Foxa2 Activity Increases Plasma High Density Lipoprotein Levels by Regulating Apolipoprotein M*

Christian Wolfrum†‡, Jessica J. Howell†§, Esther Ndungo¶, and Markus Stoffel†§*

From the †Institute of Molecular Systems Biology, Swiss Federal Institute of Technology, Eidgenössische Technische Hochschule Zürich, 8093 Zürich, Switzerland, §Competence Center for Systems Physiology and Metabolic Diseases, Eidgenössische Technische Hochschule Zürich, 8093 Zürich, Switzerland, and ¶Rockefeller University, New York, New York 10021

Obesity, diabetes, insulin resistance, and hyperinsulinaemia are frequently associated with a cluster of closely related lipid abnormalities such as low plasma levels of high density lipoprotein (HDL) and elevated levels of triglyceride, both known to increase the risk of developing atherosclerotic disease. The molecular mechanisms linking obesity, insulin resistance, and hyperinsulinaemia to low HDL levels are incompletely understood. Here we demonstrate that insulin, through a Foxa2-mediated mechanism, inhibited the expression of apolipoprotein M (apoM), an important determinant of plasma pre-β-HDL and α-HDL concentrations. Obese mice had decreased apoM expression and plasma pre-β-HDL levels due to inactivation of Foxa2 in hyperinsulinemic states. Nuclear reexpression of Foxa2 with a phosphorylation-deficient mutant Foxa2T156A (Ad-T156A) activated apoM expression and increased plasma pre-β-HDL and α-HDL levels. In contrast, haploinsufficient Foxa2+/− mice exhibited decreased hepatic apoM expression and plasma pre-β-HDL and HDL levels. The increase in plasma HDL levels and pre-β-HDL formation by Foxa2 was mediated exclusively by apoM, as constitutive active expression of Foxa2 in apoM−/− mice had no effect on plasma HDL levels. Our results identify a fundamental mechanism by which insulin regulates plasma HDL levels in physiological and insulin-resistant states and thus have important implications for novel therapeutic approaches to prevent atherosclerosis.

Epidemiological data indicate that a low level of high density lipoprotein (HDL) cholesterol is associated with increased cardiovascular morbidity and mortality (1, 2) and that genetic syndromes of high HDL are associated with longevity and decreased incidence of coronary heart disease (3). Furthermore, acute weight loss due to a hypocaloric diet frequently leads to a significant increase in plasma HDL concentrations in comparison with the base line, demonstrating that HDL metabolism is also under metabolic/hormonal control (4, 5).

Mouse models of obesity and insulin resistance have been used extensively to study the molecular and genetic mechanisms of lipoprotein metabolism and atherosclerotic plaque formation. In general, HDL levels are inversely related to atherosclerosis susceptibility in humans and mice. The protective properties of HDL are most likely due to its role in reverse cholesterol transport but also may be related to the antioxidant and anti-inflammatory effects of HDL in the arterial wall (6). In humans, low HDL cholesterol levels may be associated with defects in synthesis and/or catabolism of the major HDL apolipoprotein, apoAl (7, 8). The accelerated catabolism of HDL apolipoproteins is believed to reflect increased core lipid exchange between HDL and triglyceride-rich lipoproteins, leading to modifications of HDL composition and size that result in increased catabolism of HDL particles and reduced plasma HDL levels. HDL particles in hypertriglyceridemic subjects with obesity and insulin resistance are also frequently smaller and more susceptible to renal filtration and degradation (9), thereby contributing to increased HDL catabolism.

It is believed that HDL exerts its antiatherogenic effects through the process of reverse cholesterol transport. HDL particles comprise a heterogeneous class of lipoproteins that contain various apolipoproteins, including apoAI, -AII, -CI-IV, -E, -F, and -M (10, 11). The formation of HDL is regarded to begin with pre-β-HDL, which is produced by the liver and extracellularly during the metabolism of triglyceride-rich lipoproteins and HDL remodeling (12). Mature HDL particles are subsequently generated from pre-β-HDL, which is considered the initial extracellular acceptor of cellular cholesterol upon efflux from peripheral tissues.

There also exist significant differences in HDL metabolism between human and commonly used mouse models. Obese mice frequently have normal or elevated HDL levels, possibly because of a lack of the gene encoding cholesteryl ester transfer protein (CETP) in mice (13). Leptin-deficient ob/ob mice also exhibit defective hepatic HDL particle uptake, possibly because of a post-transcriptional decrease in hepatic SR-B1 levels, which results in decreased recycling, degradation, and selective lipid uptake (14–16). Furthermore, in human obesity and insulin-resistant states, increased activity of hepatic lipase, an enzyme involved in the hydrolysis of triglycerides and phospholipids in HDL, produces smaller HDL particles and facilitates HDL clearance (17), whereas in obese mice hepatic lipase is significantly reduced (18).

Insulin-resistant states are commonly associated with atherogenic dyslipidemia, which is characterized by low HDL
levels and elevated plasma triglyceride concentrations (19). Together with impaired glucose metabolism, this condition is believed to contribute significantly to atherosclerosis and cardiovascular disease (19). Impaired glucose and lipid metabolism coexist in the majority of subjects with “metabolic syndrome” and form the basis for the definition and diagnosis of this complex syndrome (20–22). The most fundamental defect in these patients is resistance to cellular actions of insulin. Insulin insensitivity appears to cause hyperinsulinemia, increased gluconeogenesis and glucose output from the liver (23–25), and reduced suppression of lipolysis in adipose tissue leading to a high free fatty acid flux (26). Furthermore, insulin resistance leads to increased hepatic very low density lipoprotein secretion, causing hypertriglyceridemia (27, 28) and reduced plasma levels of HDL. Although the link between insulin resistance and dysregulation of lipoprotein metabolism is well established, a significant gap exists in our knowledge regarding the underlying cellular and molecular mechanisms. Emerging evidence suggests that insulin resistance and its associated metabolic dyslipidemia result from perturbations in the insulin-signaling pathway, thereby leading to a state of mixed hepatic insulin resistance and sensitivity. We have recently identified a molecular pathway that links hyperinsulinemia to the development of hepatic insulin resistance and dyslipidemia. The Forkhead transcription factor, Foxa2, and its coactivator, Pgc1α, are powerful activators of β-oxidation (29, 30). Insulin inhibits Foxa2 through a mechanism that involves threonine phosphorylation at amino acid position Thr-156 and nuclear exclusion. In hyperinsulinemic rodent models, Foxa2 is permanently inactivated, thus leading to hepatic steatosis and insulin resistance. Nuclear reactivation of Foxa2 through a phosphorylation-deficient Foxa2 (Foxa2T156A) in obese animal models leads to reduced hepatic fat, improved hepatic insulin sensitivity, and reduced hepatic glucose production (29).

MATERIALS AND METHODS

Animal Models—All animal models were maintained on a 12-h light/dark cycle in a pathogen-free animal facility. All animal models were inbred to produce apoM null mice. Heterozygous mice were inbred to produce apoM null mice. The apoM gene was deleted in ES (Sv129) cells by homologous recombination using a targeting vector in which all coding exons of apoM were deleted and linked to a pgk-neomycin/thymidine kinase cassette (Fig. 5A). ES cell clones were screened for homologous recombination by Southern blotting using the 5′-probe following Agel restriction digests. An ES cell clone carrying the targeted allele was injected into blastocysts and used to generate chimeric male animals that passed the mutant allele to the offspring. apoM heterozygous mice were inbred to produce apoM null (apoM−/−) mice.

Isolation of Primary Mouse Hepatocytes—Primary hepatocytes were isolated from different animal models that had been fasted for 6 h prior to the experiment. Mice were anesthetized with pentobarbital. A catheter (24 gauge) was inserted into the portal vein, and the liver was perfused with a buffer containing 10 mM HEPES (pH 7.4), 143 mM NaCl, 7 mM KCl, and 0.2 mM EDTA at a flow rate of 1 ml/min. Effluent exited via the vena cava inferior. After 10 ml of perfusion, the buffer was switched to a collagenase buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCl, 7 mM KCl, 5 mM CaCl2, and 0.2% collagenase type IV (Sigma). After 6 ml of perfusion, the liver was cut out and minced in Dulbecco’s modified Eagle’s medium (with 10% fetal bovine serum) containing 4.5 g/liter glucose. Hepatocytes were released from the liver during 15 min of light shaking at 37 °C. The cell suspension was filtered through a nylon mesh (100 μm) and centrifuged at 80 × g to pellet the hepatocytes. The cell pellet was washed three times with warm media, and the cells were plated onto collagenized plates at a density of 10⁶ cells/cm². Cell viability was measured using trypan blue staining and was >90% in all experiments. Cells were allowed to attach for 3 h after which the medium was changed to serum-free Dulbecco’s modified Eagle’s medium. Cells were serum-starved overnight, and insulin was added for a total duration of 6 h at the indicated concentration.

Isolation of Primary Human Hepatocytes—Primary human hepatocytes were obtained from ZenBio. The cells used in our experiments were obtained from a male, 26-year-old, Hispanic donor, who exhibited no signs of alcohol abuse or liver disease. Cells were tested and found to be free of the following pathogens: human immunodeficiency virus I/II, human T-cell lymphotropic virus I/II, HBsAG, HBcAB, hepatitis C virus, rapid plasma regain, and cytomegalovirus. Cells were obtained from the liver after 11 h of cold ischemia by collagenase digestion. Cells were plated at a density of 10⁸ cells/cm² on collagenized plates.

Adenoviruses—The generation of recombinant adenoviruses (Ad-T156A and Ad-GFP) was described previously (13). For in vivo experiments, mice were injected through the tail vein with 1 × 10⁹ plaque-forming units of adenovirus. Empty virus expressing only GFP served as control (Ad-GFP).

Transfection and Transactivation Assays—Transfection of HepG2 cells and luciferase transactivation assays were carried out as described previously (13, 14).

Nuclear and Cytoplasmic Extracts—Primary hepatocytes were plated at 2 × 10⁶ cells/10-cm dish for 24 h prior to the experiment and serum-starved overnight followed by incubation with 0, 0.2, 1, or 20 ng/ml human recombinant insulin (Sigma) for 5 h. Cells were washed twice with cold phosphate-buffered saline, scraped, pelleted, and resuspended in hypotonic lysis buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl) containing 1 mM dithiothreitol and protease inhibitors (Complete mixture, Roche Applied Science). Lysates were incubated on ice for 15 min, and plasma membrane disruption was confirmed by trypan blue staining. Nonidet P-40 (10%) was added to a final concentration of 0.6%, and lysates were vortexed immediately for 10 s and centrifuged at 10,500 × g for 30 s at 4 °C. The supernatants (cytoplasmic extracts) were collected, and the nuclear pellets were resuspended in nuclear lysis buffer (10 mM HEPES (pH 7.9), 100 mM KCl, 3 mM MgCl2, 0.1 mM EDTA) containing 1 mM dithiothreitol and protease inhibitors. Nuclear extracts were incubated on ice for 30 min followed by the gradual addition of one-tenth volume of 4 M (NH₄)₂SO₄ over 30 min. Extracts were cleared by centrifugation at...
Foxa2 Regulates apoM

16,000 × g for 10 min at 4 °C. Protein concentrations were determined by the BCA assay (Sigma).

Quantification of Cytosolic and Nuclear Foxa2—Western blots were scanned and band intensity quantified by densitometry using Kodak imaging software. Each value was normalized to the corresponding γ-tubulin value. To account for differences in blotting and exposure, each blot contained the same standard, and all values were adjusted to the intensity measured for this standard.

Gene Expression—Total RNA was extracted from tissues by TRIzol reagents following the manufacturer’s instructions (Invitrogen). Contaminating genomic DNA was removed by using DNase I treatment (20 units/10 µl of total RNA). cDNA was synthesized by using Moloney murine leukemia virus reverse transcriptase with dNTPs and random hexamer primers (Stratagene). The cDNAs provided templates for PCRs with specific primers in the presence of dNTPs, [α-32P]dCTP, and TaqDNA polymerase. PCR was typically performed with 20–25 cycles, and amplifications were shown to be in the linear range for all primer pairs. Hypoxanthine-guanine phosphoribosyl-transferase expression was determined as a loading control. PCR products were separated by PAGE, and bands were visualized by autoradiography. Densitometry was performed using Kodak imaging software. The primer sequences used are available on request.

Chromatin Immunoprecipitation (ChIP)—ChIP analysis was carried out using primary hepatocytes from different mouse models and the ChIP assay kit (Cell Signaling) according to the manufacturer’s protocol. All proteins/chromatin complexes were precipitated using a Foxa2-specific antibody. The putative Foxa2 binding site in the apoM promoter was amplified using primers ApoMFoxa2ChIP-F (5′-CCATGCCCCAGACTGGCT-3′) and ApoMFoxa2ChIP-R (5′-CAGTGGCAGATTCTGGAAT-3′).

Separation of Lipoproteins by FPLC—Lipoproteins from plasma (10–100 µl) were separated by FPLC using two Superose-6 FPLC columns in series (HR10/30) in 0.15 M NaCl, 0.01 M Na2HPO4, 0.1 mM EDTA (pH 7.5) at 0.5 ml/min. Columns were calibrated using high and low molecular weight standards (GE Healthcare).

Detection of Lipoproteins by Native Gel Electrophoresis—Lipoproteins were separated by agarose gel electrophoresis (1% agarose in 10 mM Tris (pH 8.6)). The agarose gel was blotted by capillary transfer onto a nitrocellulose membrane in ddH2O, Na2HPO4, 0.1 mM EDTA (pH 7.5) at 0.5 ml/min. Columns were calibrated using high and low molecular weight standards (GE Healthcare).

Immunoblotting—Whole cell protein extracts from livers were separated by SDS-PAGE (11.5%) and transferred onto a nitrocellulose membrane (Schleicher & Schuell) by electroblotting. ApoM was detected with anti-ApoM antiserum (22) (1:1000); all other apolipoproteins were detected using affinitypurified antibodies (Applied Bioscience) (1:1000). Membranes were incubated with primary antibodies overnight at 4 °C. Incubations containing the secondary antibody were performed at room temperature for 1 h.

Laboratory Measurements—Blood samples were taken from mice using heparinized capillary tubes. An aliquot of each sample was treated with 1.5 mM 5,5′-dithiobis(2-nitrobenzoic acid) to inhibit lecithin-cholesterol acyltransferase activity. Plasma cholesterol, triglycerides, and phospholipids levels were determined using a colorimetric assay system (Roche Applied Science and Wako).

Statistical Analysis—Results are given as mean ± S.D. Statistical analyses were performed by using a Student’s t test, and the null hypothesis was rejected at the 0.05 level.

RESULTS

Plasma apoM Levels Are Decreased in Hyperinsulinemic Mice—It has been reported that mice with genetic mutations in leptin signaling (ob/ob and db/db) exhibit decreased circulating apoM levels (19). To test the hypothesis that reduced apoM levels are a common feature of hyperinsulinemic animals, we compared apolipoprotein levels in wild-type, ob/ob, db/db, and high fat diet-induced obese C57Bl/6 animals (HF) by measuring hepatic mRNA and protein expression as well as plasma protein levels. As seen in Fig. 1A, we measured an ~4-fold decrease in apoM mRNA in livers of obese mice compared with lean wild-type littermates, whereas no significant difference was detected in the expression of apoAI, -AII, and -CIII. A similar decrease in apoM protein levels was measured in the plasma of hyperinsulinemic mouse models compared with lean controls (Fig. 1B). Since apoM is required for the formation of pre-β-HDL (31), we tested whether pre-β-HDL levels were affected in obese/diabetic mice by analyzing the HDL particle composition by native agarose gel electrophoresis and Western blotting. Interestingly, plasma levels of pre-β-HDL migrating particles were lower in obese mice compared with their wild-type littermates (Fig. 1C). To test whether the observed effect on apoM expression was due to a direct effect of impaired leptin signaling, we analyzed both mRNA and protein expression of apolipoproteins in primary hepatocytes that were treated with leptin. Leptin treatment had no effect on apoM mRNA expression in primary hepatocytes (Fig. 1D), nor did it affect apoM secretion into the media (Fig. 1E). Similarly, no other apolipoproteins were affected by treatment of the primary hepatocytes with leptin.

Insulin Regulates apoM Expression and pre-β-HDL Formation—To analyze whether the changes in apoM expression could have been due to increased insulin levels, we cultured primary mouse hepatocytes from several animal models for 6 h in medium containing varying amounts of insulin. As seen in Fig. 2A, a dose dependence could be observed for the regulation of apoM levels. At insulin concentrations of 0.8 ng/ml or higher (equivalent to the amount observed in a fed C57Bl/6 mouse) apoM levels were decreased approximately ~1.6-fold in hepatocytes of C57Bl/6 mice. In ob/ob, db/db, and HF-fed mice, a decrease in apoM levels could first be observed at higher concentrations, while the other apolipoprotein levels remained unchanged (Fig. 2B). We have recently shown that Foxa2 is regulated by insulin-stimulated Akt phosphorylation (29). Therefore, we analyzed whether changes in apoM correlated with the subcellular localization of Foxa2 in primary hepatocytes of these mice. As seen in Fig. 2, C and D, Foxa2 localized to the cytosol in response to insulin in a dose-dependent manner. Although in hepatocytes of wild-type mice,
Foxa2 was excluded from the cytosol in response to 0.8 ng/ml insulin, in ob/ob, db/db, and HF diet-induced obese mice, we observed Foxa2 exclusion only at higher insulin levels.

To analyze whether this effect was specifically inherent to hepatocytes of mouse origin, we cultured human primary hepatocytes in the presence or absence of insulin and determined the regulation on apolipoprotein gene expression. Similar to our findings in mouse hepatocytes, insulin led to the down-regulation of apoM mRNA (0.4 ± 0.08 p ≤ 0.01, insulin versus non-insulin-treated) and protein (0.4 ± 0.08 p ≤ 0.01, insulin versus non-insulin-treated) expression without affecting apoAI or apoAII expression (Fig. 2E). Primary human hepatocytes that were treated with insulin also secreted 2.5-fold less apoM (p ≤ 0.01) compared with untreated cells (Fig. 2E).

**Foxa2 Regulates Plasma HDL Levels**—We analyzed the hepatic gene expression profiles in obese mice in which we had expressed constitutive active Foxa2 (Foxa2T156A) with a recombinant adenovirus (Ad-T156A) and compared the expression levels with those in mice infected with a control adenovirus (Ad-GFP) using Affymetrix oligonucleotide arrays (29). Strikingly, we found an ~4-fold increase in mRNA levels of apoM in Ad-T156A compared with Ad-GFP-infected mice. In contrast, no significant difference was measured in the expression of cholesterol biosynthesis genes or apolipoproteins that influence plasma HDL concentration, including apoAI, -II, -IV, and -CIII.

To validate the microarray data, we analyzed the expression of apolipoproteins from mice in the serum and liver of Ad-T156A- and Ad-GFP-infected wild-type, ob/ob, and HF-fed mice by RT-PCR. steadystate mRNA levels of apoM increased to ~5–6-fold in livers of Ad-T156A-infected animals compared with Ad-GFP mice (Fig. 3A, data not shown). In contrast, no changes were observed in mRNA levels of apoAI, apoAII, or apoCIII. Immunoblot analysis of liver homogenates and plasma confirmed the increased apoM levels in Ad-T156A-infected mice (Fig. 3, B and C). Although apoM was up-regulated 1.8–5.3-fold in both the liver and serum of Ad-T156A-infected mice, no changes were observed for apoAI, apoAII, or apoCIII in the liver of virus-infected mice. Interestingly, we measured a significant 1.4-fold increased level of circulating plasma apoAI in Ad-T156A-infected wild-type and ob/ob animals (Fig. 3C).

To test whether overexpression of constitutive active Foxa2 led to changes in plasma lipoprotein composition, we analyzed different hyperinsulinemic mouse models injected with either constitutive-active Foxa2 (Ad-T156A) or control (Ad-GFP). Interestingly, circulating cholesterol levels were increased in all mice infected with Ad-T156A compared with Ad-GFP (ob/ob, 154 ± 20 versus 252 ± 26 mg/dl; HF, 137 ± 6 versus 160 ± 8 mg/dl; WT, 123 ± 11 versus 173 ± 11 mg/dl), and preβ-HDL levels were up-regulated in Ad-T156A animals compared with Ad-GFP-treated animals (ob/ob, 2.9 ± 0.3 mg/dl, p < 0.01; HF, 3.2 ± 0.2 mg/dl, p <
Foxa2 Regulates apoM

A.

B.

C.

D.

E.

FIGURE 2. Insulin inhibits the expression of apoM in murine and human hepatocytes. A and B, hepatic apoM (A) and apoAl (B) expression in primary hepatocytes of wild-type C57Bl/6, ob/ob, db/db, and HF diet-induced obese mice that were incubated for 6 h in varying concentrations of insulin by immunoblotting. γ-Tubulin was used as a loading control. C and D, nuclear Foxa2 (C) and cytosolic Foxa2 (D) content in primary hepatocytes of wild-type C57Bl/6, ob/ob, db/db, and HF diet-induced obese mice that were incubated for 6 h in the presence varying amounts of insulin, quantified by immunoblotting. γ-Tubulin was used as a loading control. E, gene expression analysis of apolipoproteins in primary human hepatocytes cultures in the presence or absence of insulin (100 nM) by RT-PCR (left panel) and immunoblotting (middle panel). The right panel shows secreted apoAl and apoM levels in the supernatant following 6 h of incubation in the presence or absence of insulin (100 nM). Gapdh, glyceraldehyde-3-phosphate dehydrogenase.

0.01) (Fig. 3D). FPLC analysis of the plasma of these animals showed that the increase in cholesterol levels was solely because of an increase in HDL particle numbers, whereas no changes in LDL concentration were observed (Fig. 3E). We also tested the allelic effect of a loss of function mutation in Foxa2 on apolipoprotein expression and plasma pre-β-HDL concentrations. Foxa2−/− mice exhibited a significant (∼3-fold) reduction in hepatic apoM expression and plasma concentration (Fig. 3, F and G). Furthermore, we measured a >50% decrease in plasma pre-β-HDL levels (2.8 ± 0.6, p < 0.01) and α-HDL levels (1.5 ± 0.3, p < 0.05) in comparison with wild-type littermates (Fig. 3H), concomitant with a significant decrease in plasma HDL cholesterol levels (56 ± 10 versus 83 ± 9 mg/dl, p < 0.01, respectively). Insulin levels, as well as levels of circulating free fatty acid and triglyceride, were not changed between Foxa2+/− and littermate controls. Together, these data demonstrate that Foxa2 is a powerful regulator of pre-β-HDL and α-HDL particle formation.

Insulin/Phosphatidylinositol 3-Kinase/Akt-mediated Transcriptional Regulation of apoM by Foxa2—To address the question of whether apoM is a direct target gene of Foxa2, we investigated the apoM promoter for putative Foxa2 binding sites. We identified an evolutionarily conserved site at position −474 (with respect to the translation start site) that exhibits a 96% homology to the “consensus” Foxa2 binding site (Fig. 4A) (32). To analyze the transcriptional regulation of apoM, we generated reporter constructs that contained 841 bp of mouse apoM promoter with either the wild-type Foxa2 binding site (GATT-GTTTGCA) or a mutated Foxa2 site (TATTGAAGCA). As seen in Fig. 4B, Foxa2 transactivated the apoM promoter ∼10-fold in HepG2 cells. This activation was specific for Foxa2 and was not observed with the related Forkhead transcription factor Foxo1, in which activity is also known to be regulated by insulin signaling (33). Mutations in the conserved Foxa2 binding site of the apoM reporter plasmid (mut.apoM-luc) completely abolished transactivation of the apoM promoter by Foxa2 (Fig. 4B). Because Akt/Pkb regulates the activity of Foxa2, we co-transfected the apoM reporter plasmid with increasing amounts of Akt expression vector (pAkt) and Foxa2 (pFoxa2). We observed a dose-dependent inactivation of Foxa2 by Akt, resulting in decreased transactivation of the apoM promoter in vitro. This effect was not seen in co-transfections with pFoxa1 or pFoxa2T156A (Fig. 4C) (34).

To test whether Foxa2 also occupies the apoM promoter in vivo, we performed a ChIP analysis using livers from insulin-resistant/obese as well as fasted and fed wild-type control mice. Chromatin was precipitated using a specific Foxa2 antibody (35). The interaction of Foxa2 with the apoM promoter was demonstrated by PCR using primers that specifically amplify the Foxa2 binding site in the apoM promoter. Fig. 4D shows that Foxa2 binds to the apoM promoter in livers of ob/ob mice that had been infected with Ad-T156A
**FIGURE 3.** Foxa2 regulates apoM expression in different insulin-resistant mouse models. A, RNA expression of apoAI, apoAII, apoCIII, and apoM was measured using RT-PCR in livers of wild-type C57Bl/6, ob/ob, and HF-fed mice that had been injected with either Ad-GFP or Ad-T156A. B, whole cell liver lysates were prepared from wild-type C57Bl/6, ob/ob, and HF fed-mice that had been injected with either Ad-GFP or Ad-T156A. C, plasma was prepared from wild-type C57Bl/6, ob/ob, and HF-fed mice that had been injected with either Ad-GFP or Ad-T156A. D, native gel electrophoresis of plasma from wild-type C57Bl/6 and obese animals that had been injected with either Ad-GFP or Ad-T156A and immunblotted using anti-apoM antibodies. E, gel permeation of the major lipoprotein fractions from plasma of C57Bl/6, ob/ob, or HF-fed mice infected with either Ad-GFP (black) or Ad-T156A (red). Adenovirus-injected mice were studied 14 days after infection. Plasma samples (100 μl) were applied to a FPLC gel filtration column. Fractions of the eluate were assayed for total cholesterol. F and G, gene expression analysis of apolipoproteins from liver extracts of Foxa2+/− and wild-type littermates (Foxa2+/+) by RT-PCR (F) and Western blotting (G). H, native gel electrophoresis of plasma from Foxa2+/− mice and wild-type littermates following immunoblotting using anti-apoM antibodies. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.005. Gapdh, glyceraldehyde-3-phosphate dehydrogenase.
Foxa2 mediates the change in plasma lipoprotein composition are mediated by apoM—To study the effect of Foxa2 activation on plasma HDL levels and lipoprotein composition in the absence of apoM expression, we generated apoM-deficient mice. ApoM-null mice (apoM<sup>—/—</sup>) were generated by targeted deletion of all apoM coding exons, homologous recombination in ES cells, and ES cell injections into blastocyst (for details see Fig. 5A and “Materials and Methods”). No apoM mRNA or protein could be detected in apoM<sup>—/—</sup> mice by reverse transcription-PCR analysis and immunoblotting, respectively, and the plasma levels of other apolipoproteins were unaffected in apoM<sup>—/—</sup> mice compared with wild-type littermates (Fig. 5, B and C, data not shown). Gel permeation analysis (FPLC) of the plasma lipoprotein profile showed that α-HDL levels in apoM<sup>—/—</sup> mice were decreased and that a larger size HDL subfraction had been generated (Fig. 5D). One-dimensional native gel electrophoresis of circulating lipoprotein particles from apoM<sup>—/—</sup> and control litters revealed that the α-HDL content was drastically reduced in apoM<sup>—/—</sup> mice, and no pre-β-HDL expression could be detected (Fig. 5E). We also investigated whether the increase in plasma HDL levels by constitutive active Foxa2T156A in livers of apoM<sup>—/—</sup> mice did not change the plasma HDL cholesterol levels (53 versus 55 mg/dl in Ad-Foxa2T156A versus Ad-GFP, n = 5), lipoprotein profiles, or plasma apolipoprotein composition compared with Ad-GFP-injected mice (Fig. 5, F–H). These data demonstrate that Foxa2T156A-induced increase in plasma HDL was mediated through the action of apoM, most likely through increased formation of pre-β-HDL.

**DISCUSSION**

The combination of insulin resistance and compensatory hyperinsulinemia in many cases leads to the development of
glucose intolerance, high plasma triglyceride levels, and a low concentration of plasma HDL (20, 21, 23–25). Numerous studies have shown that individuals displaying this cluster of abnormalities associated with insulin resistance/compensatory hyperinsulinemia have a significantly increased risk of cardiovascular disease (2, 24). Several studies have attempted to address the functional relationship between decreased plasma HDL cholesterol levels and hyperinsulinemia/insulin resistance; however, no conclusive mechanism for this abnormal regulation exists thus far. We and others have shown previously that apoM is an important regulator of plasma HDL levels and pre-β-HDL formation (29, 51). We showed that mice deficient in the transcription factor Tcf1 lack apoM and pre-β-HDL, accumulate cholesterol in large HDL particles (HDL1), and exhibit impaired conversion of HDL to pre-β-HDL.

In this study we have demonstrated that apoM-deficient mice exhibit a similar phenotype regarding the plasma lipoprotein profile to that of Tcf1−/− mice (31, 36), thereby supporting our previous study showing that apoM expression is required for normal HDL metabolism. We also show in this study that expression of apoM is regulated by insulin at the transcriptional level through the insulin-sensitive transcription factor Foxa2. ApoM is an important factor for the generation of HDL precursors, which are the major acceptors of extrahepatic cholesterol to generate HDL for reverse transport to the liver (31). It was previously shown by Xu et al. (19) that apoM expression is decreased in ob/ob and db/db animals in which leptin signaling is impaired. Leptin treatment of those mice restored apoM expression to levels similar to those in wild-type animals, which led Xu et al. (19) to conclude that apoM is a direct target of leptin. Those data, however, conflict with results from the same group showing that apoM expression in HepG2 cells is decreased.

**FIGURE 5. Generation and characterization of apoM null mice.** A, strategy for targeted deletion of the murine apoM gene in ES cells. The genomic structure and restriction map of the apoM locus and targeting vector pPNT-2 used to delete apoM are shown. The targeting vector was constructed by deleting all exons of apoM. A 5′-probe of the left targeting arm was used for Southern blot analysis and is shown as a bar. TK, thymidine kinase. B, Southern blot analysis of transfected ES cells and apoM+/+ ES cells grown in the presence of gentamycin. Genomic DNA was digested with AgeI and hybridized with the 5′-probe. The wild-type allele shows a 12-kb band, and the targeted allele shows a 7.2-kb fragment. C, immunoblot analysis of apolipoproteins apoAI, apoAII, apoCIII, and apoM in plasma of apoM+/+ and wild-type littermate (apoM+/−) mice. *, p ≤ 0.05. D, separation of lipoprotein particles from plasma of apoM+/+ (red) and wild-type littermate (black) mice by FPLC analysis. E, one-dimensional native gel electrophoresis of plasma from apoM−/− and wild-type littermate mice followed by immunoblotting with anti-apoAI antibodies. The α-HDL and pre-β-HDL-migrating particles are indicated. F, FPLC analysis of lipoprotein particles from plasma of apoM−/− mice that were injected with recombinant adenovirus expression Foxa2T156A (red) or control adenovirus Ad-GFP (black). G, gene expression analysis of apolipoproteins in livers of apoM−/− mice infected with Ad-T156A or Ad-GFP by RT-PCR analysis. Expression was measured 7 days after adenoviral injection. Gapdh, glyceraldehyde-3-phosphate dehydrogenase. H, Western blot analysis of plasma apolipoproteins from apoM−/− mice infected with Ad-T156A or Ad-GFP.
Foxa2 Regulates apoM

upon leptin treatment (37). Furthermore, recent other data indicate that insulin can regulate apoM expression in vitro (36), suggesting that the effects of leptin on apoM expression are mediated by insulin. In this study we have provided evidence that the action of leptin is likely indirect and that apoM is a target of Foxa2, the activity of which is regulated by insulin/phosphatidylinositol 3-kinase/Akt signaling both in murine and in human hepatocytes. Chronic hyperinsulinemia in insulin-resistant states results in the permanent inactivation of Foxa2 and impaired activation of apoM transcription. Leptin treatment of ob/ob mice rapidly reduces plasma glucose and insulin levels, which could result in a relocation of Foxa2 to the nucleus and activation of apoM gene expression (29, 38).

In addition to the increase in plasma apoM levels, we found increased plasma apoAI levels in mice that had been injected with Ad-T156A. However, when measuring mRNA levels of different lipoproteins, we did not observe increased expression of apoAI in the liver of Ad-T156A-treated mice. This result confirms our recent findings demonstrating that apoM overexpression leads to an increase in plasma HDL particle numbers concomitant with an increase in circulating apoAI (31). The exact mechanism for this increase in apoAI expression is unclear; however, it can be predicted that an increase in HDL particle biogenesis triggers increased translation/secretion of apoAI, which is a major constituent of HDL. Interestingly, a recent study in humans has shown a clear correlation of plasma apoM levels with total plasma cholesterol levels as well as with LDL or HDL cholesterol (39). Furthermore, that study did not find a statistically significant correlation between apoM and body mass index (39), a well-established quantitative trait that is correlated with leptin. Therefore, our current study confirms the importance of apoM expression on HDL and total plasma cholesterol levels in humans. Future studies are warranted to address the relationship of plasma insulin levels and the effects of insulin sensitivity on plasma apoM concentrations in humans.

HDL metabolism is substantially altered in dyslipidemic states. However, the mechanism that results in low HDL dyslipidemias, which are associated with metabolic diseases such as type 2 diabetes and obesity, has proven elusive thus far. It is generally believed that the small HDL particles that result from the intravascular lipolysis of triglyceride-enriched HDL are cleared more rapidly from the circulation and that lipolysis of triglyceride-enriched HDL lowers HDL particle numbers by causing apoAI to be shed from HDL particles and cleared from the circulation. In atherogenic dyslipidemias of the metabolic syndrome and type 2 diabetes, circulating levels of large, cholesterol-rich HDL decrease (40 – 42). Furthermore, the levels of small, dense, cholesterol-poor HDL particles and their ApoAI content are either unaffected or slightly reduced (43 – 45). In contrast, familial low HDL dyslipidemia appears to be the only phenotype that is characterized by the presence of reduced concentrations of small pre-β-HDL particles (46). In obesity and insulin resistance, plasma levels of large HDL particles decrease in parallel with those of mature HDL, whereas levels of pre-β-HDL are either unchanged or elevated (47 – 49). These findings are at odds with those observed for animal models of obesity and type 2 diabetes, where little or no decrease in mature HDL is observed, and pre-β-HDL levels are reduced. This could be because of either intrinsic differences or other pathways that are deregulated in obesity and type 2 diabetes. One possible mechanism that could reconcile decreased hepatic apoM expression with increased levels of pre-β-HDL in human obesity/hyperinsulinemia is by a reduced conversion of pre-β-HDL to α-HDL. This is supported by data published recently by us and others showing that apoM-containing particles are better acceptors of peripheral cholesterol in efflux studies compared with apoM-poor or -deficient lipoproteins (31, 50). Taken together, these data warrant taking a close look at the composition of pre-β-HDL particles in obese and type 2 diabetic subjects to elucidate whether altered ApoM expression affects the formation of mature α-HDL. Under these circumstances Foxa2, by being an important regulator of apoM expression, might be a promising therapeutic target to combat atherogenic low HDL levels associated with obesity and type 2 diabetes.

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