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Insulin suppression of apolipoprotein B in McArdle RH7777 cells involves increased sortilin 1 interaction and lysosomal targeting

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ABSTRACT

Insulin suppresses secretion of very low density lipoprotein (VLDL) apolipoprotein (apo) B in primary rodent hepatocytes (RH) by favoring the degradation of B100, the larger form of apo B, through post-endoplasmic reticulum proteolysis. Sortilin 1 (sort1), a multi-ligand sorting receptor, has been proposed as a mediator of lysosomal B100 degradation by directing B100 in pre-VLDL to lysosomes rather than allowing maturation to VLDL and secretion. The purpose of our studies was to investigate the role of sort1 in insulin-dependent degradation of apo B. Using liver derived McArdle RH7777 (McA) cells, we demonstrate that insulin suppresses VLDL B100 secretion via a phosphatidylinositide 3-kinase (PI3K) dependent process that is inhibitable by wortmannin in a fashion similar to RH. Using McA cells and *in situ* cross-linking, we demonstrate that insulin acutely (30 min) stimulates the interaction of B100 with sort1. The insulin-induced interaction of sort1–B100 is markedly enhanced when lysosomal degradation is inhibited by Bafilomycin A1 (BafA1), an inhibitor of lysosomal acidification. As BafA1 also prevents insulin suppressive effects on apo B secretion, our results suggest that sort1–B100 interaction stimulated by insulin transiently accumulates with BafA1 and favors B100 secretion by default.

1. Introduction

Hypersecretion of apo B-containing very low density lipoprotein (VLDL) by the liver is a hallmark of metabolic syndrome, and a major contributor to the development of hypertriglyceridemia. Insulin negatively regulates VLDL secretion acutely [1], in part, by targeting apo B for post-translational degradation in a post-endoplasmic reticulum (ER) compartment [2]. The process requires insulin activation of phosphatidylinositide 3-kinase (PI3K) [2,3] which translocates to intracellular membranes [4]. Insulin favors the degradation of B100 over B48 [1] which are required proteins for triglyceride-rich lipoprotein (TRL) assembly and are translated from unedited and edited apo B mRNA, respectively [5]. Inhibition of VLDL secretion by insulin minimizes competition between TRL of hepatic and intestinal origin for clearance during the postprandial period [6]. Loss of insulin-mediated regulation of apo B secretion precedes dysregulation of glucose metabolism in obese males [7], and thus may play an early role in dyslipidemia associated with insulin resistance [6].

Degradation of hepatic apo B can occur through a number of processes including ER associated degradation (ERAD), post-ER presecretory proteolysis (PERPP), and receptor-mediated re-uptake [8]. ERAD typically occurs following inadequate lipidation during translation causing B100 to be ubiquitinated and degraded by the proteasome, which is particularly prominent in HepG2 cells [9]. PERPP occurs following incubation of hepatocytes with ω-3 fatty acids [8]. Re-uptake of freshly secreted apo B mainly occurs via binding to the LDL receptor (LDLR), resulting in endosomal trafficking and lysosomal degradation [10]. Insulin stimulated apo B degradation does not occur through ERAD as movement out of the ER is necessary [2], consistent with results of others [11]. Furthermore, insulin-dependent degradation of apo B is maintained in the absence of the LDLR [12], suggesting that the LDLR is not involved. Less is known about the mechanism by which apo B is degraded via PERPP, although recent studies have implicated sortilin 1 (sort1) [13–16]. Sort1 is a multi-ligand sorting receptor that binds B100 during transit through the Golgi apparatus, and also extracellularly at the plasma membrane, resulting in trafficking of B100 to lysosomes for degradation [16]. Over-expression of hepatic sort1 in wild-type mice and restoration of hepatic sort1 expression in *ob/ob* mice results in reduced apo B secretion [15,16]. Binding of sort1 is specific for B100 [14] and B100 degradation is favored with insulin [1], suggesting the possibility

Abbreviations: Apo B, apolipoprotein B; B100, apo B derived from unedited mRNA; VLDL, very low density lipoprotein; PI3K, phosphatidylinositide 3-kinase; ERAD, endoplasmic reticulum associated degradation; PERPP, post ER presecretory proteolysis; BafA1, Bafilomycin A1.

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that sort1 trafficking may be responsible for insulin-dependent B100 degradation. Data from the current study examining this hypothesis demonstrate that insulin enhances interaction of B100 with sort1 which correlates with suppressed B100 secretion. When insulin suppression of B100 is blocked by inhibiting lysosomal degradation, B100-sort1 interaction is more apparent suggesting interaction as a mechanism for lysosomal B100 targeting.

2. Materials and methods

2.1. Materials

McA cells were obtained from the ATCC (Manassas, VA). Waymouth's 752/1 medium, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, horse serum, and most other chemicals including Bafilomycin A1 were from Sigma–Aldrich (St. Louis, MO). Purified bovine serum albumin (BSA) was purchased from Serologicals Proteins Bayer Corp. (Kankakee, IL). Aprotinin solution was purchased from MP Biomedicals (Solon, OH). Sodium orthovanadate was from Fisher Scientific (Fair Lawn, NJ). FuGENE 6 was from Promega Corp. (Madison, WI). Protein A-Sepharose and dithiobis [succinimidyl propionate] (DSP) were obtained from Pierce (Rockland, IL). Protease inhibitor cocktail sets I and III, phosphatase inhibitor cocktail set II, LY 294002, and wortmannin were purchased from Calbiochem (San Diego, CA). PROTEAN®TGX™ SDS polyacrylamide gels (4–15% (w/v) acrylamide), nitrocellulose and PVDF membranes, ECL reagents, and non-fat dry milk were obtained from Bio-Rad Laboratories (Hercules, CA). Rabbit anti-sortilin was purchased from Abcam (Cambridge, MA). Rabbit anti-pAKT (S473) and anti-AKT were purchased from Cell Signaling (Danvers, MA). Mouse anti-glyceraldehyde phosphate dehydrogenase (GAPDH) antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-mouse horseradish peroxidase-linked IgG and Hyperfilm™ were purchased from GE Healthcare (Buckinghamshire, UK). Light chain-specific anti-rabbit horseradish peroxidase-linked IgG was from Jackson ImmunoResearch Labs (West Grove, PA). The plasmid p110* containing constitutively activated mutant PI3K [17] was generously supplied by Dr. Wendy Fantyl, Chiron Corp. (Emeryville, CA). pTracer™-EF/V5-His/lacZ used to assess transfection efficiency was from Invitrogen, Corp. (Carlsbad, CA).

2.2. Cell culture

Primary RH were isolated from Sprague–Dawley rat livers using recirculating collagenase perfusions and cells were cultured on collagen coated dishes as previously described [18]. Wild-type McA cells were seeded onto 100 mm dishes and cultured in complete DMEM (cDMEM) containing 10% (v/v) FBS and 10% (v/v) horse serum, penicillin (100 U/mL) and streptomycin (100 μg/mL), as previously described [19]. After reaching 70–80% confluence, McA cells were washed three times in HBSS containing 0.2% BSA followed by incubation in DMEM containing 1% (w/v) BSA (1% BSA/DMEM) overnight (14–18 h). Experiments were terminated by washing cells three times in ice-cold (4 °C) HBSS containing 0.1 mM sodium orthovanadate and immediately freezing the dishes in liquid nitrogen. McA cells were scraped/thawed on ice into ice-cold lysis buffer containing 1% (v/v) NP40, 25 mM Tris–HCl (pH 7.4), 10% (v/v) glycerol, 150 mM NaCl, 2 mM EDTA, 10 mM tetrasodium pyrophosphate, 2 mM sodium orthovanadate, 1% (v/v) aprotinin, 1% (v/v) phosphatase inhibitor cocktail II, 1% (v/v) protease inhibitor cocktail III, and 1 mM 4-(2-aminoethyl) benzene sulfonyl fluoride (AEBSF). Cellular debris was removed by centrifugation at 17,000*g* for 15 min at 4 °C, and protein concentrations of clarified supernatants were determined using the bicinchoninic acid (BCA) assay (Pierce, Rockland, IL). Lysate proteins were then solubilized in SDS by addition of 1 part 4X Laemmli's gel loading buffer [20] with freshly added DTT (final concentration, 10 mM) per 3 parts lysate followed by heating to 95 °C for 10 min. Samples were stored frozen at −80 °C until further analysis.

2.3. Immunoprecipitation

Apo B was immunoprecipitated from media or clarified cell lysates by addition of polyclonal rabbit anti-rat apo B antibody developed by our laboratory and incubation at 4 °C with end-over-end mixing. Afterwards, Protein A-Sepharose beads were added, and samples were re-incubated to collect immunoprecipitates for 3–4 h at 4 °C. Beads were washed extensively as described [3], and proteins were eluted by addition of 2X Laemmli's buffer with heating at 95 °C for 10 min [20]. Samples were centrifuged briefly to collect eluted proteins, transferred to clean tubes, and stored at −80 °C until further analysis.

2.4. Western blotting

Proteins were separated by SDS–PAGE and electrophoretically transferred to PVDF membranes in Towbin buffer [21]. Non-specific binding was blocked by incubating membranes 2–3 h at 37 °C in TBST buffer (20 mM Tris, pH 7.5, 500 mM NaCl and 0.1% (v/v) Tween 20) containing 5% (w/v) milk. Membranes were incubated with primary antibody overnight at 4 °C with agitation, and after washing membranes 3 times in TBST, were re-incubated for 1 h at room temperature with the appropriate horseradish peroxidase-linked secondary antibody. Membranes were washed twice with TBST followed by several washes with TBS. PhosphoAKT (S473) and AKT mass were evaluated by similar methods except using nitrocellulose membranes and blocking non-specific binding by incubation in 5% (w/v) BSA/TBST. Antibody binding was evaluated by chemiluminescence detection using Hyperfilm™ or imaged directly using the BioRad Laboratories ChemiDocXRS+ system (Hercules, CA). Exposures were digitized and band intensities were quantified using Image Lab 3.0.1 software (Bio-Rad).

2.5. Lipoprotein fractionation

Media lipoproteins secreted by McA cells were separated by sucrose density gradient ultracentrifugation as described [22]. Gradients were centrifuged in a Beckman SW41 Ti rotor at 29,000 rpm for 65 h at 12 °C. Fractions (1 mL) were collected from the top, and apo B in each fraction was immunoprecipitated followed by separation of B100 and B48 by SDS–PAGE and analysis by Western immunoblotting using rabbit anti-rat apo B antibody.

2.6. McArdle RH7777 cell transfections

Transient transfections of McA cells were carried out in 4-well dishes using a 4:1 mixture of plasmid p110* and pTracer™-EF/V5-His/lacZ DNA in 5% (v/v) fetal bovine serum/DMEM and a concentration of FuGENE 6 to DNA of 6:2 according to manufacturer's protocols. Six hours after transfection, medium was removed, McA cells were rinsed in 0.2% (w/v) BSA/HBSS and reincubated in cDMEM containing 1 μM wortmannin for 12 h. McA cells were rinsed three times and half of the cells were incubated in cDMEM containing either 1 μM wortmannin or equivalent volume of DMSO. Cells were terminated; media were collected, and apo B concentrations were measured by RIA. To assess transfection efficiency, in parallel incubations McA cells were terminated by washing in Ca^{2+} and Mg^{2+}-free PBS and following lysis were assayed for β-galactosidase activity using a kit assay (E2000, Promega, Corp., Madison, WI).