Circadian Regulation of Intestinal Lipid Absorption by Apolipoprotein AIV Involves Forkhead Transcription Factors A2 and O1 and Microsomal Triglyceride Transfer Protein*

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Background: The role of apoAIV in the diurnal regulation of plasma lipids is unknown.
Results: Plasma lipids, lipid absorption, and MTP, FoxO1, and FoxA2 levels are lower at night and at mealtime in apoAIV−/− mice.
Conclusion: ApoAIV increases intestinal lipid absorption by modulating the expression of MTP involving FoxA2 and FoxO1.
Significance: Inhibition of intestinal apoAIV expression might reduce plasma lipids.

We have shown previously that Clock, microsomal triglyceride transfer protein (MTP), and nocturnin are involved in the circadian regulation of intestinal lipid absorption. Here, we clarified the role of apolipoprotein AIV (apoAIV) in the diurnal regulation of plasma lipids and intestinal lipid absorption in mice. Plasma triglyceride in apoAIV−/− mice showed diurnal variations similar to apoAIV+/+ mice; however, the increases in plasma triglyceride at night were significantly lower in these mice. ApoAIV−/− mice absorbed fewer lipids at night and showed blunted response to daytime feeding. To explain reasons for these lower responses, we measured MTP expression; intestinal MTP was low at night, and its induction after food entrainment was less in apoAIV−/− mice. Conversely, apoAIV overexpression increased MTP mRNA in hepatoma cells, indicating transcriptional regulation. Mechanistic studies revealed that sequences between −204/−775 bp in the MTP promoter respond to apoAIV and that apoAIV enhances expression of FoxA2 and FoxO1 transcription factors and their binding to the identified cis elements in the MTP promoter at night. Knockdown of FoxA2 and FoxO1 abolished apoAIV-mediated MTP induction. Similarly, knockdown of apoAIV in differentiated Caco-2 cells reduced MTP, FoxA2, and FoxO1 mRNA levels, cellular MTP activity, and media apoB. Moreover, FoxA2 and FoxO1 expression showed diurnal variations, and their expression was significantly lower in apoAIV−/− mice. These data indicate that apoAIV modulates diurnal changes in lipid absorption by regulating forkhead transcription factors and MTP and that inhibition of apoAIV expression might reduce plasma lipids.

Light entrains several biological and behavioral activities by regulating a set of clock genes that are widely expressed in different tissues (1–4). Disruptions in the normal light/dark cycle as they occur in passengers on transcontinental flights and in shift workers are associated with metabolic disorders such as hormonal imbalance, sleep disorders, cancer susceptibility, obesity, and metabolic syndrome (4, 5). The most predominant behavioral effects of the light/dark cycle are wakefulness and sleep. Wakefulness is associated with eating to assimilate energy from the environment for the sustenance of biological activities. Lipids provide the highest energy per gram. As lipids do not mix with water, they are transported on special lipid-protein emulsions called lipoproteins such as chylomicrons and very low density lipoproteins. The intestine synthesizes chylomicrons to transport dietary fat into the body (6). Lipoprotein assembly and secretion is critically dependent on apolipoprotein B (apoB), a structural protein, and microsomal triglyceride transfer protein (MTP), a chaperone protein (7–11). We have shown that plasma lipids and MTP expression exhibit in circadian changes (12) and that the circadian regulation of MTP and plasma lipids is abolished in Clock mutant (ClockΔ19/Δ19) mice (13, 14). Molecular studies have revealed that Clock regulates small heterodimer partner (SHP) protein to modulate MTP expression (14). This regulatory process suppresses MTP expression and lowers plasma triglycerides at the onset of light in mice. Therefore, ClockΔ19/Δ19 and SHP knockout mice express more MTP and absorb more lipids at all times (14). Besides regulating SHP and MTP, Clock also regulates the expression of nocturnin, a deadenylase that degrades the poly(A) tail of target mRNA. Nocturnin-deficient mice are resistant to diet-induced obesity and steatosis. Mechanistic studies showed that nocturnin-deficient mice absorb fewer lipids (15). Hence, it appears that diurnal regulation of intestinal lipid absorption involves several mechanisms.

Another protein that is likely to contribute to the diurnal regulation of lipid absorption is apoAIV, a 46-kDa protein associated with regulation of lipid metabolism. ApoAIV increases intestinal lipid absorption by modulating the expression of MTP involving FoxA2 and FoxO1.
Regulation of Plasma Lipids and Intestinal MTP by ApoAIV

Cells, Animals, and Diets—Huh-7 cells, Caco-2 cells, and primary enterocytes were cultured as described previously (12–14, 40–42). Breeding pairs of apoAIV−/− mice (34) on Sv129 background were kindly provided by Dr. Jan Breslow of the Rockefeller University. They were back-crossed to C57Bl/6J more than 10 times at SUNY Downstate Medical Center and kept on a 07:00/19:00-h lighting schedule with unlimited access to water and standard laboratory chow. In this study, we used 8–10-week-old female mice. There was no significant difference in daily food intake measured on three consecutive days between apoAIV−/− and apoAIV+/+ mice. For daytime feeding experiments, mice had access to food only from 9:30 a.m. to 3:30 p.m. for 10 days, and then they were used to measure plasma lipids and MTP at the indicated times. All procedures were reviewed and approved by the Animal Care and Use Committees of SUNY Downstate Medical Center, conforming to the accepted standards of humane animal care.

Plasma Lipid Measurements—Total plasma triglyceride and cholesterol levels were measured using kits (Thermo Fisher Scientific). HDL lipid levels were measured after precipitating apoB lipoproteins. Lipids in apoB lipoproteins were determined by subtracting HDL lipids from total lipids. Human apoB and apoA1 secreted from cells were measured by enzyme-linked immunosorbent assay (43, 44).

In Vivo Absorption of Lipids—Mice were injected intraperitoneally with 0.5 ml of Poloxamer P407 in PBS (1:6, v/v) and then gavaged with 50 μl of olive oil containing 1 μCi of [3H]triolein at 12:00 a.m. or 12:00 p.m. Blood was collected from the tail, and plasma was used for liquid scintillation counting (12–14).

Determination of MTP Activity in the Intestine and Liver—Small pieces (100 mg) of liver and 1-cm segments of proximal small intestine were collected at different times of the day, homogenized in 1 ml of ice-cold 1 mM Tris-HCl (pH 7.6), 1 mM EGTA, and 1 mM MgCl2 in a glass homogenizer, and centrifuged (SW55 Ti rotor, 50,000 rpm, 10 °C, 1 h); supernatants were used for the MTP assay (45, 46) using a kit (Chylos, Inc.).

Transfection with Plasmid DNA or siRNA—Plasmids pMTP−204, pMTP−775, and pMTP−1181 expressing luciferase under different MTP promoter sequences have been described (47). Huh-7 cells were transfected with these plasmids and a Renilla luciferase construct with or without plasmids expressing apoAIV. Cells were assayed using the Dual-Luciferase reporter assay system (14).

siRNA directed against FoxO1 (sc-35382, FKHRsiRNA) and FoxA2 (sc-35569, HNF-3β) mRNA and nonspecific control siRNA were obtained from Santa Cruz. All siRNAs were introduced into Huh-7 cells plated in 12-well plates using siRNA transfection reagent (sc-29528). After 72 h, cells were harvested for RNA or protein analysis (12–14). The expression plasmids for rat apoAIV and human apoA1 have been described (48).
Caco-2 cells were plated in Transwells and allowed to differentiate as described previously (42, 47, 49). After 14 days, cells were transfected with a mixture (1/9262 g/ml) of three lentiviral vector plasmids (control shRNA plasmids, sc-108060, or apoAIV shRNA plasmids, sc-41178-SH, Santa Cruz Biotechnology). After 48 h, cells were used for different experiments.

Uptake and Secretion of Lipids by Enterocytes and Huh-7 and Caco-2 Cells—To study uptake, enterocytes were isolated (41, 50) from the duodenum and jejunum of apoAIV/H11001/H11001 and apoAIV/H11002/H11002 mice at 8:00 p.m. and were incubated in triplicate with [3H]oleic acid. At different times, enterocytes were centrifuged, washed, and counted. To measure secretion of lipids, enterocytes were incubated in triplicate with [3H]oleic acid for 1 h, centrifuged, washed, and then incubated in fresh media for different times. Media were collected and counted. In some experiments, media were subjected to ultracentrifugation to separate chylomicrons and HDL as described previously (41, 42, 51).

For secretion studies, hepatoma cells transfected with or without apoAIV were labeled with 5 μCi/ml [3H]glycerol and incubated with or without 50% serum for 2 h (14). Total lipids in cells and medium were extracted with chloroform-methanol.

FIGURE 1. Diurnal rhythms of total plasma triglyceride and cholesterol in apoAIV−/− mice. A, intestinal samples were collected at 4-h intervals over a period of 24 h from ad libitum fed female wild-type mice at the indicated times to measure changes in apoAIV mRNA levels. Values were normalized to GAPDH mRNA levels. n = 6/time point. The bars above the x axis represent when the lights were off (black) and on (white). B–K, plasma and tissues were collected from female apoAIV−/− and apoAIV+/+ mice kept in a 12-h light/dark cycle with free access to chow and water. Triglycerides were measured in total plasma (B), non-HDL apoB-containing lipoproteins (C), intestine (D), and liver (E). Intestinal MTP activity (F), protein (G and H), and mRNA (I) levels were measured. In addition, intestinal GAPDH (J) and hepatic MTP mRNA (K) levels were quantified. Line graphs and error bars represent average ± S.D.; n = 6 for each time point; *, p < 0.05; and **, p < 0.01.
Radiolabeled triglycerides and phospholipids were separated using hexanes/ethyl ether/acetic acid (80:20:2) on a thin layer chromatographic plate (12).

Differentiated Caco-2 cells, 48 h after transduction with different lentiviral plasmids were incubated in serum-free medium containing \([^{3}H]\)glycerol for 12 h. Cells were then incubated in medium containing 50% serum for 2 h. Cells were washed and then incubated in serum-free medium for 6 h. The media and cells were collected for analysis.

**Western Blot Analyses**—Tissues were homogenized in Tris-HCl buffer containing 1% Tween 20. Proteins (20 μg) were resolved on 4–20% gradient gels (Bio-Rad). A mouse monoclonal anti-MTP antibody (BD Transduction Laboratories, 08-772-1B), rabbit anti-GAPDH (ab9485), rabbit anti-apoAI (sc-30089), rabbit anti-apoAIV (sc-50376), and rabbit anti-swine apoAIV were used as primary antibodies to detect endogenous proteins. Anti-mouse or anti-rabbit secondary antibodies were used as described previously (12–14).

**mRNA Quantifications**—Total RNA from tissues and cells were isolated using TRIzol. The purity was assessed by the A260/A280 ratio, and RNAs with ratios more than 1.7 were used for cDNA synthesis. First strand cDNA was synthesized using an Omniscript RT kit (Qiagen) and then used for qPCR using SYBR Green to quantify changes in mRNA. The data were analyzed using the ΔΔC_{T} method and presented as arbitrary units as described previously (14). The primers used were mFoxO1 forward 5′-ACGAACTCGGAGGCTCCTTAG-3′ and mFoxO1 reverse 5′-GACTGGAAGTGTCGAGTTG-GACTG-3′; mFoxA2 forward 5′-GGGTCCCTGCGGTTG-GCTAG-3′ and mFoxA2 reverse 5′-GGTCCCATCTCTCTG CT GG AT TTGC-3′; hFOXO1 forward 5′-AACCTGGCAT-TACAGTTGGCC-3′ and hFOXO1 reverse 5′-AAATGCAG-GAGGCATGACTACGT-3′; and hFOX2A forward 5′-GCC-ATGCACTCGGCTTCCAGTAT-3′ and hFOX2A reverse 5′-CAGGCCCCACGTAGCGACG-3′. Primers for other genes have been described (12–14, 40).

**Chromatin Immunoprecipitation (ChIP)**—ChIP was used to study the binding of different transcription factors to the MTP promoter using goat polyclonal antibodies against FoxO1 (Santa Cruz Biotechnology, sc-35569) and FoxA2 (Millipore, 07-633). DNA samples recovered after immunoprecipitation were subjected to semiquantitative PCR or qPCR to detect coimmunoprecipitated DNA using the MTP promoter-specific primers (forward 5′-GAAAAGTATCACAACTAGGT-3′ and reverse 5′-TGGCTCCCTCTGCCACCATCCAG-3′) that flank the consensus FoxO1 or FoxA2 binding site (−507/+1 nt) in...

**FIGURE 2.** ApoAIV deficiency does not affect diurnal rhythms of intestinal circadian genes. Expression levels of various intestinal clock genes involved in circadian rhythms in apoAIV−/− and apoAIV+/+ mice (n = 6 for each time point) were measured at the indicated times. Mean ± S.D., n = 6 for each time point.
the mouse MTP promoter (52). As negative controls, ChIP was performed in the absence of antibody or in the presence of rabbit IgG (14). These experiments were repeated three to four times with similar results. Data from a representative experiment are provided.

**Statistical Analyses**—Data are presented as the means ± S.D. Unless noted otherwise, n = 5–6 for each group or condition. Statistical significance (p < 0.05) was determined using Student’s t test or one-way analysis of variance (GraphPad Prism).

**RESULTS**

**Effect of ApoAIV Deficiency on Diurnal Variations in Plasma Lipids**—ApoAIV expression shows diurnal variations (13, 38, 39). Consistent with these studies, apoAIV mRNA levels were low in the daytime and high at night in wild-type mice (Fig. 1A). To determine whether diurnal variations in apoAIV levels could influence plasma and tissue triglyceride levels, we compared changes in apoAIV+/+ and apoAIV−/− mice. No significant differences in total body, liver, and intestinal weights were observed between apoAIV+/+ and apoAIV−/− mice (data not shown). Plasma lipid analysis showed that triglyceride levels (Fig. 1B) were significantly lower in apoAIV−/− mice at night compared with apoAIV+/+ mice, mainly because of changes in apoB lipoproteins (Fig. 1C) and not in HDL (not shown). We did not find significant changes in the intestinal and hepatic triglyceride content in these mice (Fig. 1, D and E). We had shown previously that diurnal variations in plasma lipids correlate with MTP expression in the intestine and liver (12, 14). Intestinal MTP activity (Fig. 1F) and protein (Fig. 1, G and H) and mRNA (Fig. 1I) levels were low at night in apoAIV−/− mice. No significant differences were observed in intestinal GAPDH (Fig. 1J) and hepatic MTP mRNA (Fig. 1K) in apoAIV+/+ and apoAIV−/− mice. These studies indicate that apoAIV might play a role in the diurnal control of plasma lipids and intestinal MTP expression at night.

**FIGURE 3.** Effect of daytime feeding on diurnal rhythms of plasma lipids and intestinal MTP in apoAIV−/− mice. ApoAIV+/+ and apoAIV−/− female mice had food available between 9:30 a.m. and 3:30 p.m. for 10 days. Plasma samples were collected at the indicated times to measure plasma triglyceride (A), intestinal MTP activity (C), protein (E and F), and mRNA (G) as well as Gapdh mRNA (I). In addition, areas under the curve (AUC) were integrated for triglyceride (B), MTP activity (D), MTP mRNA (H), and GAPDH mRNA (J) levels. Each point (n = 6) represents the mean ± S.D. *, p < 0.05 compared with the same time point in apoAIV−/− mice.
To determine whether intestinal MTP regulation was related to changes in circadian genes, we measured the mRNA levels in the intestines of apoAIV/+/+ and apoAIV+/−/− mice. ApoAIV deficiency had no effect on the diurnal expression of various genes involved in circadian control (Fig. 2). Therefore, apoAIV is not important for diurnal expression of clock genes but is important for increases in MTP at night.

Role of ApoAIV in Food-entrained Regulation of Plasma Lipids — Food entrainment increased plasma triglyceride (Fig. 3, A and B) during the day in apoAIV/+/+ mice consistent with earlier studies (12). Also, in apoAIV+/− mice we saw a significant increase in plasma lipids at the time of food availability; however, these enhancements were lower compared with apoAIV/+/+ mice. We next studied changes in MTP expression (Fig. 3, C and D), protein (Fig. 3, E and F), and mRNA (Fig. 3, G and H). MTP expression was the highest at the time of food availability in both groups. In apoAIV+/− mice, however, increases in MTP levels were lower than in apoAIV/+/+ mice.

![Temporal differences in the absorption of lipids in apoAIV+/+ and apoAIV+/−/− mice.](image)

A–D, lipid absorption in mice at different times. Non-fasted female apoAIV/+/+ and apoAIV+/−/− mice (n = 6) were injected intraperitoneally with 0.5 ml of Poloxamer 407 in PBS (1:6, v/v) and then gavaged with 50 μl of olive oil containing 1 μCi of [3H]triolein at midday (12:00) (A and B) or midnight (24:00) (C and D). Intestinal segments (A and C) and plasma samples (10 μl) (B and D) were collected to measure radioactivity in the plasma. *, p < 0.05; **, p < 0.01. E, uptake of [3H]oleic acid by isolated enterocytes. Enterocytes were isolated from female apoAIV+/−/− and apoAIV+/+ mice at 12:00 a.m. and incubated in duplicate with [3H]oleic acid at different times. Each point represents the mean ± S.D., n = 6. *, p < 0.05. F, secretion of lipids by enterocytes. Enterocytes were isolated from apoAIV+/−/− and apoAIV+/+ mice at 12:00 a.m., incubated with [3H]oleic acid for 1 h, washed, and incubated in fresh media. At the indicated times, enterocytes were centrifuged, and radioactivity in the media was determined. Each point represents the mean ± S.D., n = 6. *, p < 0.05. G, enterocytes were incubated in triplicate with [3H]oleic acid for 1 h, washed, and incubated in fresh media containing oleic acid and taurocholate as described previously (41, 42). After 2 h, the media were subjected to ultracentrifugation, fractions were collected from the top, and radioactivity was measured (41, 42). Mean ± S.D., n = 6. *, p < 0.05.
suggest that apoAIV contributes to optimum changes in plasma lipids and MTP in response to food entrainment.

**ApoAIV**<sup>−/−</sup> Mice Absorb Fewer Lipids at Night—To explore further how apoAIV regulates lipid absorption at mealtime, we performed [3H]triolein absorption studies in mice injected with P407 (53) to inhibit lipoprotein lipase and lipoprotein clearance at midday or midnight. [3H]Triolein-derived counts at midday in the intestinal segments (Fig. 4A) and plasma (Fig. 4B) were similar in apoAIV<sup>+/+</sup> and apoAIV<sup>−/−</sup> mice. But at midnight, triolein-derived counts were higher in the proximal intestinal segments (Fig. 4C) and lower in the plasma (Fig. 4D) of apoAIV<sup>−/−</sup> mice. These studies indicate that apoAIV<sup>−/−</sup> enterocytes retain more and secrete fewer lipids at night. This could be because of increased uptake or reduced secretion. To elucidate the role of apoAIV in various steps of lipid absorption, we performed [3H]oleic acid uptake and secretion studies in primary enterocytes isolated at 8:00 p.m. Enterocytes took up similar amounts of oleic acid with time (Fig. 4E). Secretion studies showed that apoAIV<sup>−/−</sup> enterocytes secreted less lipids (Fig. 4F). Next, we studied the distribution of secreted lipids in different lipoproteins. The secreted lipids were found mainly in the chylomicron fractions, and these lipids were more abundant in media of enterocytes isolated from apoAIV<sup>+/+</sup> mice (Fig. 4G). These studies indicate that apoAIV does not play a
Regulation of Plasma Lipids and Intestinal MTP by ApoAIV

FIGURE 6. Identification of cis elements and transcription factors required for increases in MTP expression by apoAIV. A, identification of cis elements. Huh-7 cells were transfected in triplicate with plasmids expressing apoAIV or not, along with pMTP–204, −775, and −1183 promoter constructs that express firefly luciferase under the control of different human MTP promoter sequences and pCMV–Renilla luciferase. After 48 h, cells were incubated in serum-free medium for 18 h and for 2 h in medium containing 50% fetal calf serum. Cells were washed and incubated in serum-free medium for an additional 6 h and used to measure specific luciferase activities (light unit/mg/s). For control, values in control (−apoAIV) group were normalized to 1. Mean ± S.D.; *, p < 0.01. B, identification of transcription factors. Cells were transfected in triplicate with apoAIV or not and treated with serum as described in A. At the end of the experiments, the mRNA levels of different known activators and suppressors of MTP gene expression were quantified by qPCR. Data represents the -fold increase in different mRNAs in apoAIV-expressing cells compared with control cells. *, p < 0.05; **, p < 0.01. C, knockdown of FOXO1 and FOXA2 abolishes apoAIV-mediated increases in MTP promoter activity. Huh-7 cells received siControl, siFOXO1, siFOXA2, or siHNF4α in triplicate. After 24 h, cells were transfected with pMTP−1183 promoter construct expressing firefly luciferase and pCMV–Renilla luciferase (as a control vector) along with or without apoAIV. After 48 h, cells were incubated in serum-free medium for 18 h and for 2 h in medium containing 50% fetal calf serum; after 6 h, cells were used to measure luciferase activity. Data were normalized to siControl-treated cells. Mean ± S.D.; *, p < 0.05; **, p < 0.01. D, Huh-7 cells were treated with siFOXO1, siFOXA2, siHNF4α, or siControl along with or without apoAIV-expressing plasmids. After 48 h, cells were incubated in serum-free medium for 18 h and for 2 h in medium containing 50% fetal calf serum. Cells were washed and incubated in serum-free medium for 6 h before being used to measure MTP and GAPDH mRNA. *, p < 0.05; **, p < 0.01.

role in the uptake of fatty acids but is important for their subsequent secretion by enterocytes with lipoproteins. This is consistent with the observations that apoAIV−/− intestinal segments secrete fewer apoB particles (26).

Overexpression of ApoAIV Increases MTP Expression in Huh-7 Cells—The data presented thus far indicated that MTP expression was lower in the intestines of apoAIV−/− mice at night and in daytime after food entrainment. Yao et al. (35) have shown that overexpression of apoAIV increases MTP activity in pig intestinal cell line and that this increase is further enhanced after oleic acid supplementation. Therefore, we studied the effect of apoAIV expression on MTP in human hepatoma Huh-7 cells. Hepatoma cells were used because they do not express apoAIV and, therefore, are expected to provide a significant response independent of endogenous apoAIV levels. ApoAIV, but not apoAI, expression increased MTP protein (Fig. 5A), activity (Fig. 5B), and mRNA (Fig. 5C) without affecting GAPDH (Fig. 5, A and D). Next, we studied the effect of 50% serum in apoAIV-expressing and control cells. Serum enhanced MTP activity (Fig. 5E) and mRNA (Fig. 5F) but not GAPDH mRNA (Fig. 5G) in apoAIV-expressing cells. Serum-supplemented apoAIV-expressing cells secreted more apoB (Fig. 5H) but had no effect on apoAI (Fig. 5I) secretion. To study lipoprotein secretion, cells were labeled with[^3H]glycerol. ApoAIV-expressing cells secreted more newly synthesized triglyceride (Fig. 5J) and phospholipids (Fig. 5K) compared with controls. The triglyceride/phospholipid ratio was higher in apoAIV-expressing cells (Fig. 5L). These studies show that apoAIV overexpression enhances cellular MTP levels and increases secretion of apoB-containing lipoproteins, effects that are further enhanced after serum supplementation.

ApoAIV Modulates MTP Expression Involving FoxA2 and FoxO1—As we observed increases in MTP mRNA in apoAIV-expressing cells, we hypothesized that apoAIV might regulate the expression of MTP at the transcriptional level. Therefore, attempts were made to identify cis elements in the MTP promoter critical for apoAIV effect. To this end, we used constructs that express luciferase under the control of different MTP promoter sequences. Huh-7 cells were co-transfected with different pMTP-Luc constructs that express firefly luciferase under the control of −204, −775, and −1183 bp of the MTP promoter (47, 49), Renilla luciferase (a transfection control), and plasmids expressing either pcDNA (−apoAIV) or apoAIV (Fig. 6A). ApoAIV expression did not change the promoter activity of −204 bp; however, it increased expression of luciferase under the control of pMTP−775 (+196%) and
These studies indicate that apoAIV response element resides between −204 and −775 bp.

We then attempted to identify transcription factors that interact with these cis elements with the understanding that increased expression of activators or decreased expression of repressors in apoAIV-expressing cells could explain increased MTP expression. To test this theory, we measured changes in transcription factors that activate or repress MTP (54) in apoAIV-expressing Huh-7 cells. Again, apoAIV increased MTP mRNA (Fig. 6B). It had no effect on several transcription factors but increased the expression of HNF4α, FOXO1, and FOXA2 (Fig. 6B). These transcription factors are known to up-regulate MTP expression (54). To evaluate further the role of these transcription factors, we reduced their expression using siRNA in Huh-7 cells and then transfected them with pMTP1183-Luc (Fig. 6C). All of the siRNAs reduced MTTP promoter activity, underscoring their participation in gene transcription. Similar experiments were also performed in apoAIV-overexpressing cells. Expression of apoAIV increased MTP promoter activity by 2.5-fold; this increase was not seen in cells treated with siFOXA2 and siFOXO1. Promoter activity was diminished, but not abolished, in siHNF4α-treated cells (Fig. 6C).

Furthermore, we also studied changes in endogenous MTP mRNA levels in cells treated with different siRNAs (Fig. 6D). All three siRNAs reduced MTP mRNA levels in control cells. Increases due to apoAIV expression were not seen in cells expressing siFOXA2 and siFOXO1. Similar to promoter activity studies, siHNF4α diminished, but did not abolish, the increases in MTP mRNA. These studies indicate that FOXA2 and FOXO1 are critical for apoAIV-mediated MTP regulation.

FoxO1 and FoxA2 Show Diurnal Variations, and Their Levels Are Reduced in ApoAIV−/− Mice—Next we evaluated whether apoAIV plays a role in the diurnal regulation of FoxO1 and FoxA2 in mice by measuring changes in the expression of these genes in the intestines of apoAIV−/− and apoAIV+/+ mice. Intestinal FoxO1 (Fig. 7A) and FoxA2 (Fig. 7B) levels were high at night and low in the daytime in wild-type mice, indicating diurnal variations. Increases in the mRNA levels of these transcription factors at peak times were lower in apoAIV−/− mice. In contrast, mRNA levels of Hnf1α, Pparo, and SHP were higher in apoAIV−/− mice, whereas those of Hnf4α were similar to wild-type controls (Fig. 7, D and F). These studies indi-
compared with apoAIV binding of FoxA2 and FoxO1 to the vivo apoAIV binding of these factors to the mRNA levels but had no effect on GAPDH and apoB mRNA levels (Fig. 9A). Further, it reduced MTP activity by 23% (Fig. 9B). To determine whether reductions in MTP mRNA and activity are due to transcriptional repression associated with apoAIV knockdown, we used Caco-2 cells stably transfected with plasmids expressing Renilla luciferase under the control of cytomegalovirus (CMV) or MTP (-1183 bp) promoter described before (47, 49). ApoAIV shRNA significantly reduced luciferase activity expressed under the control of MTP, but not CMV, promoter (Fig. 9C). Next, we studied the effect of apoAIV reduction on apolipoprotein and lipid secretion. ApoAIV shRNA reduced media apoB (Fig. 9D), but not apoA1 (Fig. 9E), protein levels. In addition, apoAIV shRNA reduced lipid secretion (Fig. 9F) while increasing cellular triglyceride (Fig. 9G) and phospholipids (Fig. 9H) compared with controls. These studies indicate that reductions in apoAIV are associated with decreases in MTP expression and secretion of apoB-containing lipoproteins.

**Discussion**

We examined the role of apoAIV in intestinal lipid absorption, plasma lipids, and MTP expression. The apoAIV−/− mice showed modestly reduced plasma triglyceride levels relative to wild-type controls in the dark. The effects of apoAIV deficiency on plasma triglyceride and MTP activity during the dark was able to be mimicked in the light cycle by food entrainment. Further, we provide evidence that apoAIV possibly acts through FoxA2 and FoxO1 to modulate MTP expression during the fed state. FoxA2 and FoxO1 mRNA levels are elevated to a lesser degree during feeding, and their levels are lower at night in ad libitum fed apoAIV−/− mice. These changes in message levels are accompanied by changes in lower MTP promoter

**Food Entrainment of FoxO1 and FoxA2 Requires ApoAIV**

Next, we studied changes in FoxO1 and FoxA2 mRNA levels after food entrainment. FoxO1 (Fig. 8A) and FoxA2 (Fig. 8B) levels were high at the time of food availability in apoAIV+/+ mice. These increases were severely curtailed in apoAIV−/− mice. These data indicate that FoxA2 and FoxO1 mRNA levels respond to food entrainment and that apoAIV contributes to their optimal expression at mealtime.

**ApoAIV Modulates the Binding of FoxO1/FoxA2 to the Mtp Promoter**

We then asked whether apoAIV modulates the binding of these transcription factors to the Mtp promoter in vivo. Chromatin immunoprecipitation studies showed that the binding of FoxA2 and FoxO1 to the Mtp promoter is high at night and less in the daytime in apoAIV+/+ mice (Fig. 8C). The binding of these factors to the Mtp promoter at 12:00 p.m. in apoAIV−/− mice was similar to control animals. However, their binding to the MTP promoter in apoAIV−/− mice was significantly reduced at night (Fig. 8D). These studies indicate that apoAIV expression is required for optimal binding of these transcription factors to the MTP promoter at night.

**Knockdown of ApoAIV Decreases MTP Levels in Differentiated Caco-2 Cells**

The above studies used human hepatoma cells to elucidate the mechanisms regulating MTP by apoAIV. Because apoAIV is expressed mainly in the intestine, we also looked at the effects of apoAIV knockdown in differentiated human colon carcinoma, Caco-2 cells that have been extensively used as a model for intestinal lipid absorption (55–57). ApoAIV shRNA reduced apoAIV, MTP, FOXO1, and FOXA2 mRNA levels but had no effect on GAPDH and apoB mRNA levels (Fig. 9A). Further, it reduced MTP activity by 23% (Fig. 9B). To determine whether reductions in MTP mRNA and activity are due to transcriptional repression associated with apoAIV knockdown, we used Caco-2 cells stably transfected with plasmids expressing Renilla luciferase under the control of cytomegalovirus (CMV) or MTP (-1183 bp) promoter described before (47, 49). ApoAIV shRNA significantly reduced luciferase activity expressed under the control of MTP, but not CMV, promoter (Fig. 9C). Next, we studied the effect of apoAIV reduction on apolipoprotein and lipid secretion. ApoAIV shRNA reduced media apoB (Fig. 9D), but not apoA1 (Fig. 9E), protein levels. In addition, apoAIV shRNA reduced lipid secretion (Fig. 9F) while increasing cellular triglyceride (Fig. 9G) and phospholipids (Fig. 9H) compared with controls. These studies indicate that reductions in apoAIV are associated with decreases in MTP expression and secretion of apoB-containing lipoproteins.

**Discussion**

We examined the role of apoAIV in intestinal lipid absorption, plasma lipids, and MTP expression. The apoAIV−/− mice showed modestly reduced plasma triglyceride levels relative to wild-type controls in the dark. The effects of apoAIV deficiency on plasma triglyceride and MTP activity during the dark was able to be mimicked in the light cycle by food entrainment. Further, we provide evidence that apoAIV possibly acts through FoxA2 and FoxO1 to modulate MTP expression during the fed state. FoxA2 and FoxO1 mRNA levels are elevated to a lesser degree during feeding, and their levels are lower at night in ad libitum fed apoAIV−/− mice. These changes in message levels are accompanied by changes in lower MTP promoter

**ApoAIV deficiency reduces FoxO1 and FoxA2 expression after food entrainment and the binding of these transcription factors to the Mttp promoter.** A) and B) female apoAIV+/+ and apoAIV−/− mice were fed normal chow between 9:30 a.m. and 3:30 p.m. for 10 days, and then intestines were collected at the indicated times to measure FoxO1 and FoxA2 mRNA and plotted after normalizing with 18S rRNA. Mean ± S.D., n = 5–6, p < 0.05, **, p < 0.01, compared with apoAIV+/+ mice at the same time point. C) binding of FoxA2 or FoxO1 to the mouse MTP promoter in apoAIV+/+ and apoAIV−/− mice was studied by ChIP assay. Intestines collected at different times were immunoprecipitated using anti-FoxO1 and -FoxA2 antibodies and used to amplify MTP promoter sequences as described (58). A representative electrophoresis image of the semiquantitative PCR products of FoxO1 and FoxA2 binding (anti-FoxO1 and -FoxA2) and input is shown. This gel is a representative of n = 3. D, MTP promoter sequences in the immunoprecipitates from C were also quantified by qPCR. Mean ± S.D., n = 3, *, p < 0.05.
occupancy in the knock-out mice. Further, siRNA knockdown of these transcription factors blunts the activation of MTP by apoAIV overexpression in cultured cells. Hence, we suggest that apoAIV modulates diurnal as well as food-entrained regulation of intestinal lipid absorption via an effect on MTP through forkhead transcription factors A2 and O1.

These studies provide evidence that apoAIV contributes to circadian and food-entrained regulation of plasma triglyceride and intestinal lipid absorption. ApoAIV does not affect the cellular uptake of lipids, but it increases lipid secretion by enterocytes (Fig. 4). Mechanistic studies showed that apoAIV contributes to optimum lipid absorption at mealtime by enhancing the expression of FoxO1 and FoxA2 transcription factors as well as MTP. Peaks in the expression of FoxO1 and FoxA2 (Fig. 8) occurred before the maximum expression of MTP (Fig. 3) in mice fed during the day. Further, these transcription factors bind to the MTP promoter and enhance its expression. Therefore, we propose the following sequence of events to explain the role of apoAIV in intestinal lipid absorption. Temporal increases in apoAIV expression at night or at mealtime lead to augmentations in the expression of FoxO1 and FoxA2, enhanced binding of these transcription factors to the Mtp promoter, and elevated MTP expression. Higher amounts of MTP in congruence with augmentations in apoAIV after a fatty meal might help optimize packaging and secretion of lipids by enterocytes, contributing to increases in plasma triglyceride.

Recently, it was shown that apoAIV increases MTP expression in the intestinal epithelial cells; these increases were further augmented when cells were provided with oleic acid (35). Using several approaches, we identified that FOXA2 and FOXO1 are critical transcription factors that increase MTP expression after apoAIV overexpression. First, we identified that cis elements between −204 and −775 bp in the MTP promoter are critical for the apoAIV effect. It is known that FoxO1 and FoxA2 bind to the MTP promoter at −549/−433 bp and increase hepatic expression (52, 54, 58). In contrast, HNF4α binds at −150 bp (14, 47, 59). Second, apoAIV increases MTP promoter-driven expression of firefly luciferase, and knockdown of these transcription factors abolishes apoAIV-mediated augmentations in the luciferase activity (Figs. 6 and 9). Third, knockdown of FOXO1 and FOXA2 abrogates apoAIV-induced increases in MTP expression. Fourth, we have shown that FoxO1 and FoxA2 associate temporally with the Mtp promoter in mouse intestine (Fig. 8). Therefore,
we conclude that apoAIV plays a role in increasing the expression of FoxO1 and FoxA2.

Food entrainment studies suggest that apoAIV regulates MTP by increasing mRNA levels of FoxA2 and FoxO1 at the time of food availability. We also observed that the expression of FoxO1 and FoxA2 shows diurnal variations and that peaks are observed at night. Therefore, this study invokes a new hypothesis: that apoAIV might regulate lipid absorption by increasing expression of a few transcription factors in a time-dependent manner. At this time we do not know how apoAIV regulates the expression of these transcription factors. We are not aware of any evidence that apoAIV could act as a transcription factor or as an activator/repressor of gene expression. Moreover, we were unable to detect apoAIV in the nuclei (data not shown). ApoAIV is synthesized in the endoplasmic reticulum and secreted via the Golgi, involving secretory vesicles similar to other secretory proteins and lipoproteins. It is possible that apoAIV may affect the function of different transcription factors that are found embedded in the endoplasmic reticulum membrane and modulate gene expression; or alternatively, it could alter intracellular reactive oxygen species to affect gene expression. More studies are needed to shed light on the mechanisms regulating expression of transcription factors by apoAIV.

The role of FoxO1 and FoxA2 has been studied mainly in the adipose tissue, skeletal muscle, pancreas, and liver (60, 61). FoxO1 and FoxA2 are involved in insulin/IGF-1 signaling pathways and regulate glucose and lipid metabolism in the liver. In adipose tissue and skeletal muscle, FoxO1 prevents the differentiation of preadipocytes and myoblasts. In the pancreas, it inhibits β cell proliferation. Very little is known about the role of these transcription factors in the intestine. Our studies indicate that these forkhead transcription factors may play a novel role in the temporal regulation of lipid absorption.

Genetic manipulation studies have suggested that apoAIV may not play a significant role in plasma lipid levels and food intake (29, 34, 62). We speculate that the role of apoAIV in these processes has not been appreciated because these studies were performed in the daytime. Our studies also showed no effect of apoAIV deficiency on plasma lipids and food intake in the daytime. Diurnal variation and food entrainment studies presented here show that apoAIV deficiency is associated with a reduction in lipid absorption and plasma lipids only at night and at mealtime after food entrainment.

This study raises an interesting possibility that reductions in apoAIV or inhibition of apoAIV function within enterocytes could be beneficial in lowering plasma lipids. This suggestion appears counterintuitive, as transgenic expression of apoAIV has been shown to be beneficial because of large increases in plasma HDL (62, 63). However, transgenesis in these experiments resulted in enhanced expression of apoAIV in the liver, in addition to increases in intestinal apoAIV, and it is not clear from these studies whether increased intestinal expression of apoAIV would also be advantageous.

In short, this study points to a novel mechanism by which apoAIV assists in the absorption of lipids. We have shown that apoAIV increases FoxO1/FoxA2 in the intestine at mealtime. These transcription factors increase MTP expression to augment the packaging of lipoproteins and their subsequent secretion. Thus, lipids might increase apoAIV to trigger a pathway that optimizes their packaging and secretion by enterocytes.

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