The Ether Lipid Precursor Hexadecylglycerol Causes Major Changes in the Lipidome of HEp-2 Cells

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Abstract

The ether-lipid precursor sn-1-O-hexadecylglycerol (HG) can be used to compensate for early metabolic defects in ether-lipid biosynthesis. To investigate a possible metabolic link between ether-linked phospholipids and the rest of the cellular lipidome, we incubated HEp-2 cells with HG. Mass spectrometry analysis revealed major changes in the lipidome of HG-treated cells compared to that of untreated cells or cells treated with palmitin, a control substance for HG containing an acyl group instead of the ether group. We present quantitative data for a total of 154 species from 17 lipid classes. These species are those constituting more than 2% of their lipid class for most lipid classes, but more than 1% for the ether lipids and glycosphingolipids. In addition to the expected ability of HG to increase the levels of ether-linked glycerophospholipids with 16 carbon atoms in the sn-1 position, this precursor also decreased the amounts of glycosphingolipids and increased the amounts of ceramide, phosphatidylinositol and lysophosphatidylglycerol. However, incubation with palmitin, the fatty acyl analogue of HG, also increased the amounts of ceramide and phosphatidylinositols. Thus, changes in these lipid classes were not ether lipid-dependent. No major effects were observed for the other lipid classes, and cellular functions such as growth and endocytosis were unaffected. The data presented clearly demonstrate the importance of performing detailed quantitative lipidomic studies to reveal how the metabolism of ether-linked glycerophospholipids is coupled to that of glycosphingolipids and ester-linked glycerophospholipids, especially phosphatidylinositols.

Introduction

Membrane lipid composition is critical for cell signaling, intracellular signaling and cell proliferation. Lipid rafts are enriched in cholesterol and glycosphingolipids, and seem to act as signaling platforms [1]. Moreover, phosphatidylinositolphosphates (PIPs; abbreviations of lipid classes given in Methods under the heading Annotation of lipid species) are involved in recruiting a variety of cytosolic proteins involved in endocytosis and intracellular transport [2]. Furthermore, hydrolysis of lipids by enzymes such as PLA2 is important for cellular function [3]. Knowledge about the role of single lipid species and the complex interplay between these lipids and proteins is crucial for our understanding of normal cell growth, as well as changes occurring in e.g. cancer cells. Not surprisingly, alterations in lipid composition are associated with cancer, and there is evidence that the lipids in food, for instance the content of unsaturated fat, is important for the incidence of certain cancer types [4]. Attempts are being made to treat cancer with agents that modify lipid composition. For instance, inhibitors of fatty acid synthase, which can be overexpressed in cancer, are being investigated for their ability to affect cancer growth [5]. Also, inhibiting lipid anchoring of kinases such as Ras might change cell growth [6].

Cell membranes are most often described as built up of three main lipid classes, i.e. glycerophospholipids (GPs), sphingolipids and cholesterol. By such a classification the GPs include lipids synthesized by different pathways as illustrated in Fig. 1A. The most common GPs are based on the glycerol backbone in which the hydrophobic chains are fatty acyl groups. However, also ether-containing GPs are common. They often constitute 10–20% of the total GPs in cellular membranes; and even more in certain organs, e.g. approx. 1/3 of total GPs in heart and muscle, and 1/5 of total GPs in human brain. Although ether-linked lipids have a backbone and head groups similar to GPs with fatty acyl groups only, the de novo synthesis of ether-linked lipids starts by addition of an acyl group to dihydroxyacetonephosphate. The ether-containing lipids in mammalian cells contain an ether-linked alkyl or alkenyl group in the sn-1 position. The species having an alkenyl group are often referred to as plasmalogens. These ether-linked lipids are so often neglected in textbooks and scientific articles, that they even have been called the “forgotten” lipids. For general reviews of ether-linked GPs, see [7–11].

Both ester-linked and ether-linked GPs consist of a mixture of different species, i.e. molecules with a different composition of fatty acids (FAs), alkyl or alkenyl groups. Remarkably, most ether-linked PE species (which most often is the dominating ether-linked GP) have an alkenyl group, whereas most of the ether-linked PC species have an alkyl group [10–12]. Studies performed with spin-labeled lipids show that most (70–80%) of the ether-linked PE species in the plasma membrane are localized in the inner leaflet,
whereas most (70–80%) ether-linked PC species are found in the outer leaflet. Thus, the ether-linked GPs seem to have a distribution similar to the corresponding ester-linked GPs [13]. Although most ether-linked GPs are either of the PE or PC classes, whereas most (70–80%) ether-linked PC species are found in the outer leaflet. Thus, the ether-linked GPs seem to have a distribution similar to the corresponding ester-linked GPs [13]. Although most ether-linked GPs are either of the PE or PC classes, whereas most (70–80%) ether-linked PC species are found in the outer leaflet. Thus, the ether-linked GPs seem to have a distribution similar to the corresponding ester-linked GPs [13]. Although most ether-linked GPs are either of the PE or PC classes, whereas most (70–80%) ether-linked PC species are found in the outer leaflet. Thus, the ether-linked GPs seem to have a distribution similar to the corresponding ester-linked GPs [13]. Although most ether-linked GPs are either of the PE or PC classes, whereas most (70–80%) ether-linked PC species are found in the outer leaflet. Thus, the ether-linked GPs seem to have a distribution similar to the corresponding ester-linked GPs [13]. Although most ether-linked GPs are either of the PE or PC classes, whereas most (70–80%) ether-linked PC species are found in the outer leaflet. Thus, the ether-linked GPs seem to have a distribution similar to the corresponding ester-linked GPs [13]. Although most ether-linked GPs are either of the PE or PC classes, whereas most (70–80%) ether-linked PC species are found in the outer leaflet. Thus, the ether-linked GPs seem to have a distribution similar to the corresponding ester-linked GPs [13]. Although most ether-linked GPs are either of the PE or PC classes, whereas most (70–80%) ether-linked PC species are found in the outer leaflet. Thus, the ether-linked GPs seem to have a distribution similar to the corresponding ester-linked GPs [13]. Although most ether-linked GPs are either of the PE or PC classes, whereas most (70–80%) ether-linked PC species are found in the outer leaflet. Thus, the ether-linked GPs seem to have a distribution similar to the corresponding ester-linked GPs [13]. Although most ether-linked GPs are either of the PE or PC classes, whereas most (70–80%) ether-linked PC species are found in the outer leaflet. Thus, the ether-linked GPs seem to have a distribution similar to the corresponding ester-linked GPs [13].

Figure 1. Biosynthesis of ether and ester glycerophospholipids and the chemical structures of the precursors used. (A) Schematic overview of the biosynthesis of ether and ester glycerophospholipids. Note that 1-alkylglycerols such as HG (red box) may enter the pathway through phosphorylation to 1-alkylglycerol 3-phosphate by an alkylglycerol kinase. For abbreviations of the compounds and enzymes, see below. (B) The chemical structure of the compounds used in this study, HG and palmitin. DHAP; dihydroxyacetone phosphate, G3P; glycerol 3-phosphate, DHAPAT; dihydroxyacetone phosphate acyltransferase, GPAT; glycerol phosphate acyltransferase, ADHAPS; alkylglycerol kinase, PGK; phosphatidylethanolamine, PLPE; ethanolamine phosphotransferase, EPT; ethanolamine phosphotransferase, CEPT; choline/ethanolamine phosphotransferase.

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Materials and Methods

Materials

1-O-hexadecylglycerol (HG) was from Santa Cruz Biotechnology, and sn-1-palmitin was from Sigma-Aldrich. Other chemicals used were from Sigma-Aldrich unless otherwise stated.

Cell culture

In this study we analyzed HEp-2 cells because we have performed several studies on toxin transport in these cells. HEp-2 cells (obtained from ATCC/LGC) were grown at 5% CO₂ in Dulbecco’s modified Eagle medium (Invitrogen) supplemented with 10% fetal calf serum (PAA Laboratories), 100 U/mL penicillin and 100 U/mL streptomycin (Invitrogen). Cells were seeded in 6-well plates 1 day prior to experiments.

Harvesting of cells for lipidomics

For all experiments cells were treated by adding 20 μM HG (dissolved in ethanol), 20 μM palmitin (dissolved in ethanol) or 0.1% ethanol (control) 24 hours before the cells were harvested for lipid analysis. Cells were washed in warm HEPES buffer.
using bovine serum albumin as standard protein. The protein content was quantified using supplemented with a mixture of Complete protease inhibitors (Roche Diagnostics). The protein content was quantified using Pierce BCA protein assay kit as described by the manufacturer and using bovine serum albumin as standard protein.

Annotations of lipid species
The different lipid species of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylglycerol (PG) and diacylglycerol (DAG) are listed with the two fatty acyl groups separated with a slash (/) when the fatty acid positions are known (e.g. PC 16:0/16:0) and hyphen (−) when minor fractions of positional isomers may appear (e.g. PC 16:0–18:1 may include a minor fraction of PC 18:1/16:0), in accordance with IUPAC recommendations. LysoPI and lysyOPI are abbreviated as LPI and LPC, respectively. The ether-linked GPs are shown as PC O (alkyl), PC P (alkenyl), PE O (alkyl) and PE P (alkenyl). The abbreviation GP is used to indicate all glycosylcerophospholipids. The fatty acyl groups of ether-linked lipids and the N-amidated fatty acyl groups for SM, ceramide (Cer) and glycosphingolipids are shown after the slash. Abbreviations for glycosphingolipids: glucosylceramide (GlcCer), lactosylceramide (LacCer) and globotriaosylceramide (Gb3). Cholesterol esters are abbreviated CE.

Lipid extraction
Lipids were extracted from approximately 1.5 million cells containing 215–230 μg of protein using a modified Folch lipid extraction procedure [28]. Known amounts of the deuterium-labeled or heptadecanoyl-based synthetic internal standards; LPC 17:0, PC 17:0/17:0, PE 17:0/17:0, PS 17:0/17:0, PG 17:0/17:0, PA 17:0/17:0, Cer d18:1/17:0, SM d18:1/12:0 (Avanti Polar Lipids Inc.), DAG 17:0/17:0 (C/D/N Isotopes Inc.), D6-CE 18:0 (C/D/N Isotopes Inc.), D3-GlcCer d18:1/16:0, D3-Cer d18:1/16:0, and Gb3 d18:1/17:0 (Matreya LLC) were added and used for quantification of the endogenous lipid species as previously described [29,30]. The LPC standard was used to estimate the amount of all lysoPILs, whereas PI species were estimated using the PG standard. The PE and PC standards were used to estimate the amount of ether lipids with PE or PC headgroups, respectively. Following lipid extraction, samples were reconstituted in chlororform:methanol (1:2, v/v) and stored at −20°C prior to MS analysis.

MS analyses
The species of GPs, SM, DAG and CE were analyzed by shotgun analysis on a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 5500, AB SCIEX) equipped with a robotic nanoflow ion source (NanoMate HD, Advion Biosciences) [31]. Both positive and negative ion modes using multiplexed precursor ion scanning and neutral loss (NL)-based methods were used [28,32]. The identification of ether-linked species was confirmed by sequential MS/MS analyses in negative ion mode. Collision energy of 40 eV was used. The CEs were analyzed in positive ion mode [33]. Sphingolipids were analyzed by reverse phase ultra-high pressure liquid chromatography (UHPLC) as previously described [34] using an Acquity BEH C18, 2.1×30 mm column with a particle size of 1.7 μm (Waters, Milford, Massachusetts, USA) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 5500, AB SCIEX). A 25 min gradient using 10 mM ammonium acetate in water with 0.1% formic acid (mobile phase A) and 10 mM ammonium acetate in acetonitrile:2-propanol (4:3, v/v) containing 0.1% formic acid (mobile phase B) was used. Multiple reaction monitoring (MRM) was used for quantification of sphingolipids. Identification of the ether-containing species was performed according to Hsu and Turk [35]. The MS lipidomic analyses were performed in a laboratory used to work according to GLP (Good Laboratory Practice), and published validation data show less than 15% variation for most lipid species even when analyzed on three different days [30].

Data processing
The MS data files were processed as previously described [29] using Lipid Profiler™ and MultiQuant™ software for producing a list of lipid names and peak areas. A stringent cutoff was applied for separating background noise from actual lipid peaks. Masses and counts of detected peaks were converted into a list of corresponding lipid names. Lipids were normalized to their respective internal standard [29] and the concentrations of corresponding lipid names of the lipid extraction and mass spectrometry analyses [30]; these samples were mainly used to remove technical outliers and lipid species that were detected below the lower limit of quantification. The data are shown as mean values of results for two independent experiments with bars showing the standard deviation. It should be noted that the ether lipids, PI and lysyOPIs other than LPC are estimated based on using standards of other classes, whereas all other lipids are quantified using standards of their own class.

Results
More than 300 species from 17 lipid classes were quantified following extraction of lipids from HEp-2 control cells as well as from cells treated with HG and palmitin. The analyses were performed on two independently treated samples using an analytical method that has been shown to give less than 15% variation for most lipid species [30]. Similar data were obtained in an initial experiment with control cells and cells treated with HG (two independently treated samples; palmitin not included; data not shown). A full list of all quantified species can be found in the supporting information (Dataset S1). Data for 154 species are presented in Figs. 2–6. Each of these species constitutes more than 1% of the lipids in their class for ether lipids and glycosphingolipids and more than 2% of the other classes. This was done to make the data in Figs. 2–6 better readable. We confirmed the identities of 30 species of ether-linked PCs and 26 species of ether-linked PEs by using subsequent MS/MS analyses (Dataset S1). The sum of the species quantified (pmol lipid/μg protein) are shown in Fig. 2A. The relative changes of the lipid composition of the HG or palmitin-treated cells compared to the untreated cells are shown in Fig. 2B. The changes observed for the different classes and species of each class is detailed below.

The quantification of the major phosphatidylcholine (PC) and phosphatidylethanolamine (PE) molecular species are shown in Fig. 3. The data in Fig. 3A show that there was a large increase in several PC O species with 16:0 in the sn-1 position (PC O 16:0/16:1 and PC O 16:0/18:1 increased 300–400%) following treatment with HG. Interestingly, some of these species were also somewhat increased (approximately 50% for the two species mentioned above) following treatment with palmitin. Surprisingly,
there was no strong effect on PC P species upon HG treatment (Fig. 3B), and PC P 16:0/16:0 actually decreased in contrast to PC O 16:0/16:0. One of the main species with 18:0 in the sn-1 position (PC P 18:0/16:0) was in HG-treated cells reduced to approximately 60% of that present in untreated cells, whereas the level of this species was similar in palmitin-treated cells and untreated cells. For PC species (Fig. 3E) there was a reduction in most species with 16:0 in the sn-1 position following treatment with HG (PC 16:0/16:0 was down to 30% of the control cells, whereas most other of these species were reduced only 15–20%). The other PC species were unchanged. Cells treated with palmitin had no dramatic effect on the molecular PC profile.

The data for ether-linked PE species (Fig. 3C, 3D) reveal a major increase (200–300%) in cells treated with HG for some PE P species with 16:0 in the sn-1 position; also cells treated with palmitin showed increase (up to 80%) in some PE P species, i.e. these species showed a similar trend as mentioned for the PC O and PC P species. However, we did not observe any decrease of PE P species with 18:0 in the sn-1 position as observed for PC P. PE species with 16:0 in the sn-1 position decreased only slightly (Fig. 3F), in contrast to what we observed for PC species with 16:0 in the sn-1 position (Fig. 3E).

An unexpected effect was observed in the levels of ceramides (Cer) and glycosphingolipids following treatment with HG and palmitin (Fig. 4). Treatment with HG resulted in increased levels of the very long chain ceramide species Cer d18:1/24:0 and Cer d18:1/24:1, and the increase was even slightly larger (up to 35–40%) in the cells treated with palmitin (Fig. 4A). In both treatments, the short chain Cer d16:1/16:0 was less affected. This profile picture changed dramatically when looking at glycosylated synthesis products of ceramides, i.e. glucosylceramides (GlcCer), lactosylceramides (LacCer) and globotriaosylceramide (Gb3). The HG treatment resulted in a major reduction in all monitored GlcCer, LacCer and Gb3 species compared to the untreated cells (Fig. 4B–D). In contrast, the cells treated with palmitin showed somewhat higher values than the untreated cells for GlcCer, but were very similar to the untreated cells for LacCer and Gb3. Thus, these data indicate that the only effect of palmitin treatment on the levels of glycosphingolipids seems to be mediated by the increased amounts of Cer, being the substrate for GlcCer synthesis.

HG treatment also had a major effect on the level of several PI species, i.e. an increase of approximately 250% for major species such as PI 16:0–18:1, PI 18:0–18:1 and PI 18:1/18:1 (Fig. 5A). However, treatment with palmitin also resulted in increased levels of certain PI species (40–80% for the species mentioned above). Perhaps the most remarkable effect of the HG treatment was the dramatic effect on the LPI species (Fig. 5B), which increased to more than 50 times the level of that in control cells. This effect was not a general effect on lysophospholipids as there was a small decrease of LPC (Fig. 5C); the major lysophospholipid in untreated cells. Palmitin did not have any significant effect on the level of LPC. The percent decrease of LPI shown in Fig. 2B should be considered in light of the very low amount of LPI present in control cells (Fig. 2A). The data for the major species of PS, PG, PA, DAG, CE and SM did not reveal any major changes for any of these lipid classes or species following treatment with either HG or palmitin (Fig. 6).

**Discussion**

A major finding in the present study is that the alkylglycerol HG, a precursor for ether-linked GPs, gives a much larger effect on the cellular lipidome than expected. In this article, we report quantitative lipidomic analyses for 154 species of 17 lipid classes from HEp-2 control cells and from HEp-2 cells treated with HG or palmitin (a control substance for HG containing an acyl group instead of the ether group). In spite of major effects on some lipid classes following this treatment, we did not observe any changes in the ability of these cells to undergo cell division, their endocytosis of transferrin, or toxicity of the plant toxin ricin (data not shown). As ricin is endocytosed by a variety of endocytic mechanisms and transported retrogradely through the Golgi apparatus to the ER before being translocated to cytosol where it inhibits the protein synthesis [36], these data demonstrate that many cellular functions of HG-treated cells remain unchanged.

Treatment of HEp-2 cells with HG gave as expected increased levels of some ether-linked PC and PE species with 16:0 in the sn-1 position. The treatment also resulted in a decrease of the major ether-linked PC species with 18:0 in the sn-1 position. These changes are most likely due to the changed amount of available substrates (precursors) for lipid synthesis in cells incubated with HG.

Remarkably, treatment of HEp-2 cells with HG reduced the amounts of glycosphingolipids including GlcCer, although these cells have higher levels of Cer, i.e. the substrate for formation of GlcCer. The HG-treated cells also contained less of all species of LacCer and Gb3 than the untreated cells. The mechanism behind these changes is not understood. It should be noted that only minor changes were observed for the SM species (Fig. 6F).

Treatment of the HEp-2 cells with HG surprisingly gave increased amounts of PI and LPI, whereas no significant effects
were observed for PS, PG, PA, DAG, CE and SM. More than 50 times increase was observed for LPI species following treatment with HG. The formation of the LPI species is most likely caused by the action of PLA2 on PI, although it should be mentioned that the applied MS analysis is not able to discriminate between the FAs of lysophospholipids being in the \textit{sn-1} or \textit{sn-2} position.

Lysophospholipids have during recent years been shown to have a number of biological functions [37], and LPI has recently been suggested to be a specific ligand for the orphan G protein-coupled receptor GPR55 [38]. It should be noted that even though we did not measure phosphatidylinositolphosphates (PIPs), the data showing increased levels of PI and LPI suggest that HG treatment might result in important changes also for the PIPs, which are known to play important roles in a variety of cellular functions [2]. However, future studies are needed to understand the mechanisms behind the effects of HG on the metabolism of PI, LPI and possibly also PIPs. It is also important to keep in mind that GPI anchor remodeling depends on ether lipid biosynthesis [19].

The present data show that GPs with choline headgroups (including ether species), PG, PA and DAG species mainly have shorter FAs (C16–18) and very little of longer (C20–22) polyunsaturated FAs of those most commonly found in GPs, whereas PI, PE (including ether species) and PS have considerably more of longer polyunsaturated FAs. Although similar data have

\textbf{Figure 3. Quantitative analysis of glycerophospholipids after HG or palmitin treatment.} The major species of (A) PC O, (B) PC P, (C) PE O, (D) PE P, (E) PC and (F) PE, in HEp-2 cells treated with HG (20 μM), palmitin (20 μM) or ethanol (0.1%, as control) for 24 hours. The species shown here are species comprising more than 1% of the total mass of the ether lipids and more than 2% of PC and PE for at least one of the samples. doi:10.1371/journal.pone.0075904.g003
been reported in several studies [10,39,40], there is so far no mechanistic explanation for these differences. However, a functional consequence for the cell might be that the action of PLA2 on PE or PI (these classes are in the plasma membrane mainly localized in the inner leaflet) results in intracellular release of e.g. arachidonic acid, thus creating signaling substances.

We have earlier reported lipidomic data which suggest a connection between the levels of sphingolipids and ether-linked lipids [41]. Whereas treatment of HEp-2 cells with an inhibitor of GlcCer synthase (PDM) gave decreased amounts of glycosphingolipids and no observed effects on other lipid classes, major changes in the lipidome were seen following treatment of the cells with Fumonisin B1 (FB1), an inhibitor of dihydroceramide synthase. Thus, FB1 gave increased levels of short-chain species (34 or 36 carbon atoms) of PE and ether-linked PE, whereas it did not change the level of long-chain species (40 carbon atoms or more). FB1 also decreased the amounts of many ether-linked PC species (no effect of chain length), but gave no or only minor effects on PC species. It should be mentioned, that it is not known if the effects observed following treatment with FB1 are caused by metabolic alterations after inhibition of dihydroceramide synthase or if FB1 also has an effect on other enzymes. Furthermore, the changes observed in the PE species following treatment with FB1 may be related to degradation of sphingosine to ethanolamine-phosphate and hexadecenal via sphingosine phosphate lyase.

Ford and Gross performed several studies 30 years ago, of synthesis of ether-linked lipids, including perfusion experiments where several radioactively labeled precursors were shown to be rapidly incorporated into plasmalogens in rabbit hearts, and they concluded that head group remodeling of plasmalogens was much faster than de novo synthesis of these lipids [42]. Recently Wood et al. [43] reported that a 3-substituted, 1-alkyl, 2-acyl glycerol ether could be transformed into plasmalogens in vitro in lymphocytes and in vivo in mouse tissues. However, to our knowledge, it has not previously been shown that addition of a plasmalogen precursor results in changes of the metabolism of glycosphingolipids and PI/LPI.

Our data demonstrate the importance of performing quantitative lipidomics when studying the effect of addition of lipid precursors. From the data presented, it is clear that the levels of different ester-linked and ether-linked GP species as well as glycosphingolipids are controlled in a complicated way that we do not yet understand. The large improvement in MS analyses of lipids during the last years will hopefully within the next few years increase our knowledge about how synthesis and degradation of the different lipid classes and species are controlled. We strongly believe that to increase our understanding of cellular lipid metabolism we should not continue to include ester-linked and ether-linked GPs in one class, only depending upon the head-
group of the lipids. The fact that they have two very different pathways for biosynthesis, and the data presented in the present work, stress the importance of thinking about ether-linked and ester-linked GPs as completely different classes, in spite of their common head groups.

Supporting Information

Dataset S1 Quantified lipid species. (XLSX)

Figure 6. Quantitative lipid analysis of PS, PG, PA, DAG, CE and SM. The major species of (A) PS, (B) PG, (C) PA, (D) DAG, (E) CE, and (F) SM in HEP-2 cells treated with HG (20 μM), palmitin (20 μM) or ethanol (0.1%, as control) for 24 hours. The SM species labeled d18:1/23:0 and d18:1/23:1 may alternatively be d18:1/22:0(OH) and d18:1/22:1(OH). The species shown here are species comprising more than 2% of the total mass of the lipid class for at least one of the samples.

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Author Contributions

Conceived and designed the experiments: JB T. Skotland KE KS. Performed the experiments: JB T. Sylvánne HS. Analyzed the data: JB T. Skotland KE KS. Contributed reagents/materials/analysis tools: KE KS. Wrote the paper: JB T. Skotland KE KS.

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Lipidome Changes Caused by Hexadecylglycerol

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