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Insulin, glucagon and fatty acid treatment of hepatocytes does not result in phosphorylation or changes in activity of triacylglycerol hydrolase

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Abstract

It is recognized that the majority of very low density lipoprotein (VLDL) associated triacylglycerol (TG) is synthesized from fatty acids and partial acylglycerols generated by lipolysis of intra-hepatic storage rather than made de novo. Triacylglycerol hydrolase (TGH) is involved in mobilizing stored TG. Modulating the ability of TGH to hydrolyze stored lipids represents a potentially regulated and rate limiting step in VLDL assembly. Phosphorylation of lipases and carboxylesterases trigger diverse but functionally significant events. We explored the potential for regulating the mobilization of hepatic TG through phosphorylation of TGH. Insulin is known to suppress VLDL secretion from liver, and glucagon can be considered an opposing hormone. However, neither insulin nor glucagon treatment of hepatocytes led to phosphorylation of TGH or changes in its activity. Augmenting intracellular TG stores by incubations with oleic acid also did not lead to changes in TGH activity. Therefore, changes in phosphorylation state are not a mechanism for regulating TGH activity, access to TG substrate pools or for TGH-mediated contributions to VLDL assembly and secretion.

Keywords: Triacylglycerol; Triacylglycerol hydrolase; Apolipoprotein B; Phosphorylation; VLDL

1. Introduction

The assembly of very low density lipoproteins (VLDL) is a complex process, however, details regarding the molecular events that are required or regulate the production of VLDL remain poorly understood. It is recognized that the formation of a secretion competent VLDL particle is largely regulated by the provision of lipid [1–3], and that triacylglycerols (TGs) represent the largest lipid constituent [4]. If provision of neutral lipid to the main protein component of VLDL, that is apolipoprotein (apo) B, is inadequate or limiting, the nascent apoB containing entity is degraded rather than completing the secretion process [5–7]. As much as 70% of VLDL TG has been shown to originate from intracellular storage pools rather than from de novo synthesis [8–11]. The stored TG is mobilized by lipolysis, and then re-esterified within the microsomal environment to re-form TG before it is incorporated into a VLDL particle. Therefore, mobilization of stored lipid represents a potentially regulated step in VLDL production and secretion. An enzyme localized to the lumen of the endoplasmic reticulum (ER) known as triacylglycerol hydrolase (TGH) has been characterized

Abbreviations: Apo, apolipoprotein; ATGL, adipose triglyceride lipase; BSA, bovine serum albumin; BSDL, bile salt-dependent lipase; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FBS, fetal bovine serum; HS, horse serum; HSL, hormone sensitive lipase; IP, immunoprecipitation; MAP kinase, mitogen-activated protein kinase; MUH, 4-methylumbelliferyl heptanoate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PKA, cyclic adenosine monophosphate-dependent protein kinase; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TG, triacylglycerol; TGH, triacylglycerol hydrolase; VLDL, very low density lipoprotein

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[12]. Our laboratory has cloned and expressed the rat [13], mouse [14] and human [15] TGH cDNAs. TGH is a 60-kDa soluble protein belonging to the carboxylesterase family of enzymes (EC 3.1.1.1). Stable transfection of TGH cDNA in McArdle RH7777 cells resulted in increased turnover of intracellular TG and secretion of neutral lipids [13,16]. Incubation of intact primary hepatocytes as well as transfected hepatoma cells with a TGH-specific inhibitor decreased apoB and TG secretion with a corresponding increase in intracellular TG storage [17]. This specific inhibitor is also effective towards the human TGH enzyme [16]. Decrease of TGH expression was associated with reduced mobilization of stored TG for VLDL secretion [18]. In vitro, purified TGH hydrolyzes a variety of substrates, including triacylglycerols [12]. This observation has been supported by independent experiments employing TGH purified from human liver (Borg-Capra et al. personal communication) and mouse adipose tissue [19].

Phosphorylation of a number of lipases and carboxylesterases has been demonstrated, and this modification affects their subcellular localization, access to substrate or enzymatic activity [20–28]. To date, little is known about regulation of TGH activity. The phosphorylation of rat TGH on serine 506 has been described, however, this serine residue is not conserved in the human, monkey or mouse orthologs [29]. Hence, the increase of rat TGH activity by phosphorylation cannot be directly translated to these other species. VLDL secretion from liver has been shown to respond to fed state and plasma insulin levels [30–33]. In the current study, we sought to determine if human TGH undergoes phosphorylation in response to hormones including insulin and glucagon or by increasing intracellular TG content with excess fatty acids. Several consensus sequences for phosphorylation sites are present in the TGH primary amino acid sequence. Potentially, TGH activity could be sensitive to the amount of TG storage, or to extracellular signals like insulin and glucagon, and the consequent modulation of this activity may regulate the provision of TG for assembly with primordial apoB containing particles. Although it was previously suggested that intracellular hepatic TG turnover rate (lipolysis) was insensitive to either insulin or glucagon [10], these studies were performed in the presence of dexamethasone, that is under conditions when TGH expression/activity is greatly diminished [18] and hence possible regulation of TGH-dependent turnover by these hormones remains to be established.

2. Materials and methods

2.1. Materials

Oligonucleotides were synthesized by the Institute for Biomolecular Design at the University of Alberta. The plasmid pCI-neo was purchased from Promega, Madison, WI. Molecular biology reagents were from Invitrogen Canada (Burlington, Ontario, Canada), with the exception of Pwo DNA polymerase (Roche Applied Science, Indianapolis, IN). DNA sequencing was performed by the Molecular Biology Services Unit at the University of Alberta.

Dulbecco's modified Eagle's medium (DMEM), phosphate-free DMEM, fetal bovine serum (FBS), horse serum (HS), Geneticin, Lipofectamine2000 and alkaline phosphatase were purchased from Invitrogen, Canada.

4-Methylumbelliferyl heptanoate (MUH), fatty acid-free bovine serum albumin (BSA), Ponceau S protein stain, phosphatase inhibitors, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and monoclonal anti-flag antibody covalently attached to agarose beads were from Sigma-Aldrich, Oakville, Ontario, Canada. The monoclonal M2 anti-flag antibody used in immunoblots was from Stratagene, La Jolla, CA.

Polyclonal antibodies for mitogen-activated protein (MAP) kinase (also known as ERK1) and a monoclonal anti-phosphotyrosine antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody for phospho-MAP kinase was obtained from Cell Signaling Technology (Beverly, MA). Anti-rabbit secondary antibodies conjugated to the fluorescent dye IR800 were from Rockland Immunochemicals (Gilbertsville, PA), while anti-mouse secondary antibodies conjugated to Alexa Fluor 680 were from Molecular Probes (Eugene, OR).

[^{33}P] labeled orthophosphate was purchased from ICN Biomedicals, Irvine, CA, while [^{32}P] orthophosphate was from Amersham Biosciences, Baie d'Urfé, Québec, Canada.

2.2. Cell culture

McArdle RH7777 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM containing 10% FBS (v/v) and 10% HS unless specified otherwise. All incubations were performed at 37 °C, in an atmosphere enriched with 5% CO_2 and in the presence of 100 U/mL penicillin and 100 μg/mL streptomycin.

2.3. Insulin induced stimulation of MAP kinase phosphorylation

McArdle RH7777 cells on 60 mm plates were grown to approximately 70% confluence, washed with phosphate buffered saline (PBS) and incubated in serum-free DMEM for 15 min. Media were aspirated, then cells were incubated in either DMEM only (control), DMEM supplemented with 100 nM insulin, or DMEM supplemented with 10 nM glucagon. Incubation was continued for 30 min, then cells were scraped into ice-cold buffer containing 20 mM Tris pH 8, 1% CHAPS and the phosphatase inhibitors: 1 mM sodium orthovanidate, 50 mM sodium fluoride and 5 mM tetrasodium pyrophosphate. Some dishes that received insulin treatment were harvested in a similar buffer lacking the phosphatase inhibitors. Cells were disrupted by brief sonication and centrifuged at 2700×*g* at 4 °C for 5 min to pellet unlysed cells and insoluble cell debris. Supernatants were transferred to clean tubes and their protein concentrations determined using Bio-Rad protein assay reagent. Cell lysates containing 60 μg of protein were adjusted to consistent volumes with respective buffers. Aliquots from cells treated with insulin and harvested with or without the phosphatase inhibitors were treated with 20 U of alkaline phosphatase and incubated at 37 °C for 15 min. Denaturing electrophoresis sample buffer was added to all samples, which were then boiled for 5 min and subjected to electrophoresis via SDS-PAGE (10%). Proteins were transferred to a nitrocellulose membrane. Immunoblots for MAP kinase and phospho-MAP kinase were performed using corresponding antibodies in accordance to the supplier's protocols. Fluorescently labeled secondary antibodies were employed to allow quantitation of fluorescence intensity from the immunoblots using a Li-Cor Odyssey Infrared Imaging System.

2.4. Protein phosphatase-1 activity

McArdle RH7777 cells were plated on 60 mm plates and grown to 70% confluence. Cells were washed with PBS and incubated in DMEM±10 nM glucagon for 30 min. Cells were harvested in Tris buffered saline (TBS; 20 mM Tris pH 7.4, 137 mM NaCl), sonicated briefly, centrifuged to pellet insoluble cell debris and unlysed cells and protein concentrations in the homogenates were determined. Protein phosphatase-1 activity in the cell lysates was measured via the dephosphorylation of [^{32}P] labeled phosphorylase *a* using an assay described previously [34–36]. Briefly, 10 μL aliquots