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Endoplasmic reticulum-localized hepatic lipase decreases triacylglycerol storage and VLDL secretion

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ABSTRACT

Hepatic triacylglycerol levels are governed through synthesis, degradation and export of this lipid. Here we demonstrate that enforced expression of hepatic lipase in the endoplasmic reticulum in McArdle RH7777 hepatocytes resulted in a significant decrease in the incorporation of fatty acids into cellular triacylglycerol and cholesteryl ester accompanied by attenuation of secretion of apolipoprotein B-containing lipoproteins. Hepatic lipase-mediated depletion of intracellular lipid storage increased the expression of peroxisome proliferator-activated receptor α and its target genes and augmented oxidation of fatty acids. These data show that 1) hepatic lipase is active in the endoplasmic reticulum and 2) intracellular hepatic lipase modulates cellular lipid metabolism and lipoprotein secretion.

1. Introduction

Hepatic steatosis, the leading cause of abnormal liver functions, is strongly associated with obesity, insulin resistance/type 2 diabetes and cardiovascular disease [1–5]. In addition, steatosis precedes liver fibrosis, cirrhosis and hepatocellular carcinoma [1,6]. Hepatic steatosis ensues when the rate of fatty acid (FA) esterification into triacylglycerol (TG) exceeds the rate of FA output through oxidation and very-low density lipoprotein (VLDL) secretion. It is now well documented that lipolysis of hepatic TG stores by the cytosolic adipose triglyceride lipase (ATGL) provides FA for oxidation [7–10] and that attenuation of ATGL activity leads to hepatic TG accumulation [8,9,11]. On the other hand, an endoplasmic reticulum (ER)-associated carboxylesterase3/triacylglycerol hydrolase mobilizes stored TG for VLDL secretion [12–16].

Hepatic lipase (HL) is a lipase secreted from the liver. HL exhibits phospholipase A_1 and TG hydrolase activities and is involved in the metabolism of plasma lipids present in high-density lipoproteins (HDL) and in VLDL [17–20]. Aside from its lipolytic function, HL also facilitates the selective uptake of cholesteryl esters (CE) from HDL [21–24] as well as the removal of apoB-containing lipoprotein remnants via receptor mediated endocytosis [20,25–27]. These mechanisms ultimately clear excess lipid from the circulation. Yet, whether HL expedites or delays the onset of atherosclerosis remains controversial [28]. Lipids that HL accesses have been so far limited to the extracellular compartment (plasma and cell surface). However, because HL is a secreted protein it is also present in the ER and along the secretory route for a limited period of time prior to secretion. We hypothesized that HL attains its lipolytic activity intracellularly and it may therefore be capable of modulating intracellular TG pools. Several secreted and plasma membrane proteins have been already shown to play an intracellular role in TG export via VLDL, in addition to their roles in lipoprotein metabolism within the plasma compartment or at the cell surface. These include apoE [29–31], phospholipid transfer protein [32,33] and low-density lipoprotein (LDL) receptor [34]. HL is likely to gain its lipolytic activity along the secretory

Abbreviations: apoB, apolipoprotein B; ATGL, adipose triglyceride lipase; CE, cholesteryl ester; DG, diacylglycerol; ER, endoplasmic reticulum; E600, diethyl-*p*-nitrophenylphosphate; FA, fatty acid; HL, hepatic lipase; HL-R, ER-retained hepatic lipase; McA, McArdle-RH7777; PBS, phosphate-buffered saline; MTP, microsomal triglyceride transfer protein; OA, oleic acid; PC, phosphatidylcholine; PDI, protein disulfide isomerase; PL, phospholipids; Ppia, cyclophilin; R12, McArdle cells expressing HL-R; TG, triacylglycerol; VLDL, very-low density lipoprotein

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pathway [25,35]. Recent studies strongly suggest that over-expression of secretion-competent HL resulted in diminished lipid levels within the endoplasmic reticulum and in the reduction of TG in the media [36], however the precise mechanism by which the secreted HL affected cellular lipid metabolism (extracellular vs intracellular role) remains unclear. To establish whether HL promotes the mobilization of lipids intracellularly, we generated McArdle-RH7777 (McA) cells stably expressing a cDNA encoding mouse HL chimera that enforces HL retention in the ER and investigated whether the expression of this intracellularly retained HL affects lipid metabolism.

2. Theory

Secreted HL has been shown to diminish intracellular lipid levels and VLDL secretion. We hypothesize that HL becomes active in the secretory pathway and this intracellular activity diminishes substrate availability for the assembly of VLDL.

3. Materials and methods

3.1. Generation of McA cells stably expressing mouse HL cDNA

Mouse cDNAs encoding FLAG-tagged secretion-competent mouse HL (HL-S) or intracellularly retained HL (HL-R) were generated. The forward primer contained a sequence of the coding region of the cDNA and an XhoI restriction site (underlined) [5′-C CTA CTC GAG GGT AAG ACG AGA GAC ATG GGA AAT CCC CT-3′]. The reverse primer, corresponding to the complementary strand, encoded an XbaI restriction site (underlined) [5′-A CGT TCT AGA GAA TAG ACT TCT TTA TTT TTT TGC ATG GG-3′]. These primers were used to amplify the HL cDNA (~1.6 kb) from a mouse liver lgt11 cDNA library [37]. Amplification was performed at 93 °C 1 min, 60 °C 1 min, 72 °C 2 min for 30 cycles. The PCR product was digested and ligated into XhoI and XbaI sites of pBluescript II SK-plasmid (Stratagene) and the entire cDNA was sequenced. The plasmid was used as a template to generate chimeric cDNA encoding the mouse HL protein with the FLAG epitope at the extreme C-terminus (HL-secreted; HL-S) or with the FLAG epitope followed by the mammalian ER retrieval signal—HVEL (HL-retained; HL-R) at the extreme C-terminus [14,38]. The reverse primer for HL-S [5′-CT AGT CTA GAC TAG TCA CTT ATC GTC GTC ATC CTT GTA ATC TTT TTT TGC ATG GGT CTC TTG ACT CAT CTG C-3′] and for HL-R [5′-T CAT TCT AGA TCA **CAG TTC AAC ATG** CTT ATC GTC GTC ATC CTT GTA ATC TTT TTT TGC ATG GGT CTC TTG ACT CAT CTG C-3′] corresponding to the complementary strand contained the FLAG sequence (underlined), and the HL-R primer also encoded the ER retrieval signal (bold). The aforementioned forward primer was common for both chimeric HL cDNAs. The resultant PCR product was cloned into the pCR4-TOPO plasmid (Invitrogen) and sequenced. The chimeric cDNA was excised from this plasmid using XhoI and SpeI and was ligated into a XhoI and XbaI digested pCI-neo (Promega) mammalian expression vector. Wild-type McA were stably transfected [12] with pCI-neo vector (no insert, control), with pCI-neo vector encoding FLAG-tagged secretion-competent mouse HL (HL-S), or with pCI-neo vector encoding FLAG-tagged intracellularly retained HL (HL-R). Individual HL expressing clones were isolated and analyzed for HL levels by immunoblotting with anti-FLAG antibodies. Intracellular localization of mouse HL chimeras was determined by density gradient subcellular fractionation [14,39,40] and by colocalization with ER marker protein disulfide isomerase (PDI) using confocal laser scanning immunofluorescence microscopy [14,40,41]. Lipolytic activities in cell lysates, media and microsomes prepared from stable cell lines and primary mouse hepatocytes were analyzed using the TG analogue 1,2-dilauryl-*rac*-glycero-3-glutaric acid resorufin ester [42].

3.2. Generation of adenovirus encoding HL-R and infection of McA cells

Adenovirus encoding both the green fluorescent protein (GFP) and HL-R was generated (SignaGen Laboratories, Rockville, MD, USA). Adenovirus encoding only GFP was used as control in infection experiments. McA cells were infected with 100×10^6 pfu of either adeno-HLer or adeno-GFP for 24 h. Cells were then washed with DMEM and used for experiments.

3.3. Lipid synthesis and turnover

Cells were washed and incubated in serum-free DMEM for 4 h either in the absence or presence of 0.4 mM oleic acid (OA) complexed to 0.5% BSA [12]. For analyses of lipid turnover, media after the initial 4 h incubations were aspirated, cells were washed three times with serum-free DMEM containing 0.5% FA free BSA, and were incubated for an additional 16 h in serum-free DMEM. Cells and media were then collected for analyses. Lipids from cell lysates and cell-free media were extracted and quantified by gas chromatography as previously described [43].

For studies investigating lipid synthesis and turnover, cells were treated exactly as described above for lipid synthesis, turnover and secretion studies except that the initial 4 h incubations also contained either 2.5 μCi/mL [9,10(n)-^{3}H]OA or 1 μCi/mL [^{14}C]glycerol. Lipids were spotted on thin-layer chromatography (TLC) plates and resolved with a two-solvent system as described previously [13]. TG, CE, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) bands were isolated and radioactivity was determined by scintillation counting. In some experiments radioactive lipids were detected by fluorography by spraying the TLC plates with Amersham Amplify™ Fluorographic Reagent (GE Healthcare) and exposure to BioMax MR films (Kodak).

To address the participation of ER-retained HL in the turnover of preformed lipid stores, cells were first incubated with radiolabeled OA as described above but in the presence of a pan-lipase inhibitor 100 μM diethyl-*p*-nitrophenylphosphate (E600) in order to inhibit all intracellular TG turnover. The inhibitor was then removed, cells were then washed and further incubated for up to 16 h in DMEM. Lipids from cell lysates and cell-free media were extracted at various times as indicated and processed as described above.

Acid-soluble metabolites (ASM), a measurement of fatty acid oxidation was determined in media. First, 20% BSA was added to media followed by 70% perchloric acid and centrifugation at 21,000×*g* for 5 min. Aliquot of the supernatant was counted for radioactivity [39].

3.4. ApoB secretion

ApoB from culture media was immunoprecipitated with goat anti-apoB antibodies (Chemicon) followed by capture of imunocomplexes on protein A-Sepharose beads (Sigma). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with anti-apoB antibodies. ApoB concentrations were quantified by densitometry analysis using QuantityOne software (BioRad).

3.5. Gene expression analysis

Total RNA was isolated from cells using Trizol® reagent (Life Technologies, Inc.) and reverse transcribed using Superscript II (Invitrogen). Primer sets, and a corresponding probe for each gene of interest were designed using the Universal Probe Library (Roche) based on the NCBI reference nucleotide sequences for *Rattus norvegicus*. Each primer pair and probe combination were previously tested by qPCR. Forty-eight gene assays were loaded into separate wells on a 48-by-48 gene expression chip (Fluidigm). qPCR was run on the Biomark™ system (Fluidigm) for 40 cycles. Relative RNA expression for each gene in a sample was standardized to cyclophilin, and calculated using the comparative threshold