycle in detail, the comprehensive systems-scale catalog of lipids reported here is the first of its kind (Fig. 3). The combination and hierarchical clustering of the different datasets provides a powerful framework to derive novel hypotheses in the emerging field of lipid involvement during virus infections.

As a result, we present clear evidence that metabolism of ether lipids is functionally important for the production of infectious virions. Further integration of our findings with other published genomics and proteomics data (1, 3–8, 15, 22) led to a systems-scale model of host cell lipid metabolism during influenza virus infection, which will serve as a reference basis for future investigations (Fig. 4G, supplementary Table VII). Based on this analysis, we propose three major lipid metabolic pathways implicated in influenza virus replication: 1) elevated ether lipid and 2) elevated SPL biosynthesis required for influenza virus morphogenesis and 3) decreased peroxisomal β-oxidation associated with intracellular life cycle stages (Fig. 4G). Peroxisomal function is a common metabolic denominator and may therefore represent a key determinant for influenza virus replication. Our detailed analysis represents a major step forward in uncovering that peroxisomes and especially their lipid metabolism are exploited by influenza viruses. Our findings may open entirely new avenues with immediate exploitability for therapeutic interventions against influenza virus infection via peroxisome function.

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