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Acute suppression of apo B secretion by insulin occurs independently of MTP

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Abstract

Secretion of apolipoprotein (apo) B-containing lipoproteins by the liver depends mainly upon apo B availability and microsomal triglyceride transfer protein (MTP) activity and is subject to insulin regulation. Hepatic MTP mRNA expression is negatively regulated by insulin which correlates with inhibition of apo B secretion suggesting that insulin might suppress apo B secretion through an MTP-dependent mechanism. To investigate this possibility, we examined the acute effect of insulin on hepatic MTP expression and activity levels *in vivo* utilizing apobec-1^{-/-} mice. Insulin did not significantly alter hepatic MTP mRNA levels or lipid transfer activity 2 h following injection, but suppressed expression of genes important in gluconeogenesis. To study the specific role of MTP, we expressed human MTP (hMTP) in primary rat hepatocytes using adenoviral gene transfer. Increased expression of hMTP resulted in a 47.6 ± 17.9 % increase in total apo B secreted. Incubation of hepatocytes with insulin suppressed apo B secretion by 50.1 ± 10.8 % in cells over-expressing hMTP and by 53.0 ± 12.4 % in control transfected hepatocytes. Results indicate that even under conditions of increased hepatic apo B secretion mediated by MTP, responsiveness of hepatocytes to insulin to suppress apo B secretion is maintained.

Keywords

Insulin; lipoprotein secretion; forkhead transcription factor; FoxO1; MTP

1. Introduction

Increased hepatic VLDL secretion is a major determinant in the development of hypertriglyceridemia associated with metabolic syndrome and adult onset diabetes [1]. Apolipoprotein (apo) B is a necessary component of VLDL, as there is one required molecule of apo B per VLDL particle [2]. Regulation of hepatic VLDL assembly and secretion is complex and requires the synthesis of apo B and the lipid transfer and chaperone activities of microsomal triglyceride transfer protein (MTP) [3–5]. Insulin regulates the

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secretion of VLDL by favoring the degradation of apo B [6], the principle protein required for initiating assembly of VLDL with degradation occurring in a pre-secretory compartment [7,8]. Insulin-mediated suppression involves the activation of phosphatidylinositol 3-kinase (PI 3-kinase) by the insulin receptor transduced through insulin receptor substrates (IRS); a process that correlates with translocation of activated PI 3-kinase to intracellular membranes [9].

Recent studies indicate that insulin signaling negatively regulates hepatic MTP gene expression through the forkhead transcription factor, FoxO1 [10]. FoxO1 is a substrate for the serine-threonine kinase, AKT. Following insulin receptor activation, FoxO1 is phosphorylated by AKT and is excluded from the nucleus reducing transcriptional induction of key gluconeogenic enzymes [11] as well as *mtp* [10]. This cytoplasmic relocation of FoxO1 is believed to be responsible for the metabolic changes that arise in the shift from fasting to feeding [11,12]. Impaired ability to phosphorylate FoxO1, as occurs in various insulin resistance states and with constitutively active FoxO1 (CA-FoxO1), results in sustained nuclear localization and enhanced transcriptional activity. Kamagate *et al.* [10] recently showed that transgenic mice expressing CA-FoxO1 have increased levels of hepatic MTP expression that correlates with enhanced VLDL production compared with control mice. Knockdown of FoxO1 with siRNA in CA-FoxO1 expressing mice and in *db/db* mice resulted in decreased hepatic MTP expression and reduced VLDL production [10]. Since MTP is required for VLDL assembly, these data suggest that the inhibitory effect of insulin on hepatic MTP expression might be responsible for insulin-mediated suppression of apo B and VLDL secretion observed in control animals. To explore this possibility, we first examined the effect of *in vivo* insulin treatment on liver MTP expression and activity. Second, we determined the extent to which over-expression of MTP in hepatocytes by adenoviral gene transfer was able to overcome insulin-mediated suppression.

2. Materials and Methods

2.1 Materials

Ad-CMV-null and Ad-CMV-GFP adenoviral vectors (Serotype 5, E1/E3 deleted) were obtained from Vector Biolabs, Philadelphia PA. Ad-CMV-human MTP (hMTP) was provided by M. Mahmood Hussain (SUNY Downstate, Brooklyn, NY). Goat anti-human MTP polyclonal antibody was from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Mouse monoclonal anti-rat MTP was from BD Transduction Laboratories, San Jose, CA. Rabbit anti- β -actin was from Rockland, Gilbertsville, PA. Rabbit anti-GFP was from Millipore, Billerica, MA. Rabbit anti-goat horseradish peroxidase-linked IgG was from Thermo Scientific, Rockford, IL. Sheep anti-mouse and donkey anti-rabbit IgG horseradish peroxidase linked antibodies, ECL reagents, and HyperfilmTM were from GE Healthcare, Buckinghamshire, UK. Protease Inhibitor Cocktail I and III and Phosphatase Inhibitor Cocktail II were obtained from Calbiochem, San Diego, CA.

2.2 In vivo treatments

Apobec1^{-/-} deleted mice (in a C57B6 background) were fasted for 12 h and then injected intraperitoneally with saline or insulin (1 U/kg). After 2 h, mice were sacrificed and livers were harvested. An aliquot of 0.4 to 0.5 g of liver was immediately homogenized using a Polytron in 15 ml of TRIzol® Reagent (Invitrogen, Carlsbad, CA) to extract RNA. The remaining liver was snap-frozen in liquid nitrogen and stored at -80°C until further analysis. To assess insulin action *in vivo*, glucose levels were measured at 30, 60, 90, and 120 min via tail vein with a OneTouch®Ultra[®]2 blood glucose meter (Lifescan, Inc., Milpitas, CA).

2.3 RNA Analysis

Total liver RNA was isolated from TRIzol® according to manufacturer's instructions and stored in Formazol® at -80°C. First strand cDNA synthesis was performed by reverse transcription using total RNA recovered following ethanol precipitation. Real time PCR (RT-PCR) was performed using the PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA). Expression of MTP, Apo B, Apo E, FoxO1, and PGC1α were determined in triplicate using the following assays: Mm00435015_m1, Mm01545164_m1, Mm00437573_m1, Mm00490671_m1, and Mm00447183_m1, respectfully. Real time PCR results are reported as relative quantitation (RQ) where the PCR signal of the target transcript in a test group was normalized using the ratio to the untreated control and represent fold differences in mRNA abundance.

2.4 MTP activity assay

To assay triglyceride transfer activity of MTP, livers were homogenized and microsomes were isolated by centrifugation as previously described [13]. The fluorescence assay was performed in triplicate on 96 well titer plates by combining equal volumes of donor and acceptor vesicles with reaction buffer containing microsomal protein and incubating at 37°C for 30 min as described [13–15]. For total fluorescence assay, donor vesicles and isopropanol were combined and incubated for 5 min. Fluorescence was measured at 530 nm following excitation at 460 nm. Specific activity was calculated by subtracting out fluorescence due to vesicles alone and dividing sample fluorescence by total fluorescence. Values are expressed as the percent of lipids transferred/mg of protein/h.

2.5 Cell culture

Primary rat hepatocytes were isolated by collagenase perfusion of livers derived from male Sprague-Dawley rats (Harlan Teklad, Indianapolis, IN) between 0800 and 1100 in the morning [16]. Hepatocytes were purified on Percoll™ gradients, and viable hepatocytes were re-suspended and seeded in medium (2×10^6 cells/2 ml per dish) onto 60 mm dishes coated with type I collagen purchased from Becton Dickinson, Labware, Bedford, MA. Medium was Waymouth's 752/1 medium supplemented with 0.2% (w/v) bovine serum albumin (BSA), streptomycin (100 µg/ml), penicillin (100 units/ml), and gentamicin sulfate (10 µg/ml). After allowing cells to attach for 2–4 h, monolayers were rinsed three times with 0.2% (w/v) BSA/Hanks' balanced salt solution (HBSS), and fresh Waymouth's media containing 0.1 nM insulin (basal medium) was added. Rat hepatocytes were infected with 5 or 10 MOI of the following vectors: Ad-CMV-Null, Ad-CMV-GFP, or Ad-hMTP by incubation for 18–24 h. After rinsing monolayers, media were replaced with basal or Waymouth's medium containing 100 nM insulin and incubation was continued for 16 h. Incubations were terminated by collecting media and rinsing monolayers 3 times with cold (4°C) (HBSS) and freezing cells immediately in liquid nitrogen. Media were preserved by addition of Protease Inhibitor Cocktail I (final, 1%, v/v) and stored at 4°C until analysis. Frozen cells were scraped/thawed into ice-cold lysis buffer containing 10 mM tetrasodium pyrophosphate, 2 mM sodium orthovanadate, 1% (v/v) aprotinin solution, 1% (v/v) Phosphatase Inhibitor Cocktail II, 1% (v/v) Protease Inhibitor Cocktail III, and 1 mM AEBSF. Cellular debris was removed by centrifugation at 14,000 rpm, 4°C, for 15 min, and lysate protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockland, IL). Lysate proteins were completely denatured by addition of 4X Laemmli's buffer (final concentration, 62.5 mM TRIS, pH 7.5, 2.5% (w/v) sodium dodecyl sulfate (SDS), 5% (v/v) 2-ME, 10% (v/v) glycerol, 187 µM bromophenol blue, and 30 mM DTT (freshly added)). Samples were heated to 95°C for 5–10 min, cooled to room temperature, and stored at -80°C until further analysis.

2.6 Western blotting analysis

Proteins were separated by SDS-PAGE (50 µg/well) on Mini-PROTEAN®TGX™ 4–15% SDS polyacrylamide gels (Bio-Rad, Hercules, CA) and electrophoretically transferred to nitrocellulose or PVDF membranes according to manufacturer's instructions. Non-specific binding was blocked by overnight incubation at 4°C in TBS (20mM Tris, pH 7.5, 500 mM NaCl) containing 5% (w/v) non-fat milk and 0.1% (v/v) Tween 20. For immunoblotting, membranes were incubated for 1 h at room temperature with primary antibodies diluted in blocking buffer, followed by washing in TBS containing 0.1% (v/v) Tween 20. Antibody binding was detected by incubation of membranes for 1 h at room temperature with donkey anti-rabbit, rabbit anti-goat, or sheep anti-mouse horseradish peroxidase-linked IgG followed by enhanced chemiluminescence detection using Hyperfilm™. For quantitation, films were digitized and protein bands were quantitated using ImageQuant® 5.2 software (Molecular Dynamics, Buckinghamshire, UK). Protein expression levels were normalized to β-actin protein expression.

2.7 Apo B Secretion Radioimmunoassay

Media apo B concentrations were determined using a competitive monoclonal radioimmunoassay (RIA) developed against rat apo B using rat VLDL apo B as a standard as described previously [17]. Media apo B concentrations were normalized to cellular protein as determined by BCA assay on a dish-by-dish basis.

2.8 Statistical Analysis

Results from *in vivo* experiments are expressed as means ± S.D. of n mice. Results from over-expression studies are expressed as means ± S.E.M of n perfusions as they represent multiple analyses derived from multiple independent experiments. Significant differences between means were determined by Student's *t* test and P-values < 0.05 were considered significant.

3. Results

3.1 Effect of insulin on hepatic apo B, MTP mRNA levels, and MTP activity in vivo

In order to determine whether MTP expression is altered by insulin *in vivo*, apobec-1^{-/-} mice were fasted 12, and then injected with saline or insulin (1U/kg). Two h following injection, mice were sacrificed and liver MTP mRNA levels were quantified. No significant differences in MTP, apo B, or apo E mRNA levels (*P* > 0.05) were observed in mice injected with insulin compared with saline (Fig. 1A). Insulin injection decreased blood glucose levels within 30 min by 42.8 ± 12.6 % compared with saline injection. As an additional control for insulin action, specific mRNA levels known to be regulated by insulin were evaluated (Fig. 1A). Mice injected with insulin had significantly reduced levels of peroxisome proliferative activated receptor-γ co-activator 1 (PGC-1α) and FoxO1 mRNAs compared to saline injected mice (Fig. 1A). Reduced expression of PGC-1α and FoxO1 mRNAs indicate that there was sufficient time for changes in transcriptional activity to occur as a result of insulin injection. To determine whether MTP activity was altered following insulin treatment, liver MTP transfer activity was measured by fluorescence transfer assay. As with MTP mRNA levels, no significant differences were observed in MTP transfer activity in livers derived from mice treated with insulin versus saline (Fig. 1B).

3.2 Effect of hMTP expression on relevant cellular protein levels in rat hepatocytes

To address the role of MTP in regulation of apo B secretion, we expressed human MTP (hMTP) by adenoviral gene transfer. Rat hepatocytes were infected by overnight incubation with Ad-CMV-hMTP at 5 or 10 MOI, and hMTP as well as other relevant cellular proteins

were quantified by immunoblotting. hMTP protein was significantly increased following incubation (Fig. 2A). Control infections to assess transfection efficiency were carried out using Ad-CMV-GFP (Fig 2B). Relative to β -actin, protein disulfide isomerase (PDI), the small subunit of MTP, and rat MTP were not significantly altered following over-expression of hMTP or following insulin treatment (Fig. 2A). As shown previously [6] and confirmed here, cellular apo B48 was modestly reduced with insulin.

3.3 Effect of hMTP expression on insulin-mediated suppression of apo B secretion

To determine the effect of hMTP expression on apo B secretion, media apo B concentrations were determined by RIA. Rat hepatocytes expressing hMTP secreted significantly more apo B ($47.6 \pm 17.9\%$) than control cells (Fig. 3) indicating that the transgenic protein was successfully expressed and functioning to enhance lipoprotein assembly and secretion. To assess the effect of increased MTP on insulin-mediated apo B suppression, media apo B levels were assayed from transfected rat hepatocytes incubated in either basal media or media containing 100 nM insulin. Insulin significantly reduced apo B secretion by control rat hepatocytes by $53.0 \pm 12.4\%$ ($n = 4$) compared with rat hepatocytes incubated under basal conditions (Fig. 3). Similarly, insulin significantly inhibited apo B secretion by rat hepatocytes expressing hMTP by $50.1 \pm 10.8\%$ ($n = 5$). These results indicate that while apo B secretion is enhanced by over-expression of MTP, an equivalent proportion of apo B that is produced remains subject to insulin regulation.

4. Discussion

VLDL assembly and secretion requires the synthesis of apo B and the activity of MTP with MTP acting as both a lipid transfer protein and an apo B chaperone. Insulin suppresses VLDL apo B secretion *in vivo* [18,19], and it is possible that insulin could act on apo B, MTP, or other pathways required in the lipoprotein assembly and secretion process. *In vitro* studies using HepG2 cells have shown that insulin treatment leads to substantial decreases in MTP mRNA [20,21]. Recent studies suggest that the reduction in MTP mRNA occurs at the transcriptional level and involves the phosphorylation and nuclear exclusion of the transcription factor, FoxO1 [10]. Other *in vitro* studies have shown that insulin suppresses MTP transcription via the extracellular signal-regulated kinase (ERK1/2) branch of the mitogen-activated protein kinase (MAPK) cascade [20,22]. Because of the requirement for MTP activity in VLDL production, these reports suggested the possibility that the short-term reduction in VLDL secretion with insulin is mediated through a decline in MTP activity. In the present study, we found that insulin did not lead to any substantive decrease in MTP mRNA abundance while significantly reducing blood glucose levels. Previous research has also shown that *apob* and *apoe* genes are not modulated by insulin on a transcriptional level [22–26], and this is consistent with current studies showing apo B and apo E mRNA levels remained stable following insulin injection. Current results support that the acute effect of insulin on VLDL production is not associated with changes either in MTP or apo B mRNA abundance.

In vivo insulin injection resulted in changes in expected mRNA levels of the gluconeogenic regulatory proteins PCG1 α and FoxO1. PCG1 α along with FoxO1 induce transcription of the rate-limiting enzymes, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase [27]. Insulin negatively regulates gluconeogenesis by suppressing transcription of PGC-1 α , perhaps through nuclear exclusion and degradation of cAMP responsive coactivator TORC2 [28,29]. FoxO1 mRNA levels are increased with fasting and are reduced with insulin released following refeeding [11,12]. As expected, both PCG1 α and FoxO1 mRNA were significantly reduced with insulin injection providing support for insulin action at the level of the transcriptional machinery necessary to repress gluconeogenic gene expression.

A number of studies using various animal models have demonstrated that blocking MTP transfer activity with specific inhibitors results in reduced VLDL secretion and plasma lipid levels [30–32]. Thus, independently of alterations in MTP mRNA expression, insulin could conceivably suppress VLDL secretion in the short-term by decreasing MTP lipid transfer activity. In the current study we found by direct assay that insulin did not significantly alter MTP lipid transfer activity. Therefore, this mechanism is an unlikely reason for the suppressive effects of insulin on VLDL secretion. This result is consistent with *in vitro* evidence demonstrating no differences in MTP transfer activity with insulin although incubation times differed between studies [21].

After establishing that hepatic MTP mRNA levels and activity were unaffected by insulin, the possibility remains that a decrease in MTP protein could contribute to reduced VLDL secretion since MTP also acts as an apo B chaperone. MTP specifically interacts with the apo B peptide and inhibition of this protein-protein interaction can lead to a significant decrease in VLDL secretion [33]. We tested this hypothesis by over-expressing hMTP in rat hepatocytes by adenoviral transfer. The significant increase in apo B secretion with hMTP expression provided clear evidence that the transgenic protein was active in lipoprotein assembly. These results are consistent with *in vivo* studies in which C57BL6 mice infected with Ad-hMTP secreted significantly more apo B-containing lipoproteins than control mice [34]. Increased hMTP protein did not result in a decrease in endogenous rat MTP or a change in cellular PDI, the small subunit of MTP, consistent with previous reports [35]. Importantly, protein expression levels of rat and human MTP were unaltered following insulin treatment providing additional evidence that insulin did not significantly reduce MTP protein levels through a post-transcriptional mechanism. Together, these results suggest that changes in MTP protein expression are not responsible for insulin-mediated suppression of apo B-containing lipoprotein secretion by the liver. Overall, our results suggest that, short-term, insulin-mediated suppression of VLDL secretion occurs by a mechanism that is separate from factors that regulate MTP expression and activity.

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Fig. 1.

Insulin does not alter MTP transcript level or transfer activity *in vivo*. Male apobec-1^{-/-} mice fasted for 12h were injected with saline (n = 9) or insulin (1 U/kg, n = 6) and euthanized 2 h later. Total RNA was isolated for transcript analysis and remaining liver was used for MTP transfer activity assay. (A) Effect of insulin injection on relative mRNA levels of selected hepatic mRNA transcripts, mean $\pm$ S.D. (B) Effect of insulin injection on specific activity of MTP in liver homogenates, mean $\pm$ S.D. *P < 0.05 relative to saline condition. Filled and open bars represent saline and insulin conditions, respectively.

Fig. 2.

Expression of hMTP does not alter abundance of endogenous MTP, PDI, or apo B protein levels. Primary rat hepatocytes were infected with Ad-CMV-Null, Ad-CMV-GFP, or Ad-CMV-hMTP and incubated for 18–24. After washing monolayers, media was replaced with basal or Waymouth's medium supplemented with insulin (100 nM) and incubation was continued for 16 h. Lysates of rat hepatocytes were prepared and subjected to immunoblotting. (A) Protein expression levels of cells infected with Ad-Null or Ad-hMTP and subsequently incubated with basal (–) or 100 nM (+) insulin. (B) Protein expression levels following infection with Ad-GFP or Ad-hMTP (10 MOI) and treatment with insulin. Experiments were carried out in triplicate with essentially identical results.

Fig. 3.

Expression of hMTP stimulates apo B secretion, but fails to blunt insulin-mediated suppression of apo B secretion. Following infections with Ad-CMV-Null or Ad-CMV-hMTP, rat hepatocytes were incubated in basal medium or medium containing 100 nM insulin for 16 h, and media apo B concentrations were measured by RIA. (A) Effect of hMTP over-expression on apo B secretion, mean $\pm$ S.E.M. from 5 independent experiments where 4–5 plates/condition were analyzed. (B) Effect of insulin on basal apo B secretion in transfected hepatocytes, mean $\pm$ S.E.M. from 4 independent experiments where 4–5 plates/condition were analyzed. Media apo B concentrations were normalized to cellular protein and results are expressed as a percentage of the control or basal condition. * $P < 0.05$ relative to Ad-CMV-Null. † $P < 0.05$ relative to corresponding basal condition. Filled and open bars represent basal and 100 nM insulin conditions, respectively.