Mechanisms involved in vitamin E transport by primary enterocytes and in vivo absorption

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Abstract It is generally believed that vitamin E is absorbed along with chylomicrons. However, we previously reported that human colon carcinoma Caco-2 cells use dual pathways, apolipoprotein B (apoB)-lipoproteins and HDLs, to transport vitamin E. Here, we used primary enterocytes and rodents to identify in vivo vitamin E absorption pathways. Uptake of [^{3}H]α-tocopherol by primary rat and mouse enterocytes increased with time and reached a maximum at 1 h. In the absence of exogenous lipid supply, these cells secreted vitamin E with HDL. Lipids induced the secretion of vitamin E with intermediate density lipoproteins, and enterocytes supplemented with lipids and oleic acid secreted vitamin E with chylomicrons. The secretion of vitamin E with HDL was not affected by lipid supply but was enhanced when incubated with HDL. Microsomal triglyceride transfer protein inhibition reduced vitamin E secretion with chylomicrons without affecting its secretion with HDL. Enterocytes from *Mttp*-deficient mice also secreted less vitamin E with chylomicrons. In vivo absorption of [^{3}H]α-tocopherol by mice after poloxamer 407 injection to inhibit lipoprotein lipase revealed that vitamin E was associated with triglyceride-rich lipoproteins and small HDLs containing apoB-48 and apoA-I. These studies indicate that enterocytes use two pathways for vitamin E absorption. Absorption with chylomicrons is the major pathway of vitamin E absorption. The HDL pathway may be important when chylomicron assembly is defective and can be exploited to deliver vitamin E without increasing fat consumption.—Anwar, K., J. Iqbal, and M. M. Hussain. **Mechanisms involved in vitamin E transport by primary enterocytes and in vivo absorption.** ***J. Lipid Res.*** **2007.** 48: **2028–2038.**

Supplementary key words lipoproteins • tocopherol • HDL • chylomicrons • oleic acid

Vitamin E is an essential nutrient for normal growth and development and is a major lipid-soluble antioxidant in animals (1). Vegetable oils, nuts, and whole grains are the major source of vitamin E. Dietary vitamin E is present as tocopherols, mainly α- and γ-tocopherol, and synthetic tocopherol esters. The tocopherol esters are hydrolyzed in the intestinal lumen by pancreatic esterases (2–4). Tocopherols are solubilized by bile acids in the intestinal lumen, and this solubilization is important for their uptake by enterocytes. It was thought that the lumenal uptake of dietary vitamin E by enterocytes was a passive process (5). Using antibodies and chemical inhibitors of the scavenger receptor class B type I, Reboul et al. (6) were able to block the uptake of vitamin E by enterocytes. Thus, it appears that vitamin E uptake by enterocytes may in part be protein-mediated, much like cholesterol uptake (7).

After uptake, unlike cholesterol and retinol, tocopherols are not reesterified in enterocytes. Nonetheless, like the esterified forms of cholesterol and retinol, they are secreted with chylomicrons (1, 5, 89–14). The hypothesis that chylomicrons are the exclusive means of vitamin E absorption was challenged by studies in differentiated colon carcinoma (Caco-2) cells (15–17). These cells are known to behave like enterocytes and have been used extensively as a model system to study various physiologic functions of enterocytes (18–20). We provided evidence that, in addition to chylomicrons, HDLs play a significant role in the transport of vitamin E from the apical side to the basolateral side of the differentiated Caco-2 cells (16). The secretion of vitamin E via chylomicrons was dependent on the availability of oleic acid (OA) and microsomal triglyceride transfer protein (MTP). In addition, an intact secretory pathway was necessary, as disruption of these pathways by brefeldin A and monensin inhibited vitamin E secretion with chylomicrons. These studies suggested that vitamin E associates with chylomicrons within enterocytes before its secretion. Vitamin E secretion with HDL by these cells was inhibited by glyburide, an ABC transporter inhibitor, and enhanced by the exogenous HDL, and it was resilient to MTP inhibition and to inhibitors that affect secretion. Thus, it appears that this process may involve the efflux of vitamin E to an extracellular acceptor such as apolipoprotein A-I (apoA-I) and HDL. It remains to be

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Abbreviations: apoA-I, apolipoprotein A-I; FPLC, fast-performance liquid chromatography; IDL, intermediate density lipoprotein; MTP, microsomal triglyceride transfer protein; OA, oleic acid; P407, poloxamer 407; pIpC, polyinosinic-polycytidylic ribonucleic acid.

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determined whether HDL plays any role in the intestinal absorption of vitamin E. Based on these studies in Caco-2 cells, we envisioned two possibilities regarding the mechanisms involved in vitamin E absorption. First, vitamin E absorption depends exclusively on chylomicrons, and dual pathways may be specific to Caco-2 cells. Second, in vivo absorption may also involve dual pathways. To evaluate these two possibilities, we studied vitamin E absorption mechanisms in rodents.

METHODS

Materials

OA, sodium cholic acid, sodium deoxycholic acid, mono-oleoylglycerol, phosphotungistic acid, Tween-40, and polyinosinic-polycytidylic ribonucleic acid (pIpC) were from Sigma. Phosphatidylcholine was from Avanti Polar Lipids. OptiSolve was from Perkin-Elmer. D-α-[^{3}H]tocopherol (radiolabeled vitamin E; specific activity, 13 Ci/mmol; molecular weight, 432) was custom-synthesized and characterized (16). Rabbit anti-mouse apoB-48/apoB-100 and goat anti-human apoA-I were from Biodesign. The MTP inhibitor BMS197636 was a gift from Dr. David Gordon of Bristol-Myers Squibb Co. Purified HDL was a gift from Dr. Xian-Cheng Jiang at the State University of New York Downstate Medical Center. Other chemicals and solvents were from Fisher Scientific.

Animals and diets

Sprague-Dawley rats, wild-type C57BL/6J mice, and *Mttp* floxed mice [$Mttp^{tm2Sgy}/Mttp^{tm2Sgy}$ Tg(Mx1-cre)1Cgn/Tg (Mx1-cre) 1Cgn] were from the Jackson Laboratory. To obtain conditional deletion of the *Mttp* gene, $Mttp^{fl/fl}$ mice were given three intraperitoneal injections of 500 μg of pIpC on alternate days, whereas control mice received phosphate-buffered saline. These mice are LDL receptor-deficient and are transgenic for apoB-100. It was reported that plasma levels of apoB-100 in pIpC-treated mice decreased by 95% and cholesterol levels decreased by >90% compared with PBS-injected mice (21, 22). Male 8–12 week old mice and rats were used in this study. Food was removed the night before the experiments. The institutional animal care and use committee approved these studies.

Short-term vitamin E absorption

For short-term vitamin E absorption studies, age-matched male mice (n = 3/group) on chow diet were fasted overnight and gavaged with 2 μCi of [^{3}H]α-tocopherol in 15 μl of olive oil. After 2 h, plasma and livers were collected and radioactivity was determined. Studies with poloxamer 407 (P407) involved the intraperitoneal injections of 30 mg of P407 per mouse at 1 h before the oral gavages. Tissue samples (~0.1 g) were rinsed with PBS, blotted dry, transferred to glass vials containing 1 ml of OptiSolve, and incubated for 48–72 h. After complete digestion, scintillation cocktail was added and samples were counted for radioactivity.

Solubilization of vitamin E

[^{3}H]α-tocopherol was solubilized in Tween-40 as described previously (16). Stock solutions (12 mg Tween-40/ml acetone) were prepared, and 20 μl of this stock solution was added to 14 μCi of [^{3}H]α-tocopherol and dried under nitrogen. Serum-free medium (14 ml) was then added to obtain a Tween-40 concentration of 29 μg/ml and a [^{3}H]α-tocopherol concentration of 1 μCi/ml.

Isolation of primary enterocytes

Rat and mouse primary enterocytes were isolated using the EDTA treatment method initially described by Weiser (23) and elaborated by Pinkus (24), Cartright and Higgins (25), and Iqbal, Anwar, and Hussain (26). Briefly, the proximal part (one-third to one-half) of the small intestine was collected from anesthetized rats or the whole small intestine was collected from anesthetized mice, and the lumenal contents were emptied, washed with 115 mM NaCl, 5.4 mM KCl, 0.96 mM NaH_2PO_4, 26.19 mM $NaHCO_3$, and 5.5 mM glucose buffer, pH 7.4, and gassed for 30 min with 95% O_2 and 5% CO_2. The intestines were then filled with buffer containing 67.5 mM NaCl, 1.5 mM KCl, 0.96 mM NaH_2PO_4, 26.19 mM $NaHCO_3$, 27 mM sodium citrate, and 5.5 mM glucose, pH 7.4, saturated with 95% O_2 and 5% CO_2, and incubated in a bath containing oxygenated saline at 37°C with constant shaking. After 15 min, the lumenal solutions were discarded and the intestines were filled with buffer containing 115 mM NaCl, 5.4 mM KCl, 0.96 mM NaH_2PO_4, 26.19 mM $NaHCO_3$, 1.5 mM EDTA, 0.5 mM dithiothreitol, and 5.5 mM glucose, pH 7.4, saturated with 95% O_2 and 5% CO_2, and bathed in saline as described above. After 15 min, the lumenal contents were collected and centrifuged (1,500 rpm, 5 min, room temperature), and the pellets were resuspended in DMEM saturated with 95% O_2 and 5% CO_2.

Uptake and secretion studies

For cellular uptake experiments, enterocytes were incubated with 1 μCi/ml Tween-40-solubilized [^{3}H]α-tocopherol in DMEM at 37°C for different times with constant shaking. Cell suspensions were gassed at 15 min intervals with 95% O_2 and 5% CO_2. After washing with DMEM, lipids were extracted with 2 ml of isopropanol and total radioactivity was counted in a scintillation counter and normalized for protein.

For secretion studies, enterocytes were labeled with 1 μCi/ml Tween-40-solubilized [^{3}H]α-tocopherol in DMEM for 1 h at 37°C as described for the uptake experiments. After 1 h, enterocytes were centrifuged (3,000 rpm, 5 min), and pellets were washed with excess DMEM to remove external [^{3}H]α-tocopherol and then chased for 2 h with DMEM at 37°C (oxygenated at 15 min intervals) containing lipid/bile salt micelles consisting of 0.14 mM sodium cholate, 0.15 mM sodium deoxycholate, 0.17 mM phosphatidylcholine, and 0.19 mM mono-oleoylglycerol in the presence of increasing concentrations of OA. Unless stated otherwise, we used 1.6 and 2.2 mM OA along with lipid/bile salt micelles for secretion studies involving rat and mouse primary enterocytes, respectively.

For the MTP inhibitor study, during the chase, lipid micelles were supplemented with 1.6 mM OA and were incubated in the presence or absence of 1 μM BMS197636. To study the role of HDL in vitamin E efflux, micelles were supplemented with 100 μg of HDL and were incubated for 2 h as described. At the end of the incubation, the enterocytes were centrifuged (3,000 rpm, 5 min) and supernatants were used for sequential density gradient ultracentrifugation. Total [^{3}H]α-tocopherol was extracted from cell pellets using 2 ml of isopropanol incubated overnight at 4°C. The next day, isopropanol was collected and aliquots were counted for radioactivity in a scintillation counter. Protein was measured by the method of Bradford (27) using BSA as the standard.

Density gradient ultracentrifugation

Sequential density gradient ultracentrifugation was preformed to isolate large chylomicrons, small chylomicrons, and VLDLs from the enterocyte-conditioned media based on the methods used to isolate these particles from lymph and plasma (28–30) as

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described for cell culture media (15, 17, 26). This method is suitable for separating large triglyceride-rich lipoproteins based on their buoyant density (28–30). The apolipoprotein composition of different lipoproteins has been described previously (15). To the conditioned media (2 ml) was added 2 ml of d = 1.006 solution containing KBr (0.57 g/ml) to obtain a final density of 1.10 g/ml. The media were then overlaid with 3 ml each of 1.063 and 1.019 g/ml, and 2 ml of 1.006 g/ml, density solutions and subjected to sequential ultracentrifugation. Large chylomicrons [Svedberg units (S_f) > 400)] were obtained by collecting the top 1 ml after ultracentrifugation (SW41 rotor, 33 min at 40,000 rpm, 15°C). The tubes were overlaid with 1 ml of 1.006 g/ml density solution and ultracentrifuged (SW41 rotor, 3.5 h at 40,000 rpm, 15°C), and the top 1 ml fraction was collected as small chylomicrons (S_f = 60–400). After replenishing the top with 1 ml of 1.006 g/ml density solution, samples were ultracentrifuged (SW41 rotor, 17.5 h at 40,000 rpm, 15°C) again to obtain VLDL. The first 1.5 ml fraction represented VLDL (d < 1.006 g/ml, S_f = 20–60). The rest of the gradient was fractionated into seven additional 1.5 ml fractions. Aliquots from each fraction were then counted for radioactivity using a scintillation counter. Density in each fraction was measured using a refractometer (Fisher Scientific).

Fast-performance liquid chromatography

Pooled plasma samples were subjected to fast-performance liquid chromatography (FPLC) using a Superose 6 column (GE Healthcare). This procedure is not suitable for differentiating between large triglyceride-rich lipoproteins but is very useful in separating small cholesterol-rich lipoproteins. Samples were chromatographed at a flow rate of 0.2 ml/min, and fractions of 250 μl each were collected and assayed for radioactivity. Triglyceride and cholesterol levels were also measured in these fractions using commercially available kits (Thermo Trace, Melbourne, Australia).

Determination of MTP activity in mouse intestine

The proximal small intestines were washed with ice-cold PBS to remove debris, and small segments (~1 cm) of the proximal small intestine were homogenized with 1 ml of ice-cold 1 mM Tris-HCl, pH 7.6, 1 mM EGTA, and 1 mM $MgCl_2$ buffer in a glass homogenizer. The homogenates were centrifuged (SW55 Ti rotor, 50,000 rpm, 10°C, 1 h), and supernatants were used for MTP transfer assay as described (31, 32) using a kit from Chylos, Inc.

Western blot analysis

Equal volumes of selected fractions were used for the determination for apoB and apoA-I proteins (33, 34). Under reducing conditions, proteins were separated on 4–20% Tris-HCl precast gels (Bio-Rad) and transferred to nitrocellulose membranes, blocked for 1 h at room temperature with 5% nonfat dry milk in PBS containing 0.25% Tween 20 (PBST), and washed and incubated overnight with primary antibody (1:1,000) at 4°C. Next, the membranes were washed and incubated with secondary antibody conjugated with horseradish peroxidase (1:4,000) in PBST containing 1% nonfat dry milk and incubated at room temperature for 1 h. Immune reactivity was detected by chemiluminescence.

RESULTS

Vitamin E uptake by primary enterocytes

Absorption is defined as the transport of dietary fat and fat-soluble vitamins from the intestinal lumen to the plasma. An essential step in this process is the transport of these nutrients across the intestinal epithelial barrier. To understand the mechanisms involved in vitamin E absorption, studies were initiated using isolated mouse and rat primary enterocytes. First, we determined the uptake of vitamin E by these enterocytes (**Fig. 1**). Vitamin E was solubilized in Tween-40 as described (16) and was provided to cells for different times. Amounts of radioactivity in cells were determined. Cellular association of vitamin E increased in a time-dependent manner and appeared to saturate at ~30–60 min in enterocytes isolated from mouse (Fig. 1A) and rat (Fig. 1B) intestines. At 1 h, these cells took up 20–48% of the added vitamin E (data not shown). These studies indicate that the cellular association of vitamin E is time-dependent and that cells assimilate significant amounts of added vitamin E within a short period.

Secretion of vitamin E by enterocytes

To study vitamin E secretion, mouse primary enterocytes were incubated with vitamin E for 1 h as described for uptake studies (Fig. 1), washed, and incubated in media

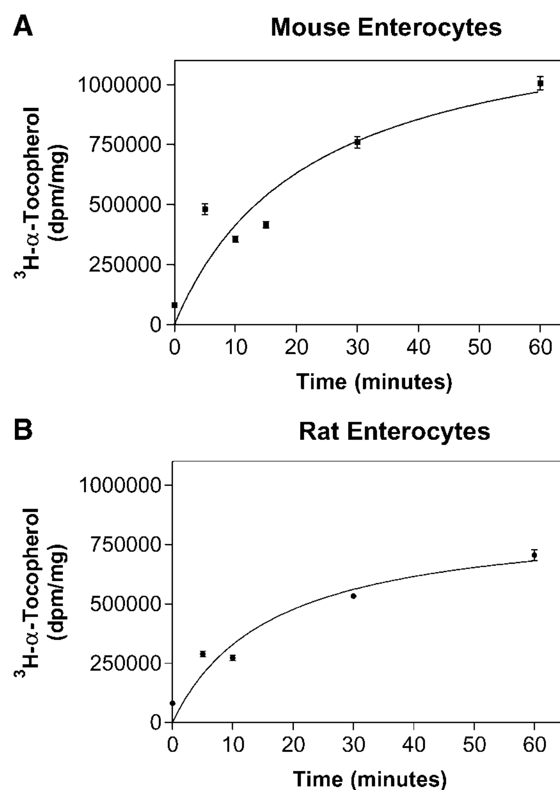

Fig. 1. Uptake of vitamin E by primary enterocytes. Mouse (A) and rat (B) primary enterocytes were incubated in triplicate with Tween-solubilized vitamin E (1 μCi/ml). At the indicated times, cells were centrifuged and the pellets were washed with excess PBS. The pellets were then resuspended in 2 ml of isopropanol to isolate lipids and incubated overnight at 4°C. The cells were centrifuged, and an aliquot of the supernatant was used for radioisotope determinations. Next, excess isopropanol was dried under nitrogen, and the pellets were incubated in 0.1 N NaOH for 5 h at 37°C. An aliquot was used for protein determination as described in Methods. The data are plotted as means ± SD. In some cases, error bars are smaller than the symbol sizes.

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with no lipid/bile salt micelles (**Fig. 2A**). The conditioned media were then subjected to density gradient ultracentrifugation, and fractions were counted for radioactivity. Under these conditions, enterocytes failed to secrete vitamin E with chylomicrons and VLDLs (Fig. 2A). However, vitamin E was found in the bottom HDL fractions (Fig. 2A, F4–F6). Enterocytes supplemented with lipid/bile salt micelles secreted vitamin E mainly in lipoprotein particles corresponding to the density of intermediate density lipoprotein (IDL) (Fig. 2B). Because there was no vitamin

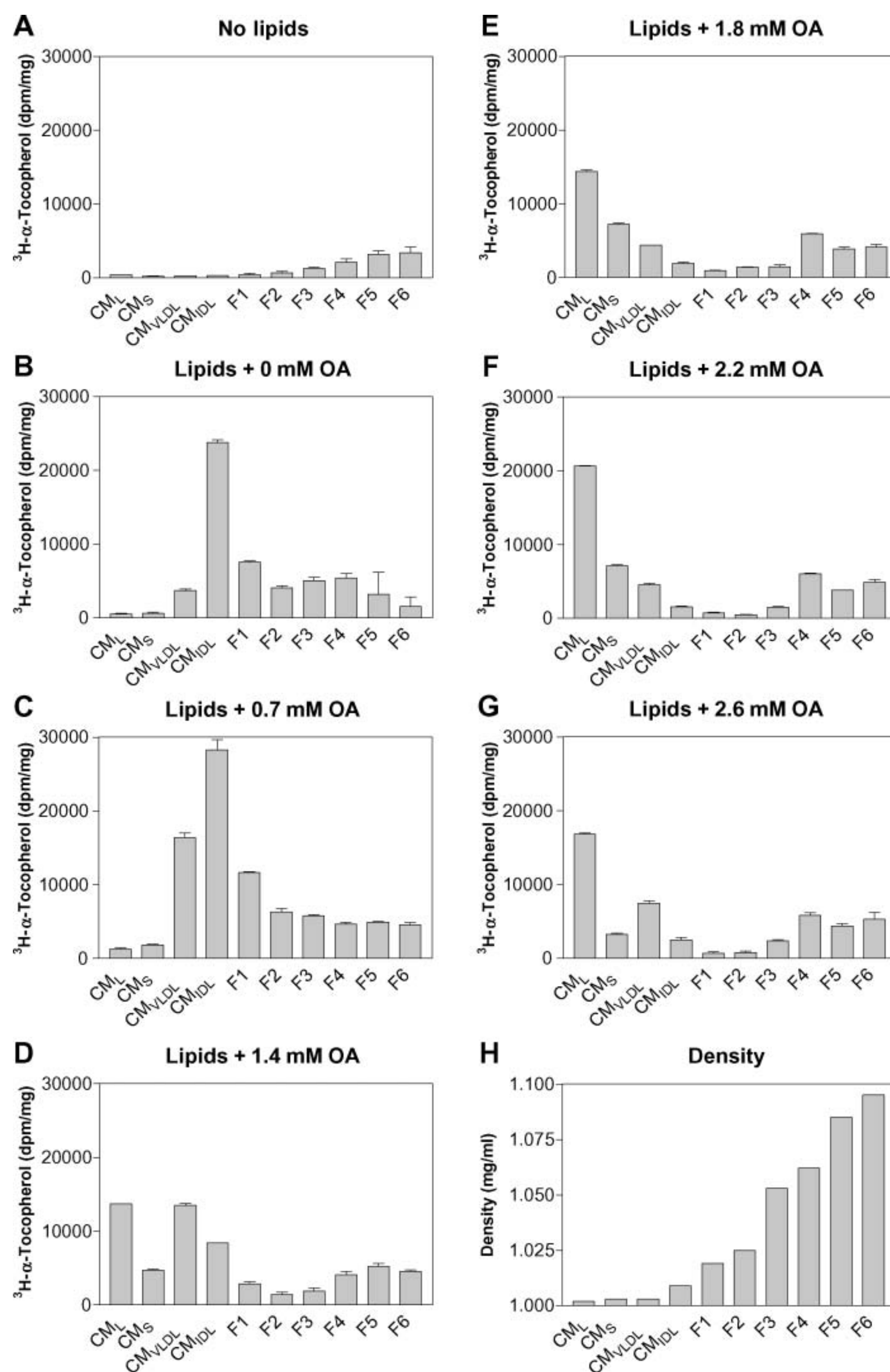

Fig. 2. Secretion of vitamin E by enterocytes. Mouse primary enterocytes were radiolabeled with vitamin E for 1 h, washed, and then incubated with serum-free media containing no lipid/bile salt micelles (A), lipid/bile salt micelles (B), or lipid/bile salt micelles supplemented with 0.7 (C), 1.4 (D), 1.8 (E), 2.2 (F), or 2.6 (G) mM oleic acid (OA) for 2 h. The conditioned media were then subjected to density gradient ultracentrifugation, and fractions were counted for radioactivity. Density in these fractions was measured using a refractometer (H). Vitamin E distribution in different fractions was quantified and normalized for proteins. CM, chylomicron; IDL, intermediate density lipoprotein. Error bars indicate ± SD.

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E in chylomicron fractions, we studied the effect of OA, which is known to induce chylomicron assembly and secretion in intestinal cells (15–17, 26). Supplementation of 0.7 mM OA induced the secretion of vitamin E with VLDL-size lipoproteins (Fig. 2C). Nonetheless, significant amounts of vitamin E were still secreted with IDL-size lipoproteins (Fig. 2C). Again, no vitamin E was found associated with chylomicrons. However, inclusion of 1.4 mM OA resulted in a significant increase in the secretion of vitamin E with chylomicrons (Fig. 2D). Under these conditions, equal amounts of vitamin E were secreted with chylomicrons and VLDL/IDL-size lipoproteins. Increasing the OA concentration to 1.8 mM (Fig. 2E) and 2.2 mM (Fig. 2F) augmented the amounts of vitamin E secreted with chylomicrons, with a concomitant decrease in its secretion with VLDL/IDL-size particles. When 2.2 mM OA was used, chylomicrons and HDLs contained 66% and 29% of total secreted vitamin E, respectively. Large chylomicrons contained 40% of the secreted vitamin E. There was no further increase in vitamin E secretion with chylomicrons when higher concentrations of OA were used (Fig. 2G). These studies indicated that lipid availability is essential for the secretion of significant amounts of vitamin E by mouse enterocytes and that OA availability determines the secretion of vitamin E with different sized triglyceride-rich particles.

Under all of the conditions described above (Fig. 2 A–G), there were significant amounts of vitamin E in the high density fractions (F4–F6) and the levels of vitamin E in these lipoproteins did not change with lipid and OA supplementation. Thus, transport of vitamin E with HDL occurs independent of lipid supply. Furthermore, in contrast to its secretion with apoB-lipoproteins, secretion of vitamin E with HDL is not affected by lipid availability.

Studies were then extended to rat primary enterocytes. Cells were incubated with radiolabeled vitamin E for 1 h and then chased for 2 h in the presence or absence of lipid/bile salt micelles (**Fig. 3A**). In the absence of added lipid/bile salt micelles, these cells secreted vitamin E with HDL (Fig. 3A, − Lipids). No vitamin E was present in chylomicron fractions. Enterocytes incubated with lipid micelles secreted vitamin E in VLDL/IDL-size chylomicrons (Fig. 3A, + Lipids). Studies were then performed to identify optimal concentrations of OA required for the secretion of vitamin E with chylomicrons. We observed that 1.6 mM OA was optimal for the secretion of vitamin E with chylomicrons (data not shown). In a separate experiment, enterocytes were labeled with α-tocopherol, washed, and incubated with media containing lipid/bile salt micelles supplemented with or without 1.6 mM OA. Again, cells incubated with lipid/bile salt micelles secreted vitamin E in VLDL/IDL-size lipoproteins (Fig. 3B, − OA). When lipid micelles were supplemented with OA, we observed a significant shift in the peaks. Now, 68% of the secreted vitamin E was with large and small chylomicrons (Fig. 3B, + OA). Under this condition, 20% of the total secreted vitamin E was present in the bottom fractions corresponding to HDL. These studies indicate that rat enterocytes secrete vitamin E mainly with triglyceride-rich apoB lipoproteins in the presence of abundant lipid supply. In addition, these cells secrete significant amounts of vitamin E with HDL.

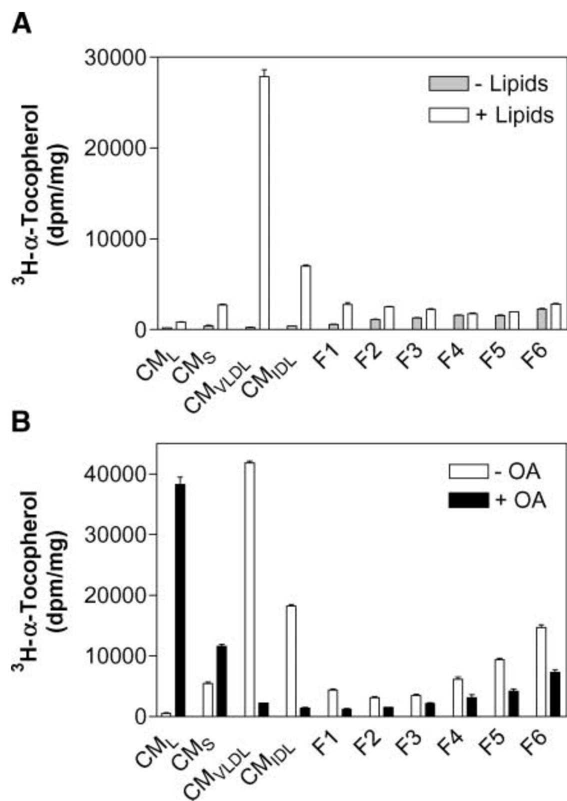

Fig. 3. Vitamin E secretion by rat primary enterocytes. A: Effect of lipids. Rat primary enterocytes were incubated with 1 μCi/ml radiolabeled vitamin E for 1 h, washed with oxygenated media, and then incubated with either serum-free media supplemented with (+ Lipids) or without (− Lipids) lipid/bile salt micelles as described in Methods for 2 h. Media were subjected to ultracentrifugation, and radioactivity was counted in each fraction and corrected for protein. B: Effect of OA. Enterocytes were pulse-labeled with radiolabeled vitamin E for 1 h, washed with oxygenated media, and then incubated with lipid/bile salt micelles supplemented with (+ OA) or without (− OA) 1.6 mM OA for 2 h. Media were subjected to ultracentrifugation, and vitamin E distribution in different fractions was quantified and normalized for protein. Error bars indicate ± SD.

Effect of HDL on vitamin E efflux by enterocytes

In the secretion studies described above, vitamin E was also found in the bottom fractions corresponding to the density of HDL. To determine whether the vitamin E in the HDL could arise from cellular efflux, enterocytes loaded with radiolabeled vitamin E were treated with or without 100 μg of purified HDL, an acceptor of lipids in the efflux pathway (**Fig. 4A**). In control cells, small amounts of vitamin E were found in HDL, consistent with the data described above (Figs. 2, 3). HDL treatment significantly increased the amounts of vitamin E in these fractions (Fig. 4A, HDL). Note that there was no vitamin E in the chylomicron fractions, because these cells were not incubated with lipid/bile salt micelles containing OA. These data indicate that enterocytes could efflux vitamin E to HDL.

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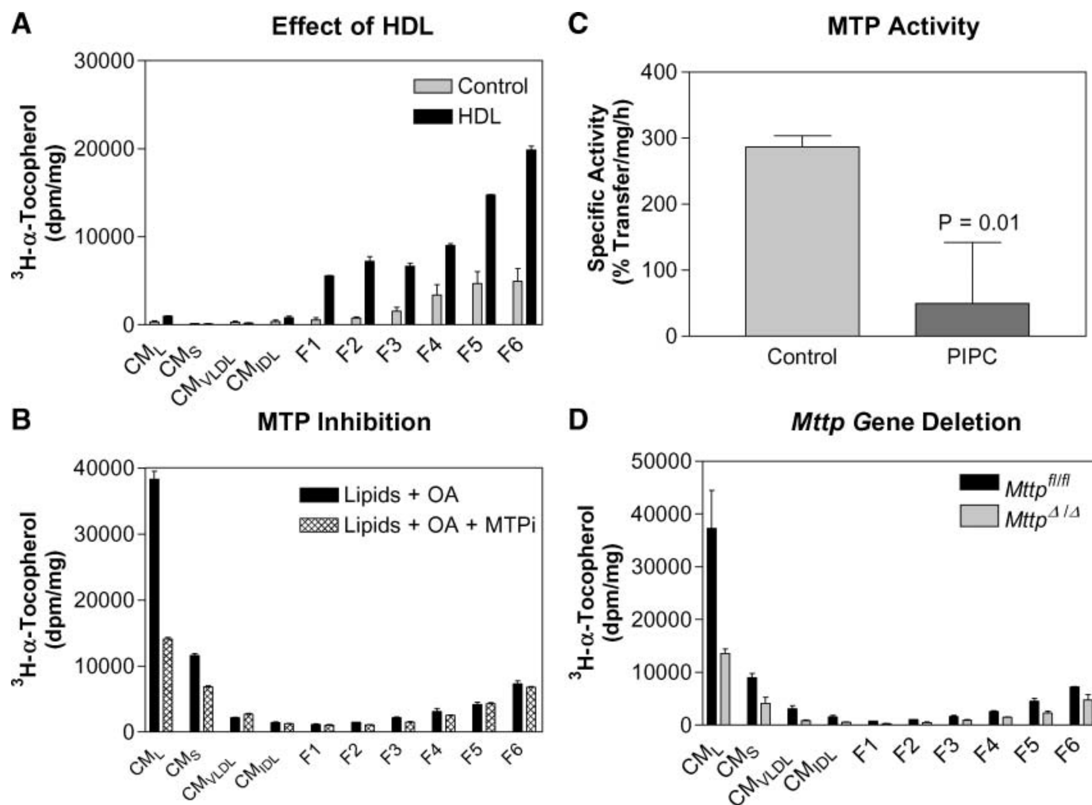

Fig. 4. Role of HDL and microsomal triglyceride transfer protein (MTP) in vitamin E secretion by enterocytes. A: Effect of HDL. Mouse enterocytes were incubated with radiolabeled vitamin E for 1 h, washed with oxygenated media, and then incubated with (HDL) or without (Control) 100 μg of HDL for 2 h. Media were subjected to ultracentrifugation, and vitamin E distribution in different fractions was quantified. B: Effect of MTP inhibition. Enterocytes labeled with vitamin E were incubated with micelles containing 2.2 mM OA in the presence and absence of MTP inhibitor [BMS197636, 1 μM (MTPi)] for 2 h. Vitamin E distribution in different fractions was quantified and corrected for protein. C: Effect of *Mttp* gene deletion on activity. The proximal small intestines from control and polyinosinic-polycytidylic ribonucleic acid (pIpC)-injected *Mttp*$^{fl/fl}$ mice were washed with ice-cold phosphate-buffered saline. Small pieces (~1 cm) of proximal small intestine were homogenized with 1 ml of ice-cold 1 mM Tris-HCl, pH 7.6, 1 mM EGTA, and 1 mM $MgCl_2$ buffer in a glass homogenizer. The homogenates were centrifuged (SW55 Ti rotor, 50,000 rpm, 10°C, 1 h), and supernatants were used to measure the triglyceride transfer activity of MTP. D: Effect of *Mttp* gene deletion on vitamin E secretion. Enterocytes from control (*Mttp*$^{fl/fl}$) and pIpC-injected (*Mttp*$^{Δ/Δ}$) mice were labeled with radiolabeled vitamin E for 1 h, washed with oxygenated media, and then incubated with lipid/bile salt micelles containing 2.2 mM OA for 2 h. Media were subjected to density gradient ultracentrifugation. Vitamin E distribution in different fractions was quantified and normalized for protein. Error bars indicate ± SD.

Effect of MTP inhibition and gene deletion on vitamin E secretion by enterocytes

It is known that the assembly of triglyceride-rich apoB-lipoproteins is critically dependent on MTP activity. Thus, we studied the effect of the MTP inhibitor BMS197636 on the secretion of vitamin E by mouse enterocytes (Fig. 4B). Enterocytes were radiolabeled with vitamin E for 1 h and chased with micelles supplemented with OA in the presence and absence of MTP inhibitor. Cells supplemented with OA secreted vitamin E mainly in large and small chylomicrons, and this secretion was significantly inhibited in the presence of MTP inhibitor. MTP inhibitor had no major effect on the secretion of vitamin E as HDL (Fig. 4B). These data suggest that vitamin E secretion is dependent on MTP activity.

To further evaluate the role of MTP in vitamin E secretion, primary enterocytes were obtained from mice that were subjected to conditional deletion of the *Mttp* gene. For this purpose, *Mttp*$^{fl/fl}$ mice were injected with pIpC. This treatment is known to induce the expression of Cre-recombinase and deletion of the *Mttp* gene (22). Injections of pIpC reduced MTP activity by 80% compared with control enterocytes obtained from mice injected with PBS (Fig. 4C). This reduction is consistent with other published studies (35, 36). Normal and MTP-deficient enterocytes were incubated for 1 h with radiolabeled vitamin E and chased in the presence of lipid/bile salt micelles containing OA. Enterocytes obtained from control mice secreted vitamin E in chylomicron-size particles (Fig. 4D, *Mttp*$^{fl/fl}$). Mice injected with pIpC also secreted vitamin E in chylomicron-size particles (Fig. 4D, *Mttp*$^{Δ/Δ}$), but the amounts were considerably lower compared with those in controls. *Mttp* gene deletion had no significant effect on the amounts of vitamin E secreted with HDL. These data suggest that reduced MTP activity results in a significant decrease in the secretion of vitamin E with triglyceride-rich lipoproteins and underscore the importance of the chylomicron pathway in vitamin E secretion.

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Vitamin E absorption in mice

To gain insight into vitamin E absorption, studies were conducted in wild-type C57BL/6J mice injected with P407 or saline (control). P407 treatment is known to inhibit plasma lipoprotein lipase, resulting in increased apoB-lipoproteins in plasma without affecting HDL (37). One hour after the intraperitoneal injection of P407 or saline, mice received oral gavages of radiolabeled vitamin E in olive oil. After 2 h, plasma and livers were collected and radioactivity was counted. Livers obtained from mice injected with P407 had significantly reduced amounts of vitamin E, suggesting that P407 inhibited lipoprotein lipase, resulting in decreased uptake of vitamin E-containing lipoproteins (**Fig. 5A**). Plasma samples (300 μl) from each group were pooled and subjected to sequential density gradient ultracentrifugation, after which fractions were collected and counted for radioactivity. Mice injected with P407 had 66% and 20% of the secreted vitamin E associated with chylomicrons/VLDLs and HDLs, respectively (Fig. 5B). These data indicate that the majority of the gavaged vitamin E enters the circulation as part of chylomicrons. In control mice, however, the majority of the vitamin E was associated with HDL and a minor peak was observed with chylomicrons.

The presence of the majority of radiolabeled vitamin E in HDL was not anticipated; thus, we next analyzed plasma from control and P407-treated mice that had received oral gavages of radiolabeled vitamin E by FPLC (Fig. 5C). In control mouse plasma, 72% of the radiolabeled vitamin E was in fractions 60–70, corresponding to small HDL (see below). In contrast, when lipase activity was inhibited by P407 injection, vitamin E was observed in fractions 22–36 and 60–70, corresponding to chylomicron/VLDL and small HDL, respectively. These lipoproteins carried 43% and 25% of the vitamin E, respectively. We interpret these data to suggest that intestinal absorption of vitamin E involves chylomicrons and small HDLs. In control animals, chylomicrons are rapidly hydrolyzed by lipoprotein lipase, resulting in the formation of HDL and chylomicron remnants. Vitamin E associated with remnant particles is removed from the plasma very rapidly. In contrast, HDL-associated vitamin E remains in the plasma for longer times.

Next, we quantified the mass of triglycerides and cholesterol present in different fractions to gain insights into the distribution of triglyceride- and cholesterol-rich lipoproteins in different fractions (Fig. 5D, E). The majority of triglycerides in P407-injected animals were in fractions 22–36, indicating that these fractions represent triglyceride-rich lipoproteins (Fig. 5D). Very low amounts of triglycerides were observed in control plasma, consistent with the understanding that triglyceride-rich chylomicrons are rapidly catabolized and cleared from the plasma. Analysis of cholesterol in different fractions revealed that the majority of cholesterol-rich lipoproteins in control mice were in fractions 42–58, representing plasma HDL (Fig. 5E). In P407-injected animals, cholesterol was present in three distinct peaks (Fig. 5E). The first peak (fractions 22–36) was the same as that seen for triglyceride-rich lipoproteins, indicating that this cholesterol was associated with them. The second peak (fractions 42–58) was similar to the HDL cholesterol present in control mice. The third small peak (fractions 60–70) was also evident (Fig. 5C). Because the majority of radiolabeled vitamin E was in this peak, we propose that this peak may represent the small HDL secreted by the intestinal cells. Thus, from the data obtained from P407-injected mice, it can be concluded that vitamin E absorption involves triglyceride-rich lipoproteins as well as HDLs. The HDL secreted by intestinal cells is distinct from the normal HDL present in the plasma.

We then tried to characterize the small HDL carrying the radiolabeled α-tocopherol. A major feature of HDL is the presence of exchangeable apolipoproteins such as apoA-I. To identify apolipoproteins present in lipoproteins that carry radiolabeled vitamin E, we performed absorption studies in P407-injected mice (Fig. 5F). Plasma from these mice was subjected to FPLC. Again, α-tocopherol was in two peaks, fractions 26–36 and 66–74, corresponding to chylomicron/VLDL and small HDL. Next, we performed Western blot analyses to test the hypothesis that these peaks contain apoB-48 and apoA-I. We observed that vitamin E-containing fractions 26–36 and fractions 66–74 had apoB-48 and apoA-I, respectively. Thus, we conclude that the two peaks carrying radiolabeled vitamin E represent apoB-48- and apoA-I-containing lipoproteins.

DISCUSSION

Vitamin E transport by primary enterocytes

The data presented here show that enterocytes use dual (chylomicron and HDL) pathways to secrete vitamin E. The chylomicron pathway requires MTP activity. Chemical inhibition and gene deletion of MTP significantly reduced the amounts of vitamin E secreted with chylomicrons. The HDL pathway was not affected by MTP inhibition or gene deletion but was enhanced in the presence of exogenous HDL. Thus, two independent, noncompensatory pathways are involved in vitamin E absorption.

The secretion of vitamin E by the triglyceride-rich chylomicrons was highly regulated and was critically dependent on the exogenous lipid supply, as very little vitamin E was secreted in the absence of lipid supply. To determine the optimal lipid concentrations required for vitamin E secretion, we studied the effect of different concentrations of OA. The total amounts of vitamin E secreted were not significantly different when incubated with different amounts of OA. However, the major effect of increasing concentrations of OA was on the size of the lipoproteins that carried vitamin E. Mouse enterocytes incubated with lipid micelles secreted vitamin E with IDL-size lipoproteins (Fig. 2). Low concentrations of OA resulted in the secretion of vitamin E with IDL- and VLDL-size particles. Higher concentrations of OA were required for its secretion with chylomicrons. This observation highlights a remarkable dependence on OA for the secretion of vitamin E by enterocytes with different sized triglyceride-rich lipoproteins. In addition, we found that the optimum amounts of OA re-

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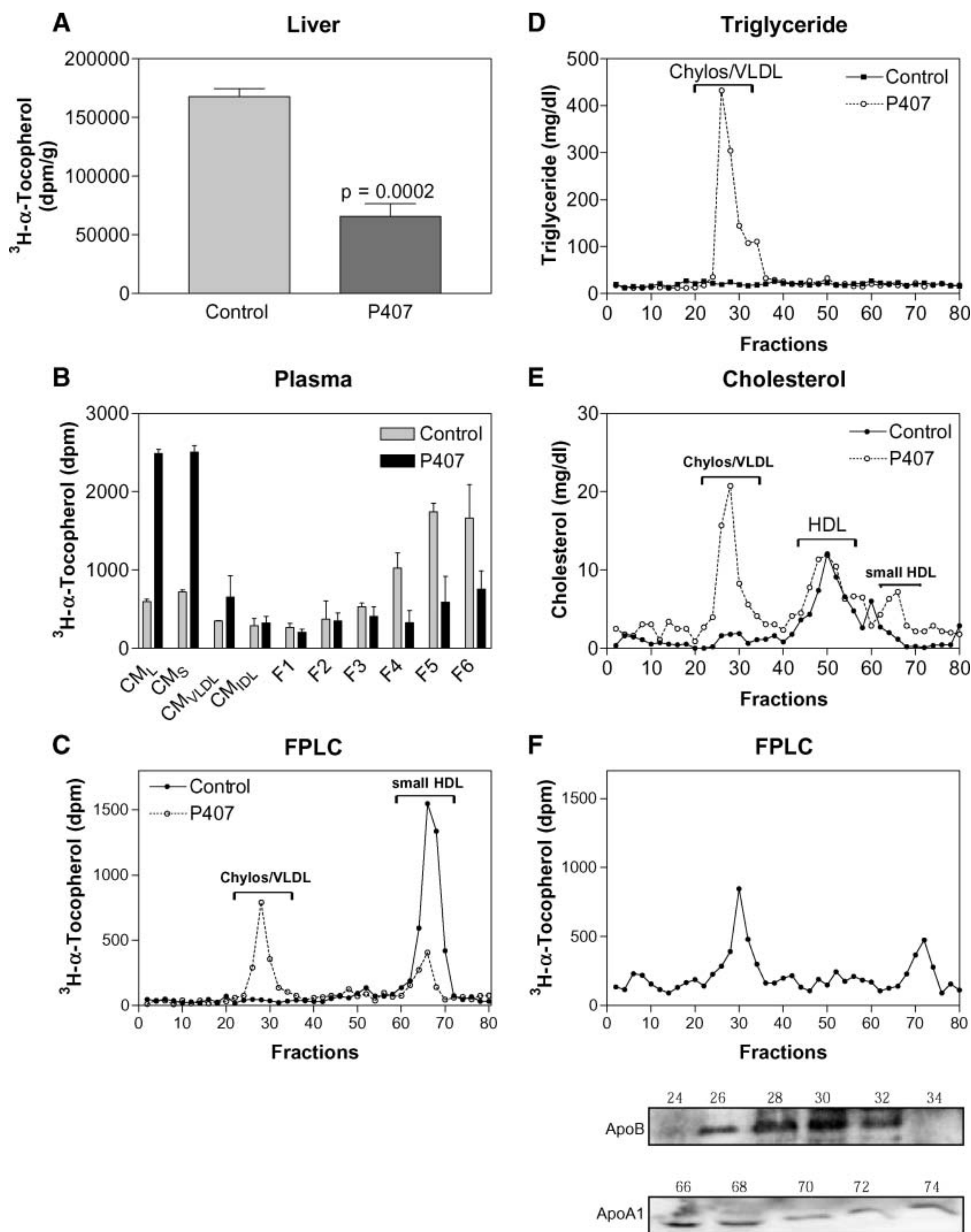

Fig. 5. Vitamin E absorption in mice. A–E: Wild-type C57Bl/6J mice were injected intraperitoneally with saline (control) or poloxamer 407 (P407; 30 mg/mouse). After 1 h, both groups received oral gavages of radiolabeled vitamin E (2 μCi) in 15 μl of olive oil. After 2 h, mice were euthanized, and plasma and liver samples were collected and radioactivity was determined. Liver samples were digested using OptiSolve and used to determine radioactivity (A). Plasma samples from each group were pooled and subjected to sequential density gradient ultracentrifugation (B). An equal amount of pooled plasma was also chromatographed on a Superose 6 column as described in Methods, and radioactivity was determined in different fractions (C). In addition, triglyceride (D) and cholesterol (E) levels were measured in these fractions. F: In a separate experiment, mice were first injected with P407 and then gavaged with radiolabeled vitamin E as described for A, and plasma was subjected to fast-performance liquid chromatography (FPLC). Odd fractions were used to measure radioactivity. Even fractions were used to visualize apolipoprotein B (apoB) or apoA-I by immunoblotting, as described in Methods. Chylos, chylomicrons. Error bars indicate ± SD.

quired for the secretion of vitamin E by rat and mouse enterocytes were different. For example, an OA concentration of 1.6 mM was adequate for the secretion of vitamin E with chylomicrons by rat enterocytes, but it was not adequate for vitamin E secretion by primary mouse enterocytes (Fig. 4B). In mouse enterocytes, 2.2 mM OA was found to be optimum for the secretion of vitamin E with chylomicrons. The requirement by mouse enterocytes for

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a higher concentration of OA to assemble and secrete triglyceride-rich lipoproteins could be the result of a faster metabolic rate. Thus, enterocytes isolated from different species require different amounts of OA to secrete vitamin E with chylomicrons.

At this time, the mechanisms by which high OA concentrations induce the secretion of vitamin E with different lipoproteins are not clear. It is possible that the availability of higher amounts of OA results in the robust synthesis of triglyceride-rich lipoproteins, resulting in the association of vitamin E with these particles. It is also possible that a protein(s) involved in vitamin E transfer is induced when enterocytes are exposed to high OA concentrations, facilitating its association with chylomicrons. Understanding how OA modulates vitamin E secretion may reveal new mechanisms involved in vitamin E targeting to triglyceride-rich lipoproteins for its secretion.

It has been proposed that chylomicron assembly involves three steps (38, 39): the formation of small primordial lipoproteins, the synthesis of lipid droplets independent of apoB-lipoprotein assembly, and the "core expansion" involving the bolus addition of lipids to primordial lipoproteins. Performed phospholipids have been suggested to mark the synthesis of primordial lipoproteins. Because retinyl esters were only secreted with larger chylomicrons (17), it has been suggested that they might serve as signposts for the synthesis of larger nascent lipoproteins (39). Given that vitamin E is secreted with lipoproteins of all sizes (Fig. 2), we propose that it probably associates with primordial lipoproteins and might serve as a marker for their assembly.

In contrast to the chylomicron pathway, the secretion of vitamin E by the HDL pathway was not affected by lipid availability. However, this pathway was enhanced when exogenous HDL was added to the media. Thus, the HDL pathway might be modulated by other mechanisms, such as the amounts of acceptors. It remains to be determined whether regulators of key proteins in the HDL pathway, such as ABCA1 and apoA-I, would affect the amounts of vitamin E transported by enterocytes. In this regard, it would be interesting to study the effects of liver X receptor/retinoid X receptor agonists on vitamin E transport via the HDL pathway. It is not clear whether the vitamin E found associated with HDL is attributable to the intracellular assembly of vitamin E with HDL or represents efflux to acceptors. Because HDL enhanced vitamin E secretion (Fig. 4A), it is likely that efflux pathways play a role in the secretion of vitamin E with HDL.

To our surprise, ablation of MTP did not enhance the amounts of vitamin E secreted with HDL. Similarly, the amounts of vitamin E secreted with HDL remained unchanged whether enterocytes secreted vitamin E with chylomicrons or not. These data indicate that these pathways do not represent compensatory pathways; rather, they signify independently regulated pathways. Thus, the HDL pathway is probably not induced under the conditions of chylomicron assembly deficiency syndromes such as abetalipoproteinemia and chylomicron retention disease. Nonetheless, we speculate that this pathway may play an important role under conditions of defective chylomicron assembly and may explain the reduction in vitamin E deficiency symptoms in abetalipoproteinemia patients provided with high doses of vitamin E (40, 41).

The observation that enterocytes use two pathways to transport vitamin E is consistent with our previous observations that Caco-2 cells use dual pathways for vitamin E secretion (16). The major difference between primary enterocytes and Caco-2 cells was the contribution of these two pathways in vitamin E transport. In enterocytes, vitamin E is preferentially secreted with chylomicrons. In contrast, Caco-2 cells use HDL as the primary vehicle for vitamin E transport. Thus, it is possible that transformed cells prefer secreting vitamin E with HDL, whereas primary enterocytes adept at chylomicron assembly prefer this pathway for vitamin E transport.

Vitamin E absorption in mice

To understand the in vivo mechanisms involved in vitamin E absorption, we inhibited lipoprotein lipase using P407, which has been speculated to coat plasma triglyceride-rich lipoproteins, thereby preventing their interaction with lipoprotein lipase (37, 42). In addition, it has been shown to exert no obvious effect on HDL metabolism (37). In P407-injected mice, triglyceride-rich lipoproteins accumulate in the plasma and the delivery of dietary lipids to the liver is inhibited (37, 42). We observed that injection of P407 decreased vitamin E content in the liver (Fig. 5A). Ultracentrifugation and gel filtration studies revealed that there was significant accumulation of vitamin E with triglyceride-rich chylomicrons and with small HDLs. Thus, we conclude that vitamin E absorption in mice occurs via the chylomicron and HDL pathways.

This is the first report recognizing the possible in vivo contribution of the HDL pathway in vitamin E absorption, and its physiologic significance remains to be fully appreciated. Although HDL contributes to a small fraction of total vitamin E absorption in mice, it might play a significant physiologic role under certain conditions. It is known that feeding high doses of vitamin E to abetalipoproteinemia patients results in the absorption of vitamin E (41) and retards vitamin E deficiency symptoms (40, 43). It has been proposed that abetalipoproteinemia patients secrete apoB-lipoproteins that are enriched in vitamin E (44); however, this remains to be supported by other studies. It should be noted that in several human studies using deuterium-labeled tocopherol, Traber and associates (41, 45–47) have observed significant amounts of vitamin E in HDL in addition to its presence in apoB-lipoproteins. In these studies, it is difficult to know the origin of the HDL-associated tocopherol. Based on the studies presented here, we propose that the HDL pathway might contribute to the low levels of vitamin E absorption in abetalipoproteinemia patients (41) and that the amounts of vitamin E absorbed via the HDL pathway might be sufficient to compensate for the loss of the chylomicron pathway and to provide vitamin E for the proper functioning of various organs. In addition, this pathway might

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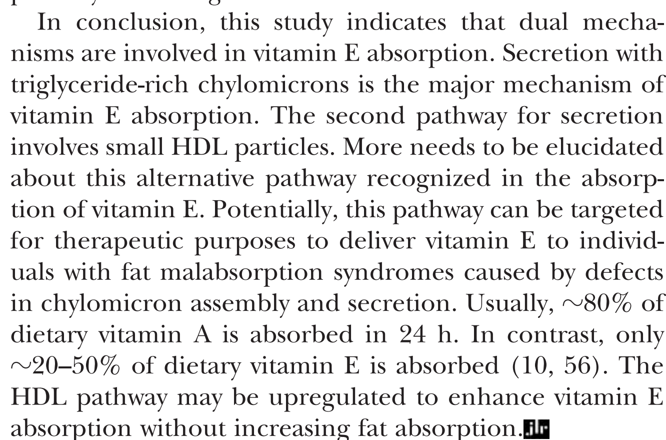
be important in the absorption of vitamin E under fasting conditions.

Based on human studies as well as those using isolated primary hepatocytes, it was generally accepted that vitamin E is resecreted from the liver as part of VLDL (45, 48, 49). However, cell culture studies using hepatoma cells suggest that vitamin E might associate with VLDL extracellularly (50–52). These suggestions were mainly based on the observation that vitamin E secretion was insensitive to brefeldin A, a potent inhibitor of the secretory pathway (51). Several in vitro studies have supported this suggestion (51, 53, 54). For example, overexpression of α-tocopherol transfer protein in hepatoma cells increases vitamin E efflux. A recent in vivo study using liver-specific *Mttp* conditional deletion mice reported that VLDL deficiency does not affect vitamin E levels in peripheral tissues (55). This could be attributable to the secretion of α-tocopherol with liver HDL. However, the data presented here about the mechanisms involved in the secretion of vitamin E by enterocytes warrants further studies in hepatocytes. It is possible that hepatocytes may also use dual pathways involving VLDL and HDL to secrete vitamin E.



In conclusion, this study indicates that dual mechanisms are involved in vitamin E absorption. Secretion with triglyceride-rich chylomicrons is the major mechanism of vitamin E absorption. The second pathway for secretion involves small HDL particles. More needs to be elucidated about this alternative pathway recognized in the absorption of vitamin E. Potentially, this pathway can be targeted for therapeutic purposes to deliver vitamin E to individuals with fat malabsorption syndromes caused by defects in chylomicron assembly and secretion. Usually, ~80% of dietary vitamin A is absorbed in 24 h. In contrast, only ~20–50% of dietary vitamin E is absorbed (10, 56). The HDL pathway may be upregulated to enhance vitamin E absorption without increasing fat absorption.

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