Biochimica et Biophysica Acta xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

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

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ARTICLE INFO

Article history:
Received 20 October 2012
Received in revised form 9 January 2013
Accepted 23 January 2013
Available online xxxx

Keywords:
Hepatic lipase
Triacylglycerol
VLDL
apoB
Fatty acid oxidation
Endoplasmic reticulum

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

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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1388-1981/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.bbalip.2013.01.017

secretory 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 $21{,}000\times g$ 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

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threshold ($\Delta\Delta C_t$) method. Values were normalized to an endogenous housekeeping gene cyclophilin (Ppia).

3.6. Statistical analysis

The results are expressed as means ± SEM. Statistical analysis was performed by a student *T*-test or one-way ANOVA with a Newman–Keuls post test using GraphPad Prism software. *P* values <0.05 were considered statistically significant.

4. Results

4.1. Intracellular HL is active

To determine the potential role of HL on intracellular lipid metabolism, we produced two cDNA constructs. The first construct, HL-secreted (HL-S), encodes the full-length murine HL cDNA with a sequence encoding the FLAG epitope (DYKDDDDK) immediately before the stop codon. The second construct, HL-retained (HL-R), encodes the full-length murine HL cDNA with an additional sequence following the FLAG epitope encoding the mammalian ER retrieval signal (HVEL), which is present in ER-localized mouse carboxylesterase 3/triacylglycerol hydrolase and carboxylesterase 1/Es-x [39,44], immediately before the stop codon (Fig. 1A and Supplemental Fig. S1A). These constructs were inserted into pCI-neo plasmid used for transfection of McA cells. Several McA clones stably expressing variable levels of HL-S and HL-R were isolated and tested for HL expression, activity and cellular lipid metabolism. Experiments were conducted with a clone R12 stably McA expressing HL-R (Fig. 1B) and a clone S7 stably expressing HL-S (Supplemental Fig. S1A). Some experiments were also performed with additional HL-S clone S1 and HL-R clone R5 (Supplemental Fig. S2).

HL-R is retained within McA cells (Fig. 1B) and is barely detectable in media following a 16 h incubation as measured by immunoblotting and lipase activity (Supplemental Fig. S1A, B). Furthermore, HL-R is distributed in a reticular pattern within the cell similar to the ER resident protein PDI (Fig. 1C). Importantly, intracellularly retained HL is catalytically active as demonstrated by a 3-fold increase of lipolytic activity in microsomes isolated from R12 cells compared to microsomes prepared from control pNEO1 (control) cells (Fig. 1D). Relatively low levels of HL-S could be detected in cell lysate from S7 cells, while high levels of HL-S were found in the media (Supplemental Fig. S1A). Media from S7 cells exhibited increased lipolytic activity compared to media from either R12 or pNEO1 cells (Supplemental Fig. S1B) and this activity was only about 20% higher than that present in media from cultures of primary mouse (C57BL/6) hepatocytes after the same incubation period (Supplemental Fig. S1C). Therefore, S7 cells appear to secrete a comparable amount of HL as primary mouse hepatocytes. Furthermore, S7 cell lysates showed a 50% increase in lipolytic activity compared to pNEO1 microsomes (Supplemental Fig. S1B), suggesting that intracellularly localized HL destined for secretion is catalytically active along the exocytotic pathway.

4.2. Intracellular HL activity prevents lipid accumulation

Because the intracellular HL-R is active, we postulated that the lipase might hydrolyze its native substrates (TG and glycerophospholipids) within the ER and therefore affect lipid storage and VLDL secretion. We performed experiments to study lipid turnover and apoB secretion in McA cells stably transfected with pNEO1 (control) and HL-R and HL-S vectors, as well as, with McA cells infected with adenovirus encoding HL-R. Cells were incubated with 0.4 mM OA for 4 h to stimulate lipid synthesis [12]. Cellular TG mass increased from 6.5 μg/mg cell protein in untreated cells (no OA supplementation) to 22 μg/mg of cell protein

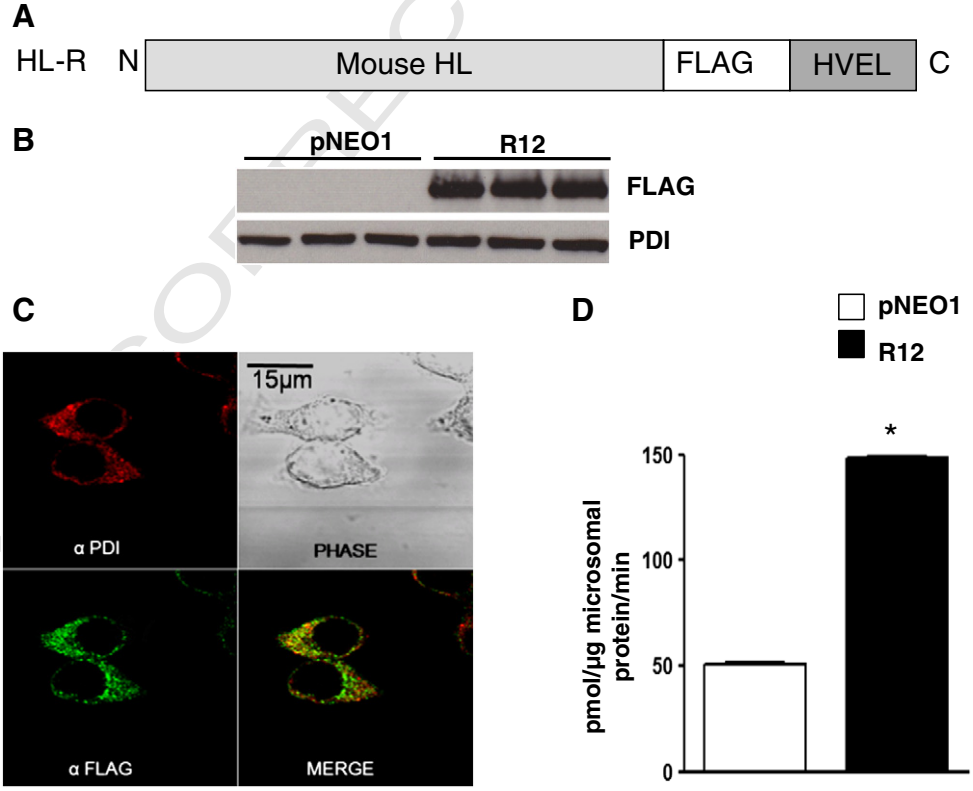

Fig. 1. Generation of McA stably transfected cell lines. (A) Schematic depiction of FLAG-tagged ER-retained HL (HL-R) chimera. (B) Immunoblot of expressed HL-R in McA cell lysates. pNEO1 are cells stably transfected with empty vector, R12 are cells stably transfected with HL-R. PDI (protein disulfide isomerase) is an ER-localized protein containing a similar ER-retention signal (KDEL) and was used as the loading control. (C) Localization of FLAG-tagged HL-R in transfected McA cells by confocal immunofluorescence microscopy. (D) Lipase activity in microsomal fractions isolated from control (pNEO1) and HL-R expressing (R12) McA cells. *$P<0.001$ versus control pNEO1.

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in oleate supplemented pNEO1 cells and from 2 μg/mg cell protein to 13 μg/mg cell protein in HL-R cells (Fig. 2A). OA supplementation did not affect PC or PE mass or the levels of unesterified cholesterol (FC) (results not shown). Although OA significantly augmented intracellular TG concentrations in both cell lines, TG mass remained significantly lower in the HL-R expressing R12 cells compared to the control pNEO1 cells (Fig. 2A). Comparably decreased accumulation of TG upon OA incubation was also observed in another HL-R clone R5 (Supplemental Fig. S2B, C). R12 cells were also significantly depleted of cellular CE relative to pNEO1 cells (Fig. 2A). This suggests that intracellular HL hydrolyzed neutral lipids formed from exogenously supplied OA. Further incubation of cells in the absence of OA (16 h) resulted in depletion of 56% of TG and 35% of CE in the control pNEO1 cells and 53% of TG and 32% of CE in R12 cells (Fig. 2A). Because the rate of depletion of preformed TG and CE in R12 cells was comparable to the control pNEO1 cells, this result suggests that the ER-localized HL did not participate in the hydrolysis of preformed stored neutral lipids. Decreased accumulation of TG in HL-R expressing cells was accompanied by diminished levels of the lipid droplet associated protein perilipin 2 (Fig. 2B).

To address the mechanism of neutral lipid lowering by intracellular HL expression, radiolabeled OA pulse/chase experiments were undertaken. Incorporation of exogenously supplied OA into glycerolipids and cholesteryl ester was significantly decreased in HL-R expressing cells, while the level of intracellular free radiolabeled fatty acid was not different between control and HL-R expressing cells (Fig. 3A, B). Interestingly, while the radioactivity in most glycerolipids in HL-R expressing cells was decreased, the cells appeared to accumulate radiolabeled monoacylglycerol (MG) during the pulse period suggesting active lipolysis of TG and DG, however the radioactivity in MG was too low for performing statistical analysis with confidence (Fig. 3A). The rate of disappearance of radioactivity from preformed OA-labeled TG pools (chase period) was similar between control and HL-R expressing cells, 70% in control cells and 75% in HL-R expressing cells, indicating that other lipases are responsible for the turnover of stored cytosolic TG pools. Although lower incorporation of OA into glycerophospholipids (PC and PE) was observed in HL-R expressing cells, the turnover of the radioactivity in these lipids was similar between control and HL-R expressing cells (Fig. 3B). However, differences were observed in the turnover of CE. Control cells turned over about 50% of radiolabel in CE, while HL-R expressing cells showed accelerated CE turnover resulting in depletion of 90% of radiolabel during the same incubation period (Fig. 3B).

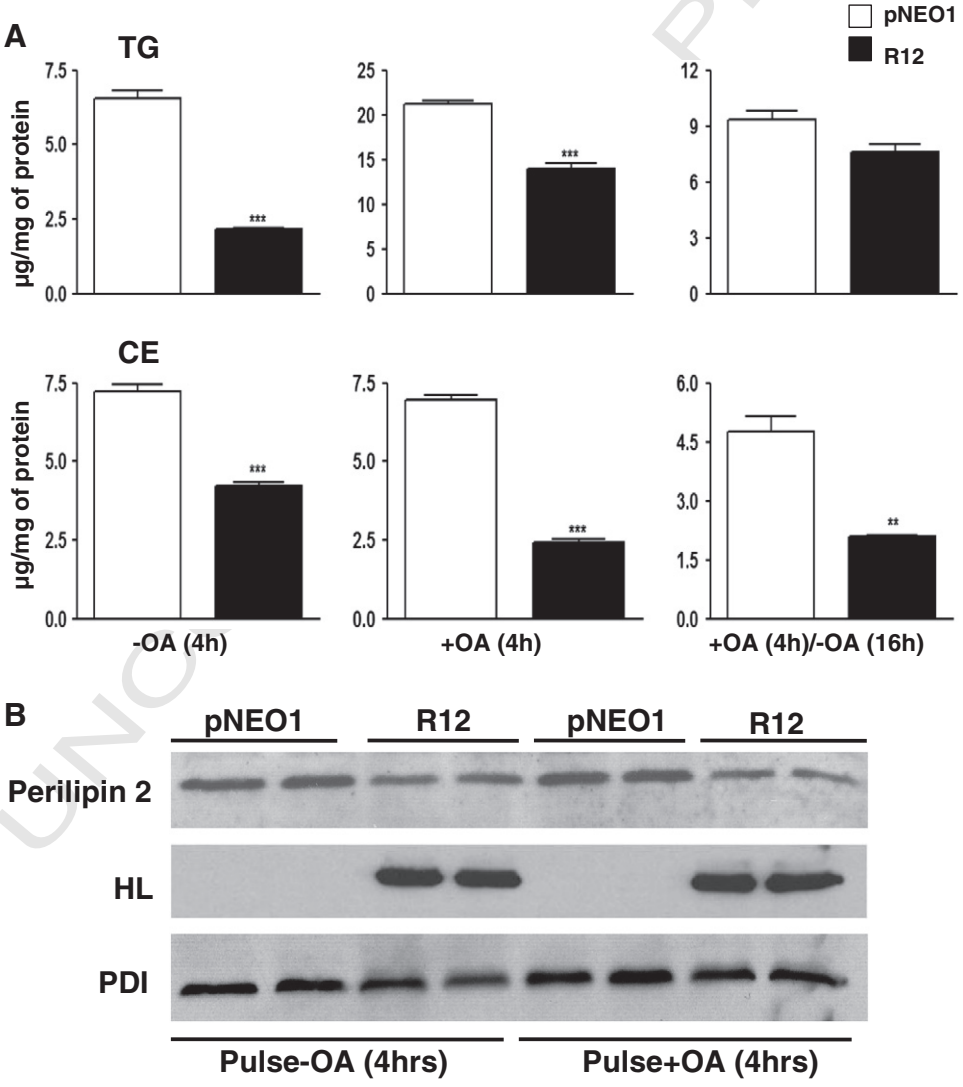

Fig. 2. HL-R depletes neutral lipid stores. (A) Analysis of cellular TG and CE mass in control and HL-R expressing McA cells grown to ~70% confluency (untreated, −OA), after incubation with OA (+OA) and after additional incubation in the absence of OA following initial OA supplementation (+OA/−OA). Data are shown as the mean ± SEM for n = 3–4 experiments performed in triplicate. *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus control pNEO1. (B) Immunoblot analysis of a lipid droplet associated protein perilipin 2 in control and HL-R expressing cells.

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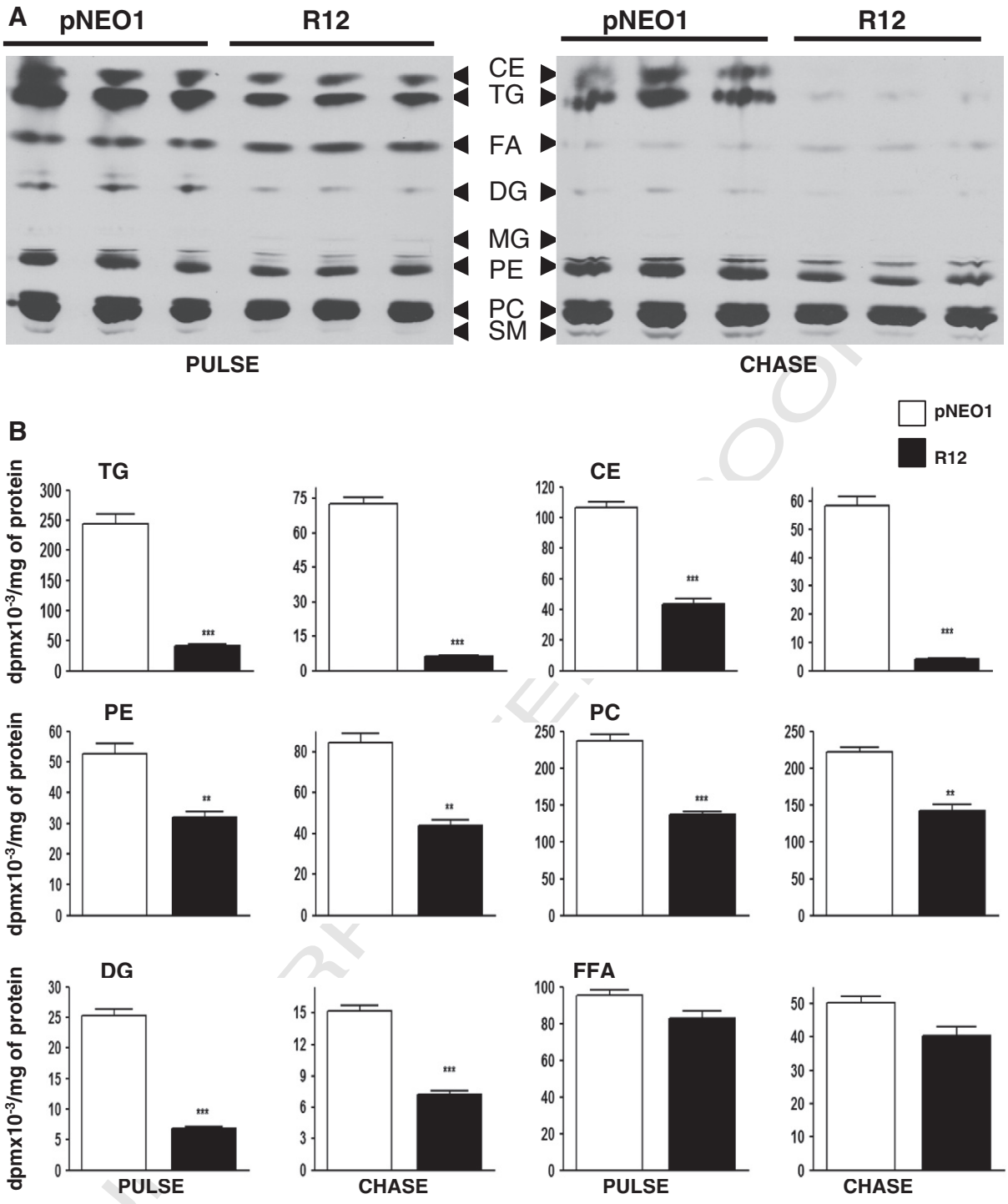

Fig. 3. HL-R decreases lipid accretion. (A) Incorporation of [^{3}H]OA into lipids determined by TLC and fluorography. SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; MG, monoacylglycerol; DG, diacylglycerol; FA, free fatty acid (i.e. OA); TG, triacylglycerol; CE, cholesteryl ester. (B) Radioactivity in lipids in cells after radiolabeled pulse with [^{3}H]OA and chase (absence of OA). Values are the mean ± SEM for n = 3–4 experiments performed in triplicate. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus control pNEO1.

In addition to cell lines stably expressing HL-R we have also performed studies in McA cells infected with HL-R encoding adenovirus. Cells infected for 24 h expressed active HL-R (Ad-HLer) intracellularly (Fig. 4A, B), though the levels of protein expression were lower compared to the R12 clone stably expressing HL-R. No immunodetectable HL-R was observed in the media of infected cells (data not shown). Similarly to cells stably expressing HL-R (R12, R5), ad-HLer significantly decreased the amounts of radiolabeled cellular glycerolipid and CE upon incubation of cells with 0.4 mM [^{3}H]OA for 4 h (Fig. 5).

Compared to the ER-retained HL (HL-R) as expected the secretion competent HL (HL-S) had a more moderate effect on the incorporation of radiolabeled OA into cellular lipids due to its transitory localization in the intracellular lipid synthetic compartment. Nevertheless, incorporation of OA into TG was decreased by 30% (Supplemental Table S1A), while incorporation of OA into PC was not statistically different. Decrease in incorporation of OA into TG in HL-S expressing cells was also observed in the presence of 50 μM poloxamer 407 (Supplemental Table S1B). This is the detergent concentration that inhibited 100% of HL activity in the media without affecting

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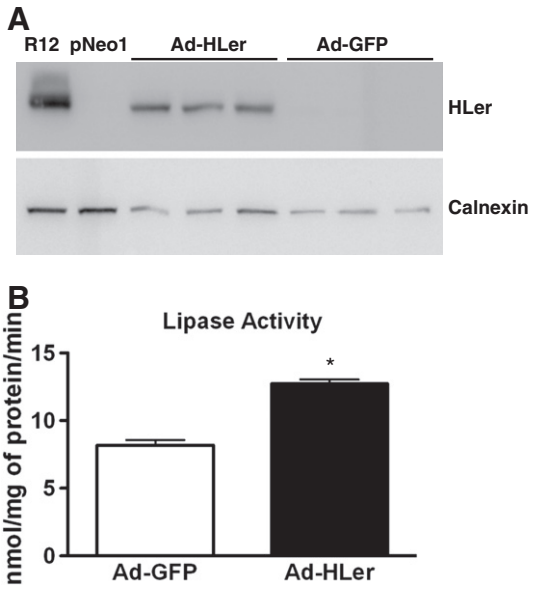

Fig. 4. Infection of McA cells with adenovirus encoding ER-retained HL (HLer). (A) Immunoblot of expressed HLer in McA cell lysates. Calnexin (an ER-localized protein) was used as the loading control. (B) Lipase activity is cell lysates against resorufin ester. *$P<0.05$ versus control pNEO1.

intracellular HL activity or cell integrity (Supplementary Fig. S3). Therefore, the lipid lowering effect of HL-S was due to the intracellular activity of the lipase.

4.3. Mobilization of preformed lipids

Lipid mass turnover and pulse/chase experiments (Figs. 2 and 3) suggested that intracellular HL does not participate in the mobilization (hydrolysis) of preformed lipids. However, due to significantly diminished generation of lipid stores in HL-R cells the rate of turnover of preformed lipids could have been influenced by decreased substrate availability. Therefore, in order to be able to make a more precise comparison of turnover of preformed lipids in the two cell lines, the cells should have equal amounts of available substrate with similar specific radioactivity. We therefore first incubated cells with OA in the presence of lipase inhibitor diethyl-*p*-nitrophenylphosphate (E600) that blocks all lipolysis [13] and thus initial neutral lipid (TG and CE) accretion in both control and HL-R expressing cells was equal upon incubation with 0.4 mM [^{3}H]OA (Fig. 6A). Similarly, OA incorporation into PE was not different between control and HL-R expressing cells in the presence of the lipase inhibitor, while the incorporation of OA into PC was significantly increased (46%). These results demonstrate that the decreased incorporation of exogenously supplied OA into lipids observed in the absence of lipase inhibitor (Figs. 2 and 3) was due to the lipolytic activity of HL-R. Incorporation of glycerol into both TG and PC was augmented in HL-R expressing cells (Fig. 6B). The rate of turnover of preformed TG during subsequent 16 h incubation after removal of E600 was similar between control and HL-R expressing cells indicating that HL-R did not access preformed lipid storage pools (Fig. 6A, B). Interestingly, CE levels increased during the 16 h "Chase" incubation when the only possible substrate would need to come from preformed labeled lipids.

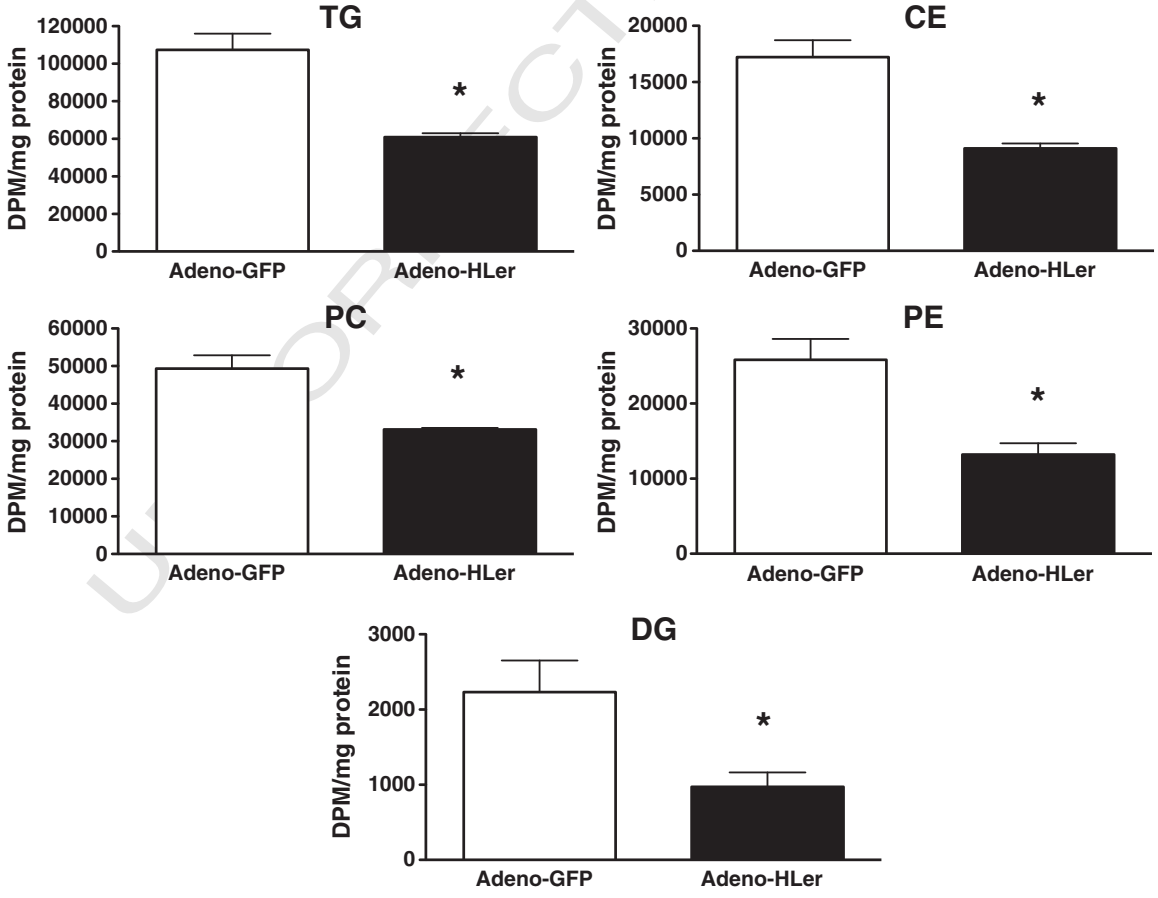

Fig. 5. Adenovirus-mediated HLer expression decreases lipid accretion. Radioactivity in cellular lipids after 4 h radiolabeled pulse with 0.4 mM [^{3}H]OA. Values are the mean ± SEM for n = 3 experiments. *$P<0.05$ versus control pNEO1.

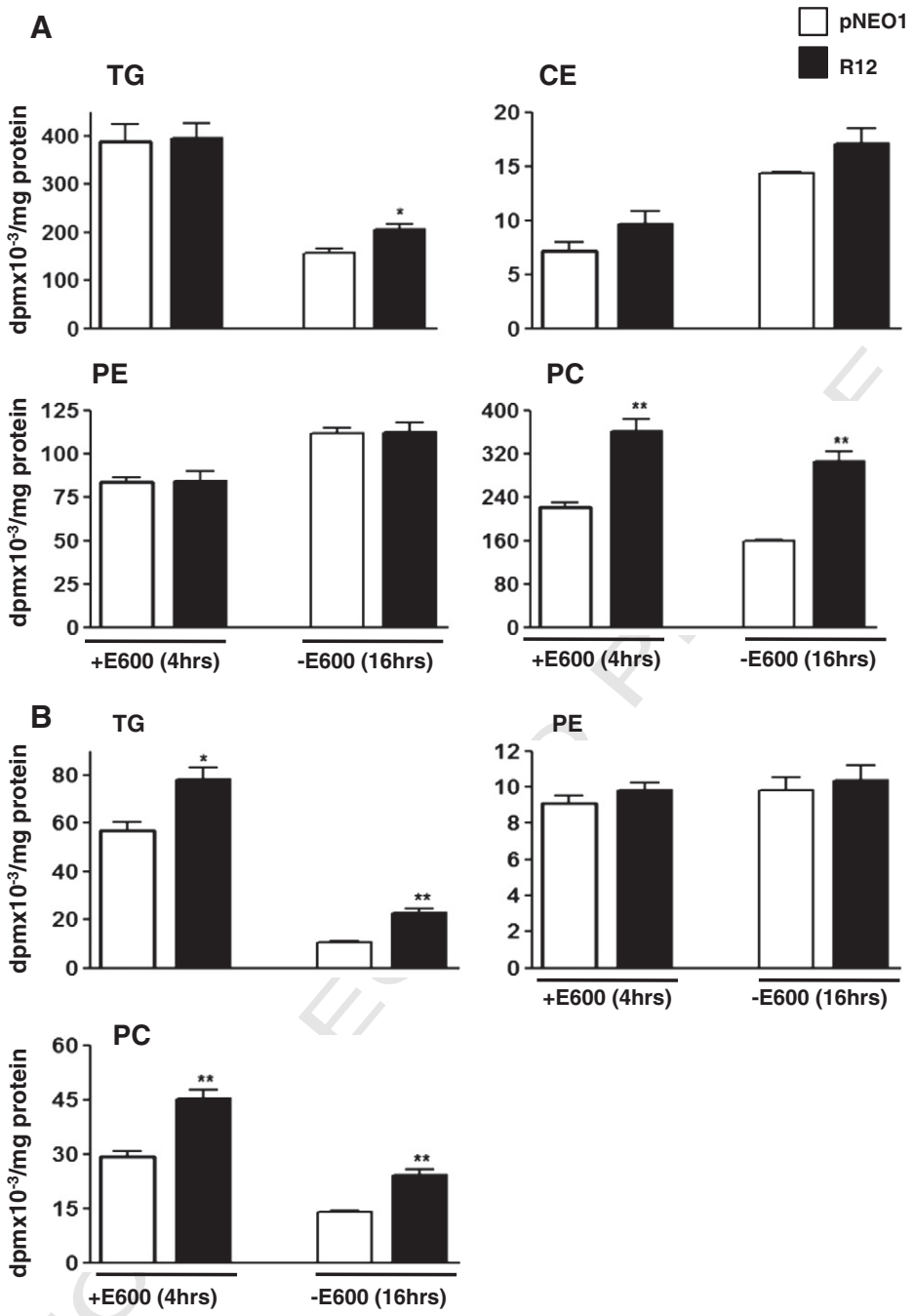

Fig. 6. HL-R does not access preformed stored lipid pools. (A) Incorporation of [^{3}H]OA into lipids in the presence of the lipase inhibitor (+E600) and turnover of preformed lipids after removal of the inhibitor (−E600). (B) Incorporation of [^{14}C]glycerol into lipids in the presence of 0.4 mM OA and the lipase inhibitor (+E600) and turnover of preformed lipids after removal of the inhibitor (−E600). Values are the mean ± SEM for n = 3–4 experiments performed in triplicate. *P<0.05, **P<0.01 versus control pNEO1.

4.4. Intracellular HL activity attenuates VLDL secretion

TG and CE are the major neutral lipid components of VLDL. Because HL-R expression reduced intracellular TG and CE levels, we hypothesized that this could lead to diminished lipid availability for VLDL assembly and reduced apoB secretion.

Even in the presence of exogenous OA-mediated TG synthesis (Pulse period), the secretion of apoB100 from R12 cells was decreased by 85% (Fig. 7A). Secretion of apoB48 from all McA cell lines was very low (Fig. 7A). No significant change in apoB secretion from HL-S expressing S7 cells was observed in the presence of OA (Supplemental Table S1C). Upon removal of OA from the culture media and cessation of TG synthesis by exogenously supplied FA (chase period), apoB secretion from HL-R expressing R12 cells was almost completely abolished (Fig. 7A), while HL-S expressing S7 cells secreted 30% less apoB100 than control cells (Supplemental Table S1C). ApoE secretion was unchanged by the expression of HL-R (Fig. 7A). These results emphasize the fact that apoB100 secretion from McA cells is dependent on sufficient availability of TG [45–47]. Expression of HL-R resulted in an 80–90% decrease of TG secretion into the media during both the pulse and the chase (Fig. 7B). HL-S expressing S7 cells also secreted diminished amounts (40%) of TG in OA-supplemented (Pulse) and after OA withdrawal (Chase) (Supplemental Table S1C). Significant decrease of TG and CE secretion was also observed in McA cells infected with ad-HLer following 4 h incubation with 0.4 mM [^{3}H]OA (Supplemental Fig. 4A). Therefore, HL has the ability to markedly

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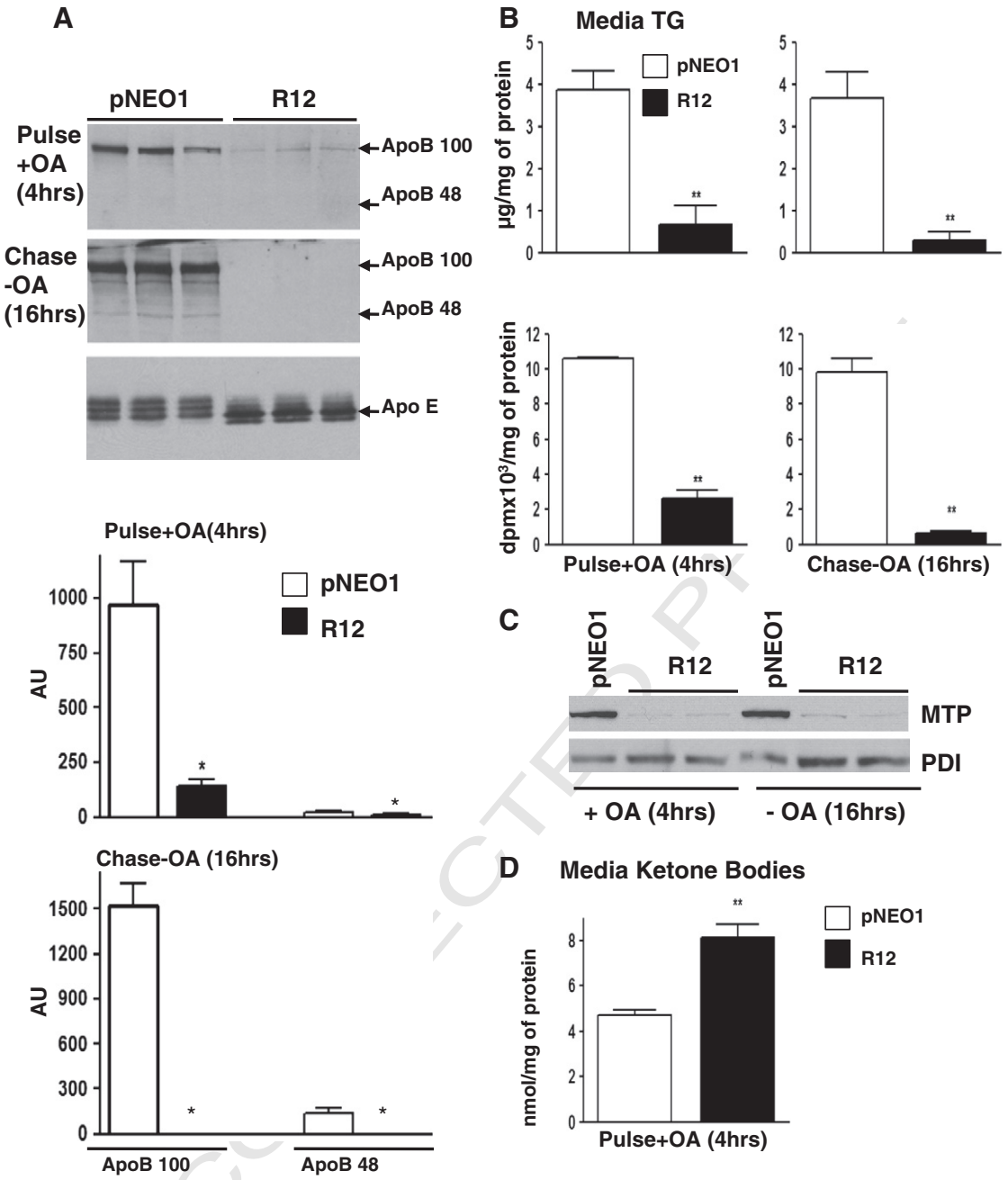

Fig. 7. HL-R attenuates VLDL secretion. (A) Immunoblot of media apoproteins and quantitation of apoB. *P<0.001 versus control pNEO1. (B) Mass and radioactivity of media TG after incubation with 0.4 mM [^{3}H]OA (Pulse + OA) followed by incubation in the absence of OA (Chase-OA). Values are mean ± SEM for n = 3–4 experiments performed in triplicate. **P<0.01 versus control pNEO1. (C) Immunoblot of MTP and a loading control protein disulfide isomerase (PDI) in McA cell lysates. (D) Ketone body accumulation in McA cell media after incubation for 4 h with 0.4 mM OA. Values are mean ± SEM (n = 3). **P<0.01 versus control pNEO1.

reduce TG secretion via its activity within the ER compartment. Microsomal triglyceride transfer protein (MTP) plays a crucial role in the initial step of apoB folding and assembly [48]. Although the *Mttp* mRNA levels in R12 cells were increased 8-fold (Table 1) the MTP protein levels were significantly diminished (Fig. 7C). These results suggest rapid degradation of the MTP in R12 cells.

4.5. Intracellular HL activity causes changes in the expression of lipogenic and oxidative genes

HL-R expressing R12 cells (OA-supplemented) exhibited significant changes in the expression of lipid uptake, lipogenic and oxidative genes compared to control cells (Table 1). The expression of key transcription factors regulating lipogenesis (LXRβ), cholesterogenesis (SREBP2) and FA oxidation (PPARα) was increased as was the expression of the respective target genes, such as LXRβ/SREBP1c targets diacylglycerol acyltransferase 1, mitochondrial glycerol-3-phosphate acyltransferase, and acetyl-CoA carboxylase; SREBP2 target LDL receptor, PPARα targets carnitine palmitoyltransferase-1 and acyl-CoA dehydrogenases (Table 1). The augmented expression of FA oxidation genes was accompanied by increased production of ketone bodies in R12 cells (Fig. 7D) and the production of acid-soluble metabolites (ASM) in ad-HLer infected cells (Supplemental Fig. S4B). This increased fatty acid oxidation may be responsible for the lack of cellular accumulation of FA released by HL-mediated lipolysis and for decreased accumulation of lipids despite significant attenuation of VLDL secretion. The most striking change in expression was observed for Pck1 gene encoding phosphoenolpyruvate carboxykinase 1 (PEPCK), which was increased

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Table 1
HL-R upregulates expression of lipogenic and oxidative genes.

Function	Gene	pNEO1	R12	*P*-value
Lipid synthesis	Mogat1	1.08 ± 0.12	1.63 ± 0.06	0.0066
	Dgat1	1.01 ± 0.09	1.29 ± 0.05	0.0351
	Gpam	1.07 ± 0.28	4.28 ± 0.65	0.0105
	Lipin2	1.05 ± 0.24	1.72 ± 0.11	0.0385
	Chka	1.03 ± 0.17	4.04 ± 0.10	<0.0001
	Acaca	1.16 ± 0.46	2.68 ± 0.11	0.0136
Lipid uptake	Scarb1	1.01 ± 0.07	1.79 ± 0.11	0.0028
	Slc27a1	1.09 ± 0.34	8.08 ± 0.52	0.0001
	Ldlr	1.19 ± 0.52	3.57 ± 0.26	0.0066
Lipid oxidation	Cpt1a	1.18 ± 0.48	3.30 ± 0.28	0.0098
	Acadl	1.10 ± 0.36	2.80 ± 0.20	0.0066
	Acadvl	1.00 ± 0.04	2.18 ± 0.18	0.0026
Lipid secretion	Mttp	1.11 ± 0.37	8.10 ± 0.51	0.0001
Gluconeogenesis/ glyceroneogenesis	Pck1	1.29 ± 0.56	101.4 ± 2.82	<0.0001
Transcription	Nr1h2/LXRb	1.01 ± 0.08	1.41 ± 0.05	0.0067
	Srebf2	1.07 ± 0.28	2.09 ± 0.24	0.0416
	Ppara	1.15 ± 0.44	4.24 ± 0.27	0.0014
ER stress	Hspa5/BiP	1.00 ± 0.07	0.22 ± 0.01	<0.0001

Values are expressed as mean ± SEM.

Abbreviations: Mogat1, monoacylglycerol O-acyltransferase 1; Dgat1, diacylglycerol O-acyltransferase 1; Gpam, glycerol-3-phosphate acyltransferase (mitochondrial); Lipin2, Lipin 2; Chka, choline kinase a; Acaca, acetyl-coenzyme A carboxylase a; Scarb1, scavenger receptor class B-member 1; Slc27a1, solute carrier family 27 (fatty acid transporter) member1/FATP-1; Ldlr, low density lipoprotein receptor; Cpt1a, liver carnitine palmitoyltransferase1; Acadl, acyl-coenzyme A dehydrogenase-long chain; Acadvl, acyl-coenzyme A dehydrogenase-very long chain; Ppara, peroxisome proliferator-activated receptor α; *Mttp*, microsomal triglyceride transfer protein; Pck1, phosphoenolpyruvate carboxykinase 1 (soluble); Nr1h2, nuclear receptor subfamily 1-group-h-member3/LXRβ, Srebf2, sterol regulatory element binding transcription factor 2; Hspa5, heat shock protein 70 kDa protein 5/BIP.

100-fold (Table 1). It is worth noting that no significant changes in the expression were observed for several key lipogenic genes including *Lxra*, *Srebp1c*, *Fas*, *Scd1*, *Dgat2*, *Mgat2*, *Agpat2*, *lipin1* and fatty acid oxidation genes *Mcad* and *Acox1*.

5. Discussion

This study was conducted to test the hypothesis that HL becomes active within the ER and is capable of modulating VLDL assembly and secretion. Surprisingly, expression of ER-targeted HL in McA cells not only decreased VLDL secretion but also lowered cellular TG accumulation. Our data are consistent with HL-catalyzed hydrolysis of *de novo* formed DG, TG and glycerophospholipids within the ER. The prolonged residence of HL-R in the ER also likely diminishes the TG pool available for bulk lipidation of lipid-poor primordial apoB particles due to diminished levels of MTP [49–51]. An attractive hypothesis is that reduced MTP- and lipid-mediated co-translational/co-translocational lipidation of apoB results in augmented apoB degradation by the proteasome or by autophagy. FA released by HL-catalyzed lipolysis do not accumulate but are oxidized and possibly also effluxed into the culture media. Importantly, our data indicate that FA generated by lipolysis within the ER bilayer can serve as ligands for PPARα activation and as substrates for mitochondrial β-oxidation. Previously, only arylacetamide deacetylase, a diacylglycerol (DG) lipase, [52] and ATGL, a lipase associated with hepatic cytosolic lipid droplets, have been shown to support this process [7,10,53]. The lipid lowering effect of HL-R appeared to be dependent on the lipolytic activity of HL since no reduction in cellular TG was observed upon incubation of HL-R expressing cells with a pan-lipase inhibitor E600. Our results are in agreement with a recent report by Bamji et al. who showed that secretion-competent human HL (analogous to HL-S in this study) was able to decrease intracellular TG levels and TG secretion in the same cell hepatoma line. Interestingly, expression of secretion-competent inactive HL (catalytic serine residue mutated to glycine) has been recently reported to diminish TG secretion without affecting intracellular lipid levels [36]. The mechanism by which inactive HL affected media TG levels is currently unclear because in addition of the lack of effect on total intracellular TG levels, the partitioning of TG into the secretory pathway (ER lumen) was also not significantly altered in cells expressing the inactive HL [36]. As in this recent report by Bamji et al. [36], we also did not observe significant differences in apoB secretion in cells expressing secretion-competent HL (HL-S) during the OA supplementation period. However, we observed diminished apoB100 secretion (30%) during the subsequent chase period in the absence of exogenously supplied OA. The observed decreased OA incorporation into glycerophospholipids in HL-R expressing cells could be partially due to the phospholipase activity of HL. However, since DG levels are diminished in HL-R expressing cells and DG is the obligate precursor for both PC and PE synthesis in these cells, the decreased glycerophospholipid synthesis is more likely due to the insufficiency of DG. Because the turnover rate of the preformed lipids appeared to be independent of HL-R expression this suggests that HL-R did not access preformed stored cytosolic neutral lipids. The increased depletion of CE in HL-R expressing cells was unexpected as a CE hydrolase activity has not been previously attributed to this enzyme. It is more likely that FA for cholesterol esterification are derived from the turnover of preformed TG storage pools ([13,39] and Fig. 6), and since these pools are diminished in HL-R expressing cells this may directly impact on CE levels. Expression of HL-R resulted in augmented expression of lipogenic and fatty acid oxidation genes. This is in agreement with Bamji et al. [36] who reported upregulation of expression of *Mttp*, *Dgat2* and *Cpt1a* in cells transfected with secretion-competent HL. However, while *Mttp* mRNA was increased 8-fold in R12 cells the MTP protein was significantly reduced suggesting rapid degradation of the protein in these cells that have greatly diminished apoB lipidation/secretion capacity. On the other hand, increased *Cpt1a* and *Ppara* mRNA expression in HL-R was accompanied with the expected increased fatty acid oxidation levels. One of the most surprising findings in our study is the 100-fold increase in the expression of *Pck1* mRNA encoding PEPCK. PEPCK is a key glyceroneogenic/gluconeogenic gene and our results suggest that one of the roles of PEPCK in R12 cells might be to provide glycerol-3-phosphate backbone for PC synthesis as indicated by increased incorporation of radiolabeled glycerol into this lipid. One possible explanation is that HL-R hydrolyzes DG within the ER, which is the obligate precursor of PC synthesis and the cells compensate by upregulating the activities of enzymes in glycerophospholipid biosynthesis in order to keep allostatic balance. The net effect is compensatory increased glycerolipid synthesis with concomitant HL-catalyzed increased degradation (futile cycle) resulting in the release of FA from newly synthesized lipids, which are subsequently targeted for oxidation.

Although the effect of HL on VLDL production in humans has not been directly studied the role of HL in this process can be inferred from several findings. HL deficiency in humans is most commonly attributed to polymorphisms in the *LIPC* (gene encoding HL) promoter [54–56], which diminish transcriptional efficacy. Less common are mutations in the coding region of the gene [57–59], which alter the structural integrity, secretion and catalytic properties of HL. Patients with HL deficiency display elevated total plasma cholesterol and TG concentrations and have a lipoprotein profile characterized by markedly increased TG- and PL-rich lipoprotein remnants and HDL [60]. It is possible that lack of HL activity would augment VLDL production. Two studies in patients with compound heterozygous mutations in the coding region of *LIPC* gene provide support for this hypothesis [58,59]. Both of these studies measured plasma lipid profiles and utilized a primed-constant infusion of deuterated leucine over a 12 h period to investigate lipoprotein turnover. In both studies the VLDL apoB100 pool size in HL deficient men were approximately three-fold of those from normotriglyceridemic men and the total plasma TG levels were also highly elevated. Decreased fractional

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catabolic rate certainly contributed to accumulation of VLDL apoB100 in HL deficient men from both studies but one study [58] also reported a three-fold higher hepatic VLDL apoB100 production rate.

In conclusion, our results in McA cells (a model cell line of hepatocytes) provide support for an anti-atherogenic role for HL. Treatments resulting in increased intracellular HL activity (gene therapy) may result in decreased intracellular TG levels and therefore in alleviation of pathologies associated not only with fatty liver conditions (i.e. non-alcoholic fatty liver disease), but also with hyperlipidemia (atherosclerosis) and hepatitis C virus infection since the infectivity of this virus appears to be dependent on the presence of intracellular TG stores and functional VLDL assembly and secretion pathway.

Acknowledgements

This work was supported by a grant-in-aid from the Heart and Stroke Foundation of Alberta, Northwest Territories and Nunavut awarded to R.L. and by operating grants from the Canadian Institutes of Health Research to R.L. and R.L.J. S.S.P. was supported by the University of Alberta Faculty of Medicine and Dentistry graduate studentship. A.D.Q. was supported by the Heart and Stroke of Canada postdoctoral fellowship. K.K. was supported by an Alexander Graham Bell Canada graduate scholarship from the Natural Sciences and Engineering Research Council of Canada and by a Walter H. Johns fellowship from the University of Alberta. R.L.J. is a Heart and Stroke Foundation of Canada Investigator and a holds a Canadian Institutes of Health Research New Investigator award. R.L. is a Scientist of the Alberta Innovates-Health Solutions. The authors thank Audric Moses for expert technical assistance in lipid analysis. Lipid analysis by gas chromatography was performed by a Lipid Analysis Core, which receives support from the Women and Children Health Research Institute.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbalip.2013.01.017.

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Please cite this article as: B. Erickson, et al., Endoplasmic reticulum-localized hepatic lipase decreases triacylglycerol storage and VLDL secretion, Biochim. Biophys. Acta (2013), http://dx.doi.org/10.1016/j.bbalip.2013.01.017