Activation of Liver X receptors (LXRs) increases sphingolipid biosynthesis in hepatic cells

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Abstract

Sphingolipids are essential components of cellular membranes that also serve as signals to mediate important biological processes such as survival, proliferation, and differentiation under both physiological and pathological conditions. In this study, we provide evidence that the nuclear hormone receptor that regulates cholesterol metabolism, Liver X receptor (LXR), also regulates cellular sphingolipid content in mouse hepatic cells. Using a lipidomics approach, we showed that LXR activation leads to a significant increase in the levels of several sphingolipid species, including ceramides and sphingomyelin. These findings on the regulation of sphingolipids by LXRs may be useful for the development of novel drug therapies in diseases associated with sphingolipid alteration.

Introduction

Sphingolipids are bioactive lipid molecules that have been implicated as key modulators of many diseases including cardiovascular, metabolic and autoimmune, and neurological disorders [1]. Of the many classes of sphingolipids, ceramide and sphingosine-1-phosphate have been shown to be an important mediators in the cellular signal transduction involved in apoptosis, proliferation, stress responses, necrosis, inflammation, autophagy, senescence, and differentiation in multiple tissues [2] [3] [4] [5] [6]. Understanding the complex regulation of sphingolipid synthesis would help to identify key ceramide, biosynthetic, and degradation pathways as potential pharmacological targets. LXRs are nuclear hormone receptors that serve as ligand-regulatable transcription factors to control the expression of genes involved in cholesterol metabolism, *de novo* lipogenesis, and anti-inflammatory pathways [7]. Oxy-cholesterols are natural ligands of LXRs; however, the synthetic ligand, GW3965, is a highly specific LXR agonist for both in vitro and in vivo activation of LXRs. Given the importance of LXRs in cellular lipid metabolism, we have investigated their role(s) in sphingolipid biosynthesis in the AML-12 mouse hepatocyte cell line.

Objective

The objective of this study was to determine the effect of LXR agonist GW3965 on cellular sphingolipid content.

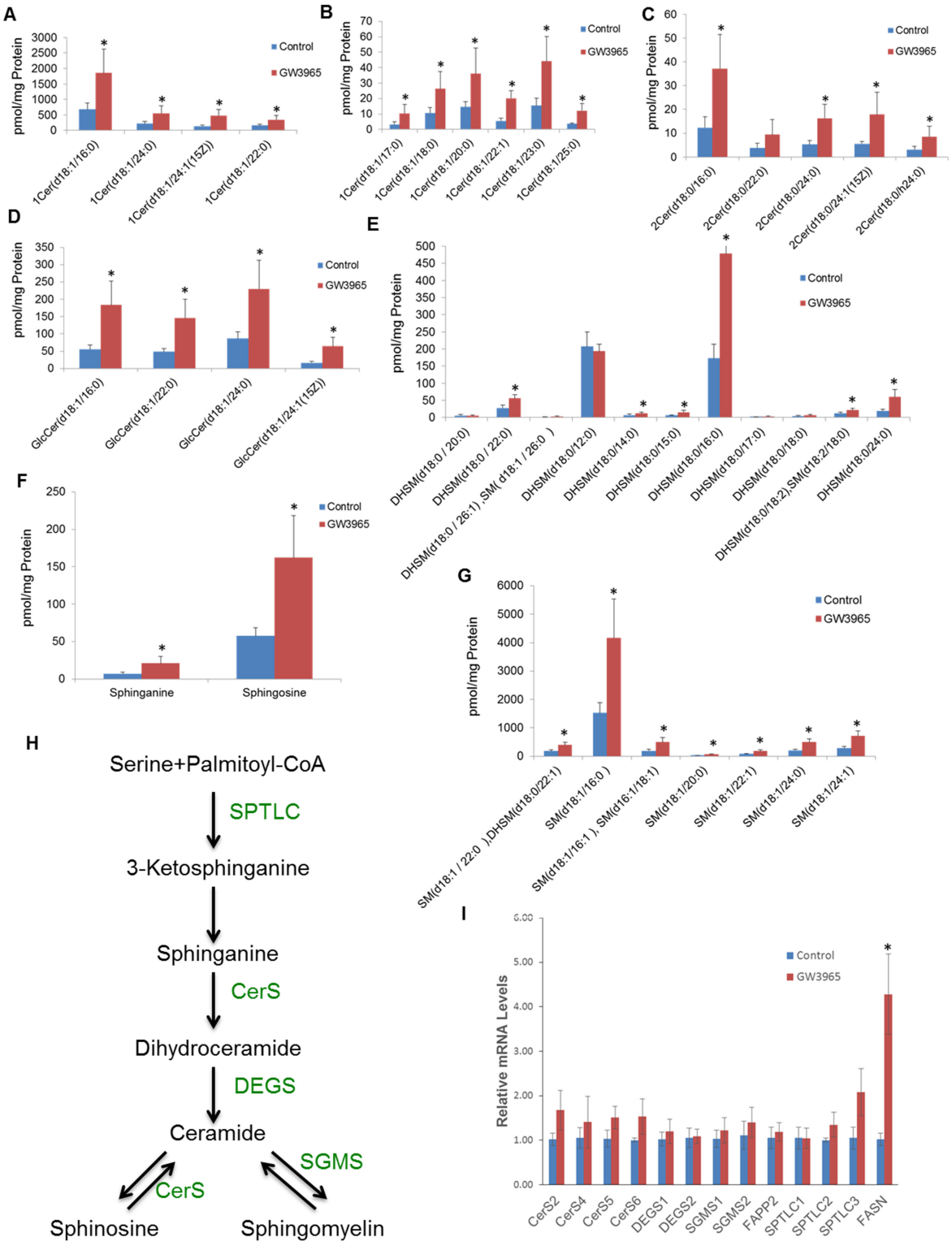

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Figure Legend

Figure 1. LXR-agonist GW3965 induces sphingolipid synthesis.

(A-G) Lipid levels of **(A,B)** Ceramides (1Cer), **(C)** Dihydroceramides (2Cer), **(D)** Glucosylceramides (GlcCer), **(E)** Dihydrosphingomyelin (DHSM), **(F) S**phinganine & Sphingosine, **(G)** Sphingomyelin (SM) in control and GW3965-treated AML-12 cells. GW3965 was added at a dose of 10 μM for 24 h. Bars represent the mean of the respective indi-

vidual ratios ±SD (n = 3, *p <0.05).
(H) Sphingolipid synthesis pathway with important regulatory genes.
(I) qRT-PCR analysis of genes involved in sphingolipid biosynthesis in AML-12 cells treated with GW3965 at a dose of 10 μM for 24 h. Bars represent the mean of the respective individual ratios ± SD (n = 3, *p <0.05).

Cell culture

AML-12 (CRL-2254) cells were maintained at 37℃ in DMEM-F12 1:1 containing 10% fetal bovine serum, 1×ITS, 10 nM dexamethasone, and 1×penicillin/streptomycin in a 5% CO_2 atmosphere. Cells were treated with GW3965 (Sigma-Aldrich) at a dose of 10 μM.

Lipidomic analysis

Sphingolipid analysis was performed as described before [9] [10]. In brief, cells were harvested, washed with ice cold phosphate-buffered saline (PBS), and spiked with an internal standard mixture (C17-sphingosine, C17-sphinganine, C17-sphingosine-1-phosphate, C17-sphinganine-1-phosphate, C12-ceramide, C12-ceramide-1-phosphate, C12-glucosylceramide, C12-lactosylceramide, C12-sphingomyelin, and 14:0 phosphocholine from Avanti Polar Lipids). Thereafter, the cells were resuspended in 900 μL ice cold chloroform-methanol (1:2) and incubated in ice for 15 min with vortexing every 5 min. 300 μL ice cold distilled water (dH_2O) and 300 μL ice cold chloroform were added to the samples, which were then vortexed and centrifuged at 8,000 *g* for 2 min at 4℃. The lower organic phase was transferred into a clean microcentrifuge tube. A second extraction was performed by adding 300 μL ice cold chloroform, and the lower organic phase was pooled with that of the first extraction. The collected samples were dried under a stream of nitrogen and stored at -80℃ until ready for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

RNA isolation and qRT-PCR

Total RNA was isolated and qPCR performed using the QuantiTect SYBR Green PCR Kit (Qiagen, 204141) in accordance with the manufacturer's instructions. Primers were purchased from Sigma-Aldrich (KiCqStart® SYBR® Green Predesigned Primers). Relative mRNA levels were assessed using 2-ddCt analysis.

Calculations and statistics

Results were expressed as mean ± SD. The statistical significance of differences (*p <0.05) was assessed by unpaired Student's-t test when comparing different groups; t(DF) = 4.

Results & Discussion

Our lipidomics analysis showed that several sphingolipid species were significantly upregulated by GW3965. Sphingolipids such as sphinganine, dihydroceramides, ceramides, glucosyl-ceramides, dihydrosphingomyelins, sphingomyelins, and sphingosine were significantly induced by GW3965 (Fig. 1A–G). There are 3 major pathways for sphingolipid biosynthesis [8] as shown in figure 1H: (1) the sphingomyelinase pathway, which uses sphingomyelinase to break down sphingomyelin in the cell membrane and release ceramide; (2) the *de novo* pathway, which synthesizes ceramide from palmitate and serine; and (3) the salvage pathway, which can generate ceramide through breakdown of complex sphingolipids that are ultimately converted into sphingosine, which then undergoes reacylation to form ceramide. Therefore, we next looked if the expressions of the key genes (Fig. 1H) regulating sphingolipid synthesis are affected by GW3965. Surprisingly, unlike its effect on the sphingolipid levels, GW3965 had only a modest effect on the expression of sphingolipid biosynthesis genes. Using fatty acid synthase (Fasn) expression as a positive control for LXR transcriptional activity, the relative expression levels of genes involved in the *de novo* and salvage pathways of ceramide synthesis (e.g., serine palmitoyl transferase [SPTLC1-3], ceramide synthase [CerS2-5], and dihydroceramide desaturase [DEGS1-2]) showed a tendency to increase with LXR activation but did not reach significance (Fig. 1I). Similarly, the level of sphingomyelin synthase (SGMS1-2) also did not change. Additionally, GW3965 did not affect the gene expression of phosphatidylinositol-4-phosphate adaptor-2 (FAPP2), a protein needed for intracellular ceramide transport (Fig. 1I). These results suggest that LXRs-mediated increase in sphingolipid content is possibly independent of the regulation of its biosynthetic genes by LXRs. Therefore, it is possible that LXR agonist may either

increase the flux of substrate (i.e., palmitate) via the activity of endogenous Fasn activity or alter the activities of sphingolipid synthesis enzymes. The effect of GW3965 on Fasn was LXR-dependent as assessed by LXRalpha knockdown (Suppl. data). Further studies are needed to determine the specific mechanism(s).

Conclusions

Our data conclusively show that LXR agonists are potent inducers of cellular sphingolipid synthesis. This is an important finding because it implies that LXR agonism or antagonism may be able to correct sphingolipid imbalances in specific disease contexts.

Limitations

Further studies involving other cell types and in vivo experiments, involving disease models will be needed for further validation.

Additional Information

Methods

Cell culture

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Supplementary Material

Please see https://sciencematters.io/articles/201611000022.

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Ethics Statement

Not applicable.

Citations

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