Host lipidome analysis during rhinovirus replication in HBECs identifies potential therapeutic targets

An Nguyen,†‡ Anabel Guedán,†‡ Aurelie Mousnier,‡ David Swieboda,† Qifeng Zhang,† Dorottya Horkai,* Nicolas Le Novere,‡ Roberto Solari,‡ and Michael J. O. Wakelam†‡

Babraham Institute,* Babraham Research Campus, Cambridge CB22 3AT, United Kingdom; and Medical Research Council and Asthma United Kingdom Centre in Allergic Mechanisms of Asthma,† Airway Disease Infection Section, National Heart and Lung Institute, Imperial College, London, London W2 1PG, United Kingdom

Abstract In patients with asthma or chronic obstructive pulmonary disease, rhinovirus (RV) infections can provoke acute worsening of disease, and limited treatment options exist. Viral replication in the host cell induces significant remodeling of intracellular membranes, but few studies have explored this mechanistically or as a therapeutic opportunity. We performed unbiased lipidomic analysis on human bronchial epithelial cells infected over a 6 h period with the RV-A1b strain of RV to determine changes in 493 distinct lipid species. Through pathway and network analysis, we identified temporal changes in the apparent activities of a number of lipid metabolizing and signaling enzymes. In particular, analysis highlighted FA synthesis and ceramide metabolism as potential anti-rhinoviral targets. To validate the importance of these enzymes in viral replication, we explored the effects of commercially available enzyme inhibitors upon RV-A1b infection and replication. Ceranib-1, D609, and C75 were the most potent inhibitors, which confirmed that FAS and ceramidase are potential inhibitory targets in rhinoviral infections.‡ More broadly, this study demonstrates the potential of lipidomics and pathway analysis to identify novel targets to treat human disorders.—Nguyen, A., A. Guedán, A. Mousnier, D. Swieboda, Q. Zhang, D. Horkai, N. Le Novere, R. Solari, and M. J. O. Wakelam. Host lipidome analysis during rhinovirus replication in HBECs identifies potential therapeutic targets. J. Lipid Res. 2018. 59: 1671–1684.

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Rhinoviruses (RVs), members of the picornavirus family, are the causative agent of the common cold. Like other picornaviruses, they have a single positive-strand RNA genome of about 7.5 kb. Following infection and entry into the cytoplasm, the viral genome is translated and posttranslationally cleaved into four capsid proteins and seven nonstructural proteins that participate in viral replication. All known picornaviruses use the cytoplasmic surface of ER/Golgi membranes for genome replication, and the morphology of these membranes is greatly remodeled by the virus into replication organelles or complexes. The viral nonstructural proteins, 2B, 2C, and 3A, associate with the ER and Golgi membranes and are thought important in remodeling through recruitment of host proteins. In addition to morphological changes to the ER and Golgi, viral replication induces changes to the lipid composition of cellular membranes. A number of host factors have been identified as being involved in membrane remodeling and viral replication, including Golgi brefeldin A-resistant guanine nucleotide exchange factor 1 (GBF1)/ADP-ribosylation factor 1 (Arf1) (1), phosphatidylinositol-4-kinase (PI4K) (2, 3),

Abbreviations: Arf1, ADP-ribosylation factor 1; Cer, ceramide; CL, cardiolipin; DG, diacylglycerol; HBEC, human bronchial epithelial cell; hpi, hours post infection; LPC, lysophosphatidylcholine; LPCAT, lysophosphatidylcholine acyltransferase; LPI, lysophosphatidylinositol; MOI, multiplicity of infection; OSBP, oxysterol binding protein; PA, phosphatic acid; PC, phosphatidylcholine; PC-PLA, phosphatidylcholine phospholipase A; PC-PLC, phosphatidylcholine-specific phospholipase C; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PI4K, phosphatidylinositol-4-kinase; PIP, phosphoinositide phosphatase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PIP4K, phosphatidylinositol-4,5-bisphosphate kinase; PIP5K, phosphatidylinositol 5-kinase; PKD, protein kinase D; PLD, phospholipase D; PS, phosphatidylserine; RV, rhinovirus; S1P, sphingosine-1-phosphate; SPHK, sphingosine kinase; S1P, sphingosine-1-phosphate; TG, triglyceride; VAP-A, VAMP-associated protein-A.

†Present address of A. Guedán: The Francis Crick Institute, 1 Midland Road, London NW1 1AT, UK.
‡Present address of A. Mousnier: Centre for Experimental Medicine, School of Medicine Dentistry and Biomedical Sciences, Queen’s University Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland.
§To whom correspondence should be addressed.
e-mail: Michael.wakelam@babraham.ac.uk
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oxysterol binding protein (OSBP) (4–6), and protein kinase D (PKD) (7); inhibitors of these targets inhibit replication of a number of picornaviruses. This suggests that membrane remodeling is important for picornaviral replication and represents potential for the discovery of novel anti-viral targets.

In this study, we have performed an open platform unbiased analysis of changes to the host lipidome during a single replicative cycle of RV in primary human bronchial epithelial cells (HBECs). We analyzed lipid samples extracted from synchronously infected cells over a time course of 6 h and found evidence of multiple lipid metabolic pathways being altered with an unexpected and remarkable complexity of changes in both lipid class and the length and saturation of acyl chains. These studies identified lipid-modifying enzymes as potential anti-viral targets, and we tested these with chemical inhibitors demonstrating that particular lipid-modifying enzymes may represent potential anti-viral drug targets.

**MATERIALS AND METHODS**

**Cells**

HBECs (CC2540; Lonza) were cultured following the manufacturer’s recommendations. HeLa H1 (ATCC CRL-1958) and HeLa Ohio (European Collection of Authenticated Cell Cultures 930021013) were used to produce RV1B and to perform viral end-point titer determination, respectively.

**Virus infections**

HRV-A1b stocks (ATCC) were produced by infection of HeLa H1 and were titered on HeLa Ohio cells to determine the TCID$_{50}$ per milliliter.

A number of compounds were tested for their anti-viral effect at a single concentration of 10 μM. Compounds showing activity were subsequently tested for their anti-viral activity over concentration response curves ranging from 0.5 to 20 μM. For the single dose experiments, a dose at which highest virus replication inhibition is achieved without cytotoxicity was used (10 μM for Ceranib 1 and D609; 20 μM for 3-Omethyl-SM, SK-I, SK-II, VU 0155069, and C75). HBECs were pretreated with the specified concentration of the compounds or the vehicle alone for 1 h at 37°C. RV-A1b was added to the cells at multiplicity of infection (MOI) 5, incubated for 1 h at room temperature to obtain a synchronous infection, washed with BEBM to remove unbound virus, and further incubated in fresh medium containing the compound or the vehicle for 7 h at 37°C. At the end of the infection/replication period, cells were harvested for further analysis by quantitative (q)RT-PCR, Western blot, or endpoint titer determination by TCID$_{50}$. The cytotoxicity of the compounds at 9 h was determined by using the Viral ToxGlo assay (Promega) according to the manufacturer’s instructions.

**qRT-PCR**

After the 7 h incubation period, cells were lysed with RLT buffer (Qiagen) supplemented with β-mercaptoethanol (Sigma) at 1:200 dilution. mRNA was extracted by using the RNase minikit (Qiagen) according to manufacturer’s instructions. One microgram of RNA was reverse-transcribed for cDNA synthesis for 1 h at 37°C by using the Omniscript RT kit (Qiagen). Quantification of the levels of viral RNA was conducted by using specific primer (Invitrogen) and probe (Eurofins) sequences as follows: RV forward primer 5′-GGAGAGGCTCCCTGCTGCT-3′ (50 nM), RV reverse primer 5′-GGCTSCAGTTGAAGGTAGGC-3′ (300 nM), HRV probe 5′-TGAATTCCCGCGGCTTC-3′ (100 nM); 18S forward primer 5′-GCCTGCTAGCTGTTAATTG-3′ (300 nM), 18S reverse primer 5′-CATTTCGTCCGAATGGTC-3′ (300 nM), and 18S probe 5′-ACCGCAGCAAGCGGACCAGA-3′ (100 nM). Analysis was performed by using Quantitect Probe PCR master mix (Qiagen) and the LightCycler 480 real-time PCR system (Roche). For absolute quantification, the level of each gene was normalized to the level of 18S rRNA, and the exact number of copies of the gene of interest was calculated by using a standard curve generated by the amplification of plasmid DNA.

**Western blotting**

Cells were lysed in ice-cold RIPA buffer (Sigma) supplemented with protease (Roche) and phosphatase (Sigma) inhibitors (according to the manufacturers’ instructions), and their protein content was measured by the bicinchoninic acid assay (Thermo Scientific). Equal amounts of protein were loaded onto 4–12% Bis-Tris SDS-PAGE gels (Life Technologies) followed by transfer onto PVDF membranes (Life Technologies). Membranes were blocked in PBS supplemented with 5% BSA and 0.1% Tween 20 for 1 h at room temperature. Primary antibodies were incubated overnight at 4°C, and secondary antibodies were incubated for 1 h at room temperature followed by the addition of ECL reagent and data collection on a Fusion FX7 image analyzer (Vilber Lourmat). Analysis of quantified images was performed by using ImageJ. The primary and secondary antibodies used were: rabbit anti-RV 2C [generated and used as previously described (8)], rabbit anti-lamin B1 (Proteintech), and donkey anti-rabbit conjugated to HRP (Jackson ImmunoResearch).

**LDL uptake**

HBECs were grown on coverslips and pretreated with vehicle or compound for 1 h at 37°C. Human nonacetylated LDL conjugated to BODIPY™ FL (Thermo Fisher Scientific) was added at a final concentration of 25 μg/ml, also in the presence of vehicle or compound, and cells were incubated for 1 h at 37°C. Cells were fixed with 4% paraformaldehyde, washed with PBS, permeabilized, and then stained with a mouse anti-GM130 antibody (BD Pharmigen) followed by a donkey anti-mouse antibody coupled to Alexa Fluor 546 (Jackson ImmunoResearch). LDL quantification is shown as the fluorescence ratio between LDL and GM130. Cells from five different fields of each condition and from three independent experiments were quantified using ImageJ.

**Virus endpoint titer determination (TCID$_{50}$/ml)**

Cells and supernatant were scraped, frozen-thawed twice, and centrifuged at 10,000 g for 5 min at 4°C to remove cell debris, and the supernatant containing the viral particles was used to perform TCID$_{50}$ titration assays as follows. HeLa Ohio cells were incubated in 96-well plates in DMEM (supplemented with 2% FBS and 1% penicillin-streptomycin) with 8-fold dilutions of the virus in six replicates for 5 days. Titration was assessed by the presence or absence of cytopathic effect in each well by using an RV-A1B stock as a positive control.

**Lipidomics**

Cells were harvested, washed twice in ice-cold PBS, and then flash-frozen in liquid nitrogen. Cell pellets were washed twice with cold PBS and resuspended in 1.5 ml methanol, and 40 μl of lipid standards were added. This sample of lipid standard contained 17:0:cholesterol ester (400 ng), cholesterol47 (1,000 ng), 17:1/17:1-triaclylglycerol (800 ng), 17:0/18:1-diaclylglycerol (DG;
200 ng), 17:0-monoacylglycerol (100 ng), 17:0-FFA (400 ng), 17:0-fatty acyl CoA (100 ng), 17:0-fatty acyl carnitine (50 ng), 17:0/18:1-phosphatidic acid (PA; 50 ng), 17:0/18:1-phosphatidylcholine (PC; 400 ng), 17:0/18:1-phosphatidylethanolamine (PE; 200 ng), 17:0/18:1-phosphatidylycerol (PG; 50 ng), 17:0/20:4-phosphatidylinositol (PI; 400 ng), 17:0/18:1-phosphatidylserine (PS; 200 ng), 14/140/140/140-cardiolipin (CL; 200 ng), C17-platelet-activating factor (50 ng), C17-2-hsoplatelet-activating factor (50 ng), 17:0-2-hsophosphatidyl acid (50 ng), 17:2-hsophosphatidylcholine (LPC; 100 ng), 17:1-2-hsophosphatidylethanolamine (100 ng), 17:12-hsophosphatidylglycerol (50 ng), 17:1:2-hsophosphatidylinositol (LPI; 100 ng), 17:1:2-hsophosphatidylserine (50 ng), C17-ceramide (Cer; 50 ng), C17-sphingosine (SG; 50 ng), 12:0-Cer-1-phosphate (50 ng), C17-SG-1-phosphate (SIP; 50 ng), C17-SM (400 ng), C17-SG-phosphocholine (50 ng), and C17-monosulfogalatosyl Cer (50 ng). LC-MS-grade water (1.5 ml) and 3 ml of chloroform were added. The mixture was subjected to Folch extraction. After collection of the lower phase, the upper phase was reextracted with 3 ml of synthetic lower phase (chloroform/methanol/water at a volume ratio of 2:1:1, using the lower phase for reextraction of lipid). The lower phase from both extractions was combined and dried under vacuum at 20°C with a SpeedVac (Thermo) and redissolved in 100 μl of chloroform.

Seven microtiter plates were loaded for LC-MS/MS analysis. A Thermo Orbitrap Elite system (Thermo Fisher) hyphenated with a five-channel online degasser, four-pump, column oven, and autosampler with cooler Shimadzu Prominance HPLC system (Shimadzu) was used for lipid analysis as previously described (9, 10). In detail, lipid classes were separated on a normal-phase Cogent silica-C column (150 x 2.1 mm, 4 μm, 100 Å; MiscoSol Technology) with hexane/dichloromethane/chloroform/methanol/acetonitrile/water/ethylamine solvent gradient based on the polarity of the head group. High resolution (400)/accurate mass resolution, 240 k at m/z 400). Event 2: mass range, 240 k at m/z 400). B. For negative ion analysis, the %data were added. Seven hundred and fifty microliters of extraction mixture was combined and dried under vacuum at 20°C with a SpeedVac (Thermo) and redissolved in 100 μl of chloroform.

Folch extraction. After collection of the lower phase, the upper phase was reextracted with 3 ml of synthetic lower phase (chloroform/methanol/water at a volume ratio of 2:1:1, using the lower phase for reextraction of lipid). The lower phase from both extractions was combined and dried under vacuum at 20°C with a SpeedVac (Thermo) and redissolved in 100 μl of chloroform.

Calculation of Z-score. To calculate the Z-score, the probability of x was considered in an experiment that consisted of a large number of independent trials approximated by a normal probability density function, \( \frac{1}{\sqrt{2\pi} \sigma} e^{-\frac{(x-\mu)^2}{2\sigma^2}} \).

A normal distributed variable, x, was standardized by subtracting the mean and dividing by the standard deviation of the experiment, \( Z = \frac{x-\mu}{\sigma} \). This Z-value or z-score described how many standard deviations (σ) of the experimental result (x) diverged from the mean of population (μ). The larger the z-score, the less likely the experimental result is due to chance. The probability can be computed from the cumulative standard normal distribution function, \( \Phi(z) = \int_{-\infty}^{z} \frac{1}{\sqrt{2\pi} \sigma} e^{-\frac{x^2}{2\sigma^2}} \, dx \). That gives the probability, P, that an experimental result with a z-score less than or equal to that observed is due to chance. Subtracting P from one, \( Q = 1 - P \), gives \( Q \), the probability that the observed z-score is due to chance. This is, by definition, a P-value. Consequently, z-score can be calculated from P-value by taking the inversed function, \( Z = Q^{-1}(1-\Phi) \).

Calculation of Z-scores for the determination of active pathways was performed as follows. Let \( A = \{A_1, A_2, ..., A_k\} \) for the pathway of interest, where \( A_i \) (i = 1, 2, ..., k) are metabolites. The scoring scheme can be described in two steps. First, compute a weight vector, \( \omega = [\omega_1, \omega_2, ..., \omega_k] \), where \( \sum_{i=1}^{k} \omega_i = 1 \). As a result, the weight for the reaction in A will therefore indicate the shift toward more product or reactant in infected cells than in uninfected cells. For each weighted edge of the pathway, we perform a Student’s t-test between the infected and baseline samples, from which a P-value is obtained (the alternative hypothesis is that the mean in infected samples is greater than that in normal samples). Assuming that the t-distribution can be approximated by a normal distribution, therefore, the P-value can be converted to Z-score by taking \( Z =CDF^{-1}(1-P) \), where CDF is the cumulative distribution function. By doing this, each edge, i, is assigned a Z-score, \( Z_i \) (i = 1, 2, ..., k). The score for pathway A is computed as follows:

\[ Z_A = \frac{1}{k-1} \sum_{i=1}^{k-1} Z_i. \]

As a result, \( Mean(Z_A) = \frac{1}{k-1} \sum_{i=1}^{k-1} Mean(Z_i) = 0 \),

\[ Var(Z_A) = \frac{1}{k-1} \sum_{i=1}^{k-1} Var(Z_i) = 1. \]

This means that \( Z_A \) also follows a normal distribution. In order to check to determine whether pathway A is active (significant) in infected over healthy cells, we chose the significance level (P-value) to be 0.05; as a result, the corresponding Z-score is computed to be 1.645. Thus, if \( Z_A > 1.645 \), then A is classified as active.
Network analysis was performed as follows: A complex metabolic network extracted from three databases, including iRefIndex (14), HMBD (15), and Recon2 (16), was constructed. Prizes were assigned to metabolites (nodes) computed from statistical significance levels. We also assigned negative prizes (penalties) to highly connected nodes to avoid less reliable highly degree connected nodes. Costs were assigned to interactions (edges) derived from the interaction probabilities. The optimization algorithm was run with different parameter sets and obtained optimal subnetworks, which maximize the subnetwork robustness, and the optimal subnetworks were merged into a unique optimal subnetwork.

In order to obtain independent pathways, an extra node was added to the network. This node was then connected to all terminals via edges with the same weight, \( \omega \). We used the message passing approach (17) to find an optimal forest solution, \( F \), with nodes and edge weights, by minimizing the sum of the total cost of all edges in the tree and the total penalties of all nodes that are not contained in the tree. The objective function for optimization is

\[
f(F) = \beta \sum_{v \in V_F} b(v) + \sum_{e \in E_F} c(e) + \omega k,
\]

where \( b(v) \) is the prize assigned to each node, \( v \in V_F \), \( c(e) \) is the cost of each edge, \( e \in E_F \), and \( k \) represents the number of trees in the forest \( F \). We computed the prize for each node as \(-\log(P)\) of the significance levels of their alteration in the infected cells by two-tailed Student’s t-test. The cost, \( c(e) \), is one minus the edge weight. Here, the edge weights were computed using the MScore algorithm (18). This algorithm considers the number of publications, type of interaction, and experimental methods to compute confidence scores for molecular interactions. The parameters, \( \beta \) and \( \omega \), were used to control the size of the resultant forest and the number of trees in the forest solution, respectively. In order to avoid including highly connected nodes (hub nodes), which may provide less insight information of altered pathways, we updated the prizes for all nodes in the network as follows: \( b(v) = \beta b(v) - \mu \text{degree}(v) \). Here \( \mu \) is the parameter that controls the degree-based negative prizes. We fixed the value of \( \omega \) at 50 while varying the values of \( \beta \) and \( \omega \) to adjust the effect of terminal nodes and hub nodes in the final forest. Increasing \( \beta \) promoted more terminals to be included in the optimal network, while increasing \( \omega \) weakened the hub nodes in the optimal solution. We considered different values of \( \beta \) and \( \omega \) in the ranges of 1–20 and 0.05–0.4, respectively, based on the input terminal sets.

For a given set of parameters \(( \beta, \mu, \omega )\), we also performed perturbation analysis to determine the robustness of the optimal forest \(( G_i )\) by generating a noisy optimal forest \(( T_i )\) obtained after adding random noise to edge weights. We then calculated a robustness score for forest \(( G_i )\) by determining a fraction of overlapping nodes between \(( G_i )\) and \(( T_i )\) as follows:

\[
f_{n_i} = \begin{cases} 1, & \text{if } n_j \in G_i \cup T_i \\ 0, & \text{otherwise} \end{cases},
\]

where for some optimal network \( G_i \) with \( N_{G_i} \) nodes, \( R_{G_i} \) shows the robustness score of forest \( i \). After this step, all optimal forests were sorted in descending order of their robustness scores. We then used some top (or all) optimal resultant forests and merged them into a final optimal network. This optimal network therefore showed the complex interconnection of metabolic pathways. In the next step, we calculated a robustness score for each node in the final optimal network.

\[
f_{n_i} = \frac{\sum_{j=1}^{M} f_{n_i,j}}{\sum_{j=1}^{M} f_{n_i,j}},
\]

where for a family of \( M \) optimal forests with \( N \) nodes, \( R_{n_i} \) represents the robustness score of node \( n_i \) \((i = 1, 2, ..., N)\) and \( f_{n_i,j} \) represents nodes in forest \( j \). As a result, the robustness score of a particular node is therefore proportional to the number of times that node was selected to include in the optimal forests. After this step, all enzymes will be sorted in descending order of their robustness scores. We then chose some of the top enzymes for consideration. In order to compute robustness scores for edges of the network, we considered both frequency of edge chosen for optimal networks and the probability of interaction given by that edge. The scores for edges were therefore calculated as follows: \( f_{e_{ij}} = f_{e_{ij}} + \beta_{ij} \sum_{j=1}^{M} f_{n_{ij}}, \)

where for a family of \( M \) optimal forests with \( T \) edges, \( R_{e_{ij}} \) represents the robustness score of edge \( e_{ij} \) \((i = 1, 2, ..., T)\), \( p_{e_{ij}} \) is the probability of interaction given by \( e_{ij} \), and \( F_j \) represents nodes in forest \( j \). The edge’s robustness scores therefore show how important that edge is in our network. As a result, this measurement was used to determine whether a particular interaction should be highly considered over others.

**RESULTS**

To quantify changes in the lipidome of a relevant host cell during infection with the RV-A1b strain of RV, we established conditions where cultures of primary HBECs were synchronously infected with RV-A1b. Using a high MOI of 20 and monitoring viral replication by expression of the viral 2C protein by confocal microscopy (Fig. 1), we showed that a time course up to 6 h post infection (hpi) produced a consistent high-percentage (>80%) infection of HBECs without a substantial cytopathic effect.

**Lipidomic analysis**

Lipid extracts were prepared from uninfected cells and HBECs infected with RV-A1b at 2, 3, 4, 5, and 6 hpi. All cultures were performed in triplicate and each experiment was performed three times. Thus, data collected at each time point represents the mean of nine samples from three independent experiments. The lipidomics analysis identified changes not only in the amount of a lipid class as a whole, but also changes in acyl carbon chain length and saturation of individual lipid species. The analysis demonstrated changes in phospholipids, lysophospholipids, FAs, and inositol phospholipids, in particular, a significant decrease in PC species and a substantial decrease in unsaturated acyl chains (acyl chains with 5, 6, 7, or 8 double bonds) at 6 hpi (Fig. 2A). We also observed changes in PA, DG, triglycerides (TGs), FA, fatty acyl CoA, and fatty acyl carnitine. There were changes in FA species at later stages of the infection, particularly those of long fatty acyl chains (>C16) with decreases in the level of unsaturation (Fig. 2B). This implies alterations in PA, DG, TG, and FA metabolism. PI is a functionally important membrane phospholipid, which can be phosphorylated to PI 4-phosphate (PIP4P) by PI4K and subsequently to PI 4,5-bisphosphate (PIP2) by PI4P 5-kinase. Many studies have reported the critical role of PI4K as one of the host lipid-regulatory proteins required for the viral life cycle. We observed that PI species
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Pathway analysis

Simply examining amounts of lipid species has limited benefit in understanding changes in the cell; thus, we adopted a previously described method (19, 20) to search for active pathways in infected cells. This method calculates statistical z-scores for all possible lipid pathways to judge whether a particular pathway is active or not. Observation of changes in the acyl chain length and number of double bonds of FAs suggested changes in FA elongation and desaturation during the viral replication cycle. Thus, we searched for active pathways of FA synthesis for which elongation and desaturation processes will be highlighted. Figure 3A shows FA synthesis pathways in which potential active pathways have been highlighted in red boxes, clearly highlighting the activation of both elongation and desaturation processes. We found no sign of active elongation and desaturation at 2 hpi, whereas both processes were clearly activated at 3 hpi; at later time points, only the elongation process was activated. A complete set of results for active pathways of FA synthesis is summarized in Table 1, and pathway maps for the other time points are in supplemental Fig. S1. The early activated desaturation pathways (comparing 3 hpi to 2 hpi) correlated with a reduction in the degree of saturation of FAs observed (Fig. 2). Additionally, the activated elongation pathways confirmed the steady increase of acyl carbon chain length seen at later stages of infection (see Fig. 2 at 4, 5, and 6 hpi).

Pathway analysis was also utilized to examine changes in lipid classes by searching for all possible active lipid pathways. Active pathways were computed for each time point as shown in Fig. 3. Pathway maps for all time points are in supplemental Fig S2. Table 2 shows all potentially activated and inactivated pathways in infected cells over the time course. The observed alterations in lipid pathways suggest host enzyme activities that have changed during the viral replication cycle at different time points, such as PI4K being activated early (2 hpi). We also observed shutting off of SG-1-phosphate (S1P) synthesis and the synthesis of PC from both PE and LPC, suggesting virus-induced alteration in membrane structure. Additionally, DG, which can recruit PKD to membranes, was elevated by 2 hpi with a close to significant elevation at all other time points. We repeated the lipid composition analysis comparing each time point to the control rather than comparing each time point to the previous time point. This showed a consistent increase in LPC acyltransferase (LPCAT) activity at all time points, whereas the S1P pathways were activated after 3 hpi. Interestingly, PI4K activity was not consistently activated post infection, the increase at 2 hpi was followed by a reduction in activity, though the enzyme was active again at 6 hpi (Table 2). These analyses also pointed to the potential activation of Cer synthase (LASS1) and PC-specific phospholipase C (PC-PLC) activities.

Network optimization

We adopted a further method, independent of pathway analysis, termed the Prize-collecting Steiner tree problem (21, 22). This aims to find an optimal forest of trees that highlights enzymes associated with potential changed pathways. In contrast to the lipid pathway analysis where only lipid classes were analyzed, the Prize-collecting Steiner tree problem takes all lipid species into a complex metabolic network, which includes nodes detected in experiments (terminals) and nodes that were not detected (Steiner nodes). Those nodes (metabolites or proteins) are connected via edges representing interactions between proteins as well as substrate-enzymes and product-enzyme associations. The inputs to the

Fig. 1. Infection of primary HBECs with RV-A1b. Confluent primary HBECs were infected with RV-A1b at an MOI of 20 for 1 h. After unbound virus removal, replication proceeded for up to 6 hpi. Cells were fixed and processed for confocal microscopy by staining with anti-RV 2C protein. Each column shows the 2C staining, the phase contrast, and merged images. Scale bar represents 50 μm.
network are metabolomics scores that differ between the infected and uninfected cells. The algorithm searches an underlying database and outputs an optimal subnetwork, which shows altered viral infection-associated pathways.

The output is listed in supplemental Table S2. As an example, Fig. 4 shows part of an optimal subnetwork for the 2 hpi time point compared with the uninfected baseline time point for which associated enzymes have been highlighted. This analysis showed potential changes in the activities of the acyl transferases, lysophosphatidylglycerol acyltransferase, lysocardiolipin acyltransferase 1 (LCLAT1), and 1-acyl-sn-glycerol-3-phosphate acyltransferase (AGPAT1-6), and in ectonucleotide pyrophosphatase/phosphodiesterase 2 and phospholipase A2 group VII, which generate the lysophospholipid substrates for the acyltransferases. There were also potential changes in SG kinase (SPHK)1, SPHK2, S1P phosphatase 1, Cer kinase, Cer synthase (LASS1), SM phosphodiesterase 1, and SM phosphodiesterase 2 enzymes regulating the concentrations of Cer, SG, and S1P. Notably, the enzymes highlighted from this

Fig. 2. Changes in PC (A) and FA (B) structures during the 6 h viral replication time course. The figure shows changes in both acyl chain length and saturation of PC and FA, concentrations were normalized in each group to cell number. Each time point represents the mean ± SEM from three independent experiments performed in triplicate (n = 9) per time point. *P < 0.05 by Student’s t-test.
B

Biological validation

The bioinformatics analysis identified host lipid-modifying enzymes whose activity changed during a single RV replication cycle. To test to determine whether these were important for viral replication, we selected 18 inhibitors of enzymes and pathways identified as being of interest through our lipidomics and bioinformatics analysis. In a primary screen, these compounds were tested at a final concentration of 10 μM in an RV-A1b replication assay in HBECs by end point titer determination (see supplemental Fig. S3). Eight compounds were identified as potential inhibitors of viral replication; these appeared to focus on the Cer, S1P, PA, and FAS pathways. These were further analyzed by quantification of inhibition of viral replication by end point titer determination over a full concentration range (Fig. 5) and in parallel assessed for cytotoxicity. This identified Ceranib 1, D609, and C75 as producing the most profound inhibition of viral replication at noncytotoxic concentrations. To determine where in the viral life cycle the compounds were acting, they were tested at a single maximal noncytotoxic concentration in

Fig. 2. Continued.
infection assays measuring viral genome replication by qRT-PCR and viral protein synthesis by Western blotting with an anti-2C antibody and further confirmation by end point titer determination (Fig. 6). These data show that each selected compound significantly inhibited viral genome replication and protein synthesis, indicating action early in the replication cycle. To determine whether the compounds were affecting viral entry by clathrin-mediated endocytosis, their effects upon LDL uptake by the cells were analyzed as a control; however, none of the compounds had an effect (Fig. 6D).

**DISCUSSION**

Many viruses remodel host cell intracellular membranes as part of their replicative cycle. The aim of our study was to identify RV-induced remodeling of cellular lipids in airway epithelial cells. RV is a member of the picornavirus family that includes important pathogens in animals and man, including polioviruses, foot and mouth disease virus, Coxsackie virus, and hepatitis A virus. RV infections cause the common cold, which, in healthy patients, is relatively
TABLE 1. Active pathways of FA synthesis

| Time Point (Current hpi/Previous hpi) | Pathway | Z-Score | Processes |
|--------------------------------------|---------|---------|-----------|
| 2/0                                  | 18:0-FA→18:1-FA→18:2-FA→18:3-FA→20:3-FA→20:4-FA→22:4-FA | −2.82 | Decreases in elongation and desaturation |
|                                      | 20:4-FA→22:4-FA | −2.39 | |
|                                      | 16:0-FA→16:1-FA | −2.10 | |
|                                      | 18:1-FA→18:2-FA | −1.85 | |
|                                      | 20:0-FA→20:1-FA | −1.75 | |
|                                      | 18:0-FA→18:1-FA→18:2-FA→18:3-FA→20:3-FA→20:4-FA→22:5-FA | −1.66 | |
| 3/2                                  | 16:1-FA→18:1-FA→18:2-FA→18:3-FA→20:3-FA→20:4-FA→22:4-FA | 2.80 | Changes in elongation and increase in desaturation |
|                                      | 20:4-FA→22:4-FA | 1.86 | |
|                                      | 18:0-FA→20:0-FA | 1.86 | |
|                                      | 18:1-FA→18:2-FA | 1.75 | |
|                                      | 24:0-FA→26:0-FA | −1.97 | |
|                                      | 20:0-FA→22:0-FA | −10.4 | |
|                                      | 20:1-FA→22:1-FA | −1.65 | |
| 4/3                                  | 18:1-FA→20:1-FA→22:1-FA→24:1-FA | 1.93 | Increase in elongation |
|                                      | 16:0-FA→18:0-FA | 1.71 | |
| 5/4                                  | 18:1-FA→20:1-FA | 2.22 | Increase in elongation |
|                                      | 18:0-FA→20:0-FA | 2.21 | |
| 6/5                                  | 22:0-FA→24:0-FA→26:0-FA | 2.70 | Increase in elongation and decrease in desaturation |
|                                      | 22:1-FA→24:1-FA | 2.35 | |
|                                      | 18:1-FA→20:1-FA | 2.22 | |
|                                      | 24:0-FA→26:0-FA | 1.95 | |
|                                      | 16:0-FA→16:1-FA→18:1-FA→18:2-FA→18:3-FA→20:3-FA→20:4-FA→20:5-FA→22:5-FA | −3.07 | |
|                                      | 16:1-FA→18:1-FA→18:2-FA→18:3-FA→20:3-FA→20:4-FA→22:5-FA | −2.60 | |
|                                      | 18:0-FA→18:1-FA→18:2-FA→18:3-FA→20:3-FA→20:4-FA→20:5-FA→22:5-FA | −2.46 | |
|                                      | 16:0-FA→16:1-FA→18:1-FA→18:2-FA→18:3-FA→20:3-FA→20:4-FA→22:4-FA | −2.12 | |
|                                      | 18:2-FA→18:3-FA | −1.97 | |
|                                      | 20:0-FA→20:1-FA→22:1-FA | −1.92 | |

The data represents three independent experiments each performed in triplicate. FA composition at each time point was compared with the previous time point and pathway analysis performed. Pathways with a Z-score >1.645 are considered active.

trivial, but in patients with asthma or COPD, this can provoke serious acute worsening of their disease. We aimed to describe changes in host lipid metabolism during RV replication and use this information to identify targets in the host genome that may offer novel therapeutic targets for both RV and other RNA viruses that remodel intracellular membranes. While the literature contains reports of virus-induced changes in lipids, for example, H1N1 influenza infection of mouse lung tissue (23) and rotavirus-infected MA104 cells (9), no study has utilized pathway analysis and

TABLE 2. Active lipid metabolic pathways

| Time Point (Current hpi/Previous hpi) | Pathway | Z-Score | Predicted Enzymes Activities |
|--------------------------------------|---------|---------|------------------------------|
| 2/0                                  | PA→PI→1LPI | 2.53 | Increase in LPCAT, PI4K, PI-PLA, PA phosphatase, PS synthase, and PTEN activities, and decrease in PI 3-kinase and DG kinase activities. Potential increase in PC-PLC. |
|                                      | PI → PIP | 2.46 | |
|                                      | PA→PI→PIP→PIP2 | 2.21 | |
|                                      | LPC→PC→DG | 2.30 | |
|                                      | PA→PS→PE→PC→DG | 2.91 | |
|                                      | PI→LPI | 1.78 | |
|                                      | DG→PA | −1.73 | |
|                                      | PIP2→PIP3 | −2.51 | |
| 3/2                                  | LPI→PI | 2.46 | Increase in LPIAT, PIP phosphatase, DG kinase, and LPCAT activities, and decrease in PI-PLA, PC-PLA, SG kinase, and PGP synthase activities. |
|                                      | LPC→PC | 2.24 | |
|                                      | DG→PA | 1.96 | |
|                                      | PA→PG | −1.78 | |
|                                      | SG→S1P | −1.84 | |
| 4/3                                  | PI→LPI | 1.77 | Probable increase in PI 3-kinase and Cer synthase activities. |
|                                      | PIP2→PIP3 | 1.54 | |
|                                      | SG→Cer | 1.54 | |
| 5/4                                  | PIP2→PIP3 | −1.55 | Decrease in PS synthase 1 and PI 3-kinase. |
| 6/5                                  | PC→PS | −1.97 | |
|                                      | LPC→PC→PA→PI→PIP→PIP2→PIP3 | 2.58 | Increase in PI 3-kinase, LPCAT, PLD, and SM synthase activities. Potential increase in PC-PLC. |
|                                      | LPC→PC→DG | 2.40 | |
|                                      | LPC→PC→PA→PI→LPI | 2.23 | |
|                                      | LPC→PC→PA→PG | 2.00 | |
|                                      | PC→PA | 1.87 | |
|                                      | LPC→PC→PA→PS→PE | 1.76 | |
|                                      | LPC→PC→PA→DG→TG | 1.67 | |
|                                      | PC + Cer → SM + DG | 1.66 | |

Lipid composition at a time point was compared with the previous time point and pathway analysis performed. A pathway Z-score >1.645 is significantly upregulated, while a negative Z-score less than −1.645 is significantly downregulated. Data represent three independent experiments performed in triplicate.
other bioinformatics approaches to identify potential molecular targets.

Picornaviruses induce massive remodeling of intracellular membranes into clusters of double membrane bound vesicles with a diameter of 200–400 nm resembling autophagosomes (24–27). Despite differences between members of the picornavirus family, there are common features, with polioviruses being the best studied. Polioviruses disrupt traffic through the secretory pathway, and the 2B and 3A nonstructural viral proteins, when expressed individually, disrupt the morphology of the ER and Golgi and influence the transit of protein cargo (8, 28–30). The profound remodeling of host membranes has suggested that lipid-modifying enzymes might be promising anti-viral targets (31). Several examples support this concept. This may, in part, be through altering the function of Arf1 in brefeldin A, a drug that inhibits the GTP exchange factor required for Arf1 activation (GBF1) and alters COP-I recruitment to membranes (32, 33) and impedes the replication of several picornaviruses (34, 35), including RV (data not shown). Phospholipase D (PLD), an enzyme whose activity is also regulated by Arf1 and is implicated in the regulation of membrane trafficking (36), was one of the enzymes identified by our pathway analysis as activated by RV (Table 2), thereby adding additional mechanistic insight into the role of Arf1 in viral replication. Positive-strand RNA viruses, such as enteroviruses and flaviviruses, recruit host PI4K IIIβ, and the viral RNA polymerase (3Dpol) binds PI4P and assembles on PI4P-enriched membranes (2). The picornaviral 3A protein can associate with the Golgi proteins, ACBD3 (GCP60) and PI4KIIIβ (37–40), and PI4KIIIβ has been shown to be the target of certain anti-polio drugs (41–43). A number of additional host proteins interacting with the picornavirus 3A protein have been identified including VAMP-associated protein-A (VAP-A) (37) an ER protein essential for the stimulation of SM synthesis by 25-hydroxycholesterol (44, 45) while depletion of VAPs by RNAi reduces the levels of PI4P, DG, and SM in the Golgi membranes (46). VAP-A binds OSBP, which are a family of lipid transfer proteins that control cholesterol/PI4P exchange at ER-Golgi membrane contact sites and also bind Arf1. OSBP are also targeted by certain anti-picornaviral drugs (4, 6, 47), providing further evidence for the requirement for lipid homeostasis at the ER/Golgi interface for viral replication. Additionally, the anti-viral effector protein, interferon-inducible transmembrane protein 3, interacts with VAP-A and prevents its association with OSBP, thereby disrupting intracellular cholesterol homeostasis and inhibiting viral entry (48). Thus, multiple lines of evidence point to PI4P being a pivotal ER/Golgi phospholipid in viral replication. Our data confirm that viral replication influences PI4K’s activity (Fig. 3, Table 2), but, notably, our analysis highlights distinct phases of activation at both 2 and 6 hpi, with no increase during the intervening period, pointing to a more complex regulation of phosphoinositide signaling than previously.

Changes in other lipids are implicated in viral replication; for example, poliovirus stimulates PC synthesis through activation of CTP:phosphocholine cytidylyltransferase (49) and inhibitors of phospholipid biosynthesis, such as cerulenin, block poliovirus replication (50). Recently, it has been shown that picornavirus infection induces FA import, which is linked to PC synthesis and the formation of replication complexes. This is linked to long chain fatty acyl CoA synthetase activity, a host factor required for poliovirus replication (51). Early events, such as viral entry to cells, induce activation of sphingomyelinase and the creation of Cer-rich patches on the plasma membrane (52), and picornaviruses also cause redistribution of cellular cholesterol to replication complexes, which is critical for completion of the viral life cycle (53).

In our unbiased lipidomics approach, we have performed a time course of a synchronous infection of RV in primary HBECs. The results show widespread perturbation of lipid metabolic pathways with clear effects of increased FA elongation and desaturation with time and increases in long chain FAs (C22, C24), particularly at 5 and 6 hpi (Fig. 2). Pathway analysis also suggests that between 2 and 3 hpi, there are increases in lysocardiolipin acyltransferase (LPIAT), phosphoinositide phosphatase (PIP), DG kinase, and LPCAT activities and decreases in phosphoinositide phospholipase A (PI-PLA), PC phospholipase A (PC-PLA), SPHK1, SPHK 2, and phosphatidylglycerophosphate synthase activities. Between 3 and 4 hpi, there are probable increases in PI3K and LASS1 activities. Between 4 and 5 hpi, there are decreases in PS synthase-1 and PI3K, and between 5 and 6 hpi, there are increases in LPCAT and PLD activities. The clear changes detected at 2 hpi, in particular, strongly suggest that the virus is affecting enzyme activities directly; while at later times, it is probable that there are additional effects, including upon enzyme synthesis, presumably mediated through regulation of SREBP activity. The number and complexity of the observed changes does not clearly lead to a single mechanistic understanding of how
or why viral replication is inducing such profound changes in the host lipidome. However, it does generate a number of testable hypotheses.

We have previously shown that PKD is activated late in the RV replication cycle and that PKD inhibitors reduce RV replication (7). Because PKD is a DG-activated kinase, our time course and pathway analyses showing changes in flux through DG pathways are consistent with our previous observations: our data show an activation of DG kinase activity in which DG, a recruiter of PKD, was elevated by 2 hpi.

To test to determine whether the highlighted lipid signaling and metabolic pathways were important for viral replication and not merely a consequence of replication, we chose chemical inhibitor tools for target validation rather than siRNA, as we find that administration of RNA or DNA into cells activates the innate anti-viral host response, which profoundly interferes with RV replication. The validation studies identified Ceranib 1, D609, and C75 as significant RV replication inhibitors at concentrations not cytotoxic to the host cells. D609, while reported to be

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Fig. 5. Quantification of RV-A1b replication inhibition over full drug concentration-response curves. A, C, E: HBECs were pretreated for 1 h with increasing concentrations of compounds (0.1–20 μM) or DMSO followed by infection with RV-A1b at an MOI of 5 for 1 h. Replication proceeded for 7 h and cell lysates with supernatants were harvested and processed to measure virus titers by TCID_{50}. The graphs show means (± SEM) of three independent repeats, each performed in duplicate. B, D, F: HBECs were incubated with drugs for 9 h and cell viability was determined using the ToxGlo assay. The graphs show the percent of cell viability compared with the control from three independent experiments. Viability of untreated cells is represented by the dotted line.
nonspecific, inhibits PC-PLC and SMS (54), which both produce DG from PC, consistent with a role for DG and, thus, potentially PKD in RV replication. Our pathway analysis shows a potential increase in PC-PLC activity as well as activation of SM synthase activity (at 6 hpi) producing DG and SM. On the other hand, our network optimization shows potential changes in SM phosphodiesterase, which could suggest changes in SM synthase activity as well.

The lipidomics revealed substantial FA modifications, both acyl chain length and saturation; and in keeping with this, C75, a FAS inhibitor, was an effective inhibitor of RV replication (Fig. 5). Nchoutmboube et al. (51) showed that picornavirus induces the uptake of FAs that have 16C or 18C and that this matches the membrane properties of the replication complexes. Furthermore, FAS has been described as an anti-viral target for Coxsackie virus B3 (55). Indeed, amentoflavone, a FAS inhibitor, reduced Coxsackie virus B3 replication (56). Therefore, our observation for the importance of FAS in RV replication is consistent with studies on other viruses, including picornaviruses. Ceranib 1 inhibits ceramidase, which cleaves FA from Cer, producing SG, which is phosphorylated by SG kinase leading to S1P. Our pathway analysis shows potential activation of Cer synthase activity at 4 hpi, whereas the network optimization method suggests possible changes in Cer kinase and Cer synthase (LASS1) across all time points. In addition, when comparing later time points with the baseline time point, there were consistent increases in S1P phosphatase and potentially in Cer synthase activities. Our findings are consistent with observations from other viruses. For example,
inhibitors of SM biosynthesis affect HCV replication (57), and SM localizes to WNV replication complexes and viral replication is moderately affected by SM biosynthesis inhibition (58). Cer has been shown to redistribute to WNV replication complexes and the inhibition of Cer synthesis impairs replication (59). Thus, lipidomics with pathway analysis identified novel therapeutic targets to antagonize RV infection. The inhibitors we have identified blocked replication of the viral genome (Fig. 6A) and, consequently, production of viral proteins (Fig. 6B); thus, they are acting early in the viral replication cycle. Importantly, the active inhibitors were not preventing viral entry, as assessed by monitoring clathrin-mediated endocytosis; thus, the site of action is predicted to be between viral entry and genome replication, so confirming the importance of lipid metabolism in this process. It will be important, nevertheless, to determine the roles these enzymes and associated lipid changes play in viral infection and replication.

In summary, we have performed an unbiased lipidomic study of primary HBECs infected with RV and analyzed changes in lipid composition during a single round of viral replication. We have identified significant alterations in multiple pathways, which change with time in a complex fashion; these have only been revealed by performing a time course in synchronously infected cells. Based on our network and pathway analysis, we identified a number of potentially important enzymes involved in the observed lipid metabolic changes; and using chemical inhibitors, we have shown that PC-PLC, SMS, and FAS are of interest in this respect and worthy of further study as potential therapeutic targets.

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