Transport of vitamin E by differentiated Caco-2 cells

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Abstract In hepatocytes, vitamin E is secreted via the efflux pathway and is believed to associate with apolipoprotein B (apoB)-lipoproteins extracellularly. The molecular mechanisms involved in the uptake, intracellular trafficking, and secretion of dietary vitamin E by the intestinal cells are unknown. We observed that low concentrations of Tween-40 were better for the solubilization and delivery of vitamin E to differentiated Caco-2 cells, whereas high concentrations of Tween-40 and sera inhibited this uptake. Vitamin E uptake was initially rapid and then reached saturation. Subcellular localization revealed that vitamin E primarily accumulated in microsomal membranes. Oleic acid (OA) treatment, which induces chylomicron assembly and secretion, decreased microsomal membrane-bound vitamin E in a time-dependent manner. To study secretion, differentiated Caco-2 cells were pulse-labeled with vitamin E and chased in the presence and absence of OA. In the absence of OA, vitamin E was associated with intestinal high density lipoprotein (I-HDL), whereas OA-treated cells secreted vitamin E with I-HDL and chylomicrons. No extracellular transfer of vitamin E between these lipoproteins was observed. Glyburide, an antagonist of ABCA1, partially inhibited its secretion with I-HDL, whereas plasma HDL increased vitamin E efflux. An antagonist of microsomal triglyceride transfer protein, brefeldin A, and monensin specifically inhibited vitamin E secretion with chylomicrons. These studies indicate that vitamin E taken up by Caco-2 cells is stored in the microsomal membranes and secreted with chylomicrons and I-HDL. Transport via I-HDL might contribute to vitamin E absorption in patients with abetalipoproteinemia receiving large oral doses of the vitamin.—Anwar, K., H. J. Kayden, and M. M. Hussain. **Transport of vitamin E by differentiated Caco-2 cells. *J. Lipid Res.* 2006.** 47: **1261–1273.**

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Vitamin E is a major lipid-soluble antioxidant and is an essential nutrient for normal growth and development. Its deficiency results in neurological dysfunction, muscular weakness, and reproductive failure (1–3). Although a diet rich in vegetable oils and whole grains is a sufficient source of vitamin E in normal people (4), deficiency can result because of lipid malabsorption syndromes such as abetalipoproteinemia, a disease in which apolipoprotein B (apoB)-containing lipoproteins (chylomicrons, very low density lipoproteins, and low density lipoproteins) are absent in the plasma (1, 2). High oral doses of vitamin E ameliorate the deficiency and normalize plasma and adipose tissue levels of the vitamin in these patients (5). The mechanisms involved in vitamin E absorption in the absence of apoB-lipoprotein assembly are not known.

Current knowledge of intestinal vitamin E absorption is based on human and animal studies involving oral or intraduodenal administration of vitamin E followed by lymph and plasma analysis (1–3, 6–9). Vitamin E uptake by the intestinal cells is believed to be less efficient than that of other fat-soluble vitamins. In most studies, ~20–50% of dietary vitamin E is believed to be absorbed (4, 10). In contrast, ~80% of dietary vitamin A is absorbed in 24 h. The reasons for the poor absorption of vitamin E are unknown. Intestinal absorption of vitamin E requires the presence of bile acids, as children with cholestatic liver disease poorly absorb vitamin E as well as the other fat-soluble vitamins, as a result of insufficient production and secretion of bile into the intestines (2).

After uptake, vitamin E is secreted with chylomicrons by enterocytes into the lymphatic system. The importance of chylomicron assembly in the secretion of vitamin E is underscored by the absence of vitamin E in abetalipoproteinemia patients who lack microsomal triglyceride transfer protein (MTP) (11–13). However, the molecular mechanisms involved in the targeting of vitamin E to chylomicrons within enterocytes are unknown (14) because of the lack of intestinal cell culture systems that mimic vitamin E secretion with chylomicrons. Traber et al. (15) studied vitamin E uptake by incubating Caco-2 cells for 1 h in the presence of bile acids and fatty acids. They observed increased cellular uptake of vitamin E after the addition of increasing amounts of oleic acid (OA). However, no

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Abbreviations: apoB, apolipoprotein B; I-HDL, intestinal high density lipoprotein; MTP, microsomal triglyceride transfer protein; OA, oleic acid; SFM, serum-free medium; TC, taurocholate; αTTP, α-tocopherol transfer protein.

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attempts were made to study the secretion of vitamin E by these cells.

Vitamin E that reaches plasma with chylomicrons is partly released during the hydrolysis of these particles by lipoprotein lipase. The lipoprotein lipase-mediated hydrolysis has been shown to be important for the uptake of vitamin E by peripheral cells (15, 16). However, the majority of the vitamin E reaches the liver as part of chylomicron remnants. It has been assumed that vitamin E is resecreted as part of VLDL by the liver. However, recent studies indicate that the association of vitamin E with VLDL may be extracellular and probably occurs in the space of Disse (17). The evidence that vitamin E is secreted independent of VLDL comes from the studies of Arai and colleagues (18). They studied the secretion of vitamin E in McA-RH7777 cells overexpressing α-tocopherol transfer protein (αTTP). αTTP is a 31 kDa protein mainly expressed in the liver and is defective in patients with the genetic disorder ataxia with vitamin E deficiency. Overexpression of αTTP resulted in increased secretion of tocopherol by the transfected cells (18, 19). Brefeldin A inhibited triglyceride secretion by these cells but had no effect on tocopherol secretion. These data were interpreted to suggest that hepatic cells secrete α-tocopherol independently of VLDL secretion and that vitamin E associates with these lipoproteins extracellularly. More evidence for this possibility came from the studies of Oram, Vaughan, and Stocker (20). They showed that a brefeldin A-insensitive vitamin E secretion pathway could be ABCA1-mediated efflux. Furthermore, ABCA1 ablation in mice has been shown to result in vitamin E deficiency along with the deficiency of other fat-soluble vitamins (21). Recently, Qian at al. (22) also confirmed that vitamin E is secreted by hepatocytes by a non-Golgi αTTP-mediated pathway that involves the ABC transporters. Thus, it appears that αTTP may deliver vitamin E to ABCA1 for secretion. Subsequently, secreted vitamin E associates extracellularly with VLDL and is transported to plasma.

It is not known whether such a mechanism exists in the intestine for vitamin E secretion. To study vitamin E transport by intestinal cells, we used differentiated Caco-2 human colon carcinoma cells. These cells spontaneously differentiate after reaching confluence in culture and exhibit enterocyte-like features, including expression of sucrase-isomaltase, apical and basolateral membrane demarcation by tight junctions, and development of transmembrane epithelial resistance. Hughes et al. (23) first demonstrated that these cells synthesize lipoproteins. Subsequently, these cells have been used extensively as a model system to study intestinal absorption (for reviews, see 24–27). We showed that these cells secrete chylomicron-like particles after supplementation of OA (28). Furthermore, they have been shown to secrete retinyl esters with chylomicrons (29). The assembly and secretion of chylomicrons by these cells has been shown to be sensitive to Pluronic L81 (28, 29), as in animals (30). Recently, we showed that Caco-2 cells secrete cholesterol by two independent, differentially regulated mechanisms (31). Here, we report that the mechanisms of vitamin E secretion by these cells are distinct from that observed in hepatocytes and involve two independent pathways.

MATERIALS AND METHODS

Materials

OA, taurocholate (TC), Tween-40, brefeldin A, monensin, and glyburide were from Sigma. D-[^{3}H]α-tocopherol (radiolabeled vitamin E; specific activity, 13 Ci/mmol; molecular weight, 432) was custom synthesized (Amersham Pharmacia Biotech, Buckinghamshire, England). The radiochemical purity of the product was determined to be 97.9% by high-performance liquid chromatography. The purity determined by thin-layer chromatography on silica gel ranged between 97% and 99%, depending on the solvent system used. The MTP inhibitor BMS197636 was a gift from Dr. David Gordon of Bristol-Myers Squibb Co. To prepare OA:TC (20 × 1.6:0.5 mM) stocks, 97.4 mg of OA was added to 10 ml of 10 mM TC solution, mixed by gentle swirling, and incubated at 37°C until a clear solution was achieved. Glyburide stock (500 mM) was prepared by dissolving 247 mg in 1 ml of dimethylsulfoxide. For experiments, 20 μl of the stock was added to 10 ml of serum-free medium (SFM).

Solubilization of vitamin E

To solubilize [^{3}H]α-tocopherol with TC and OA:TC, required amounts of [^{3}H]α-tocopherol in toluene were dried under nitrogen in sterile tubes, followed by the addition of appropriate amounts of TC or OA:TC. The tubes were incubated for 30 min at 37°C and swirled gently. SFM was then added to obtain the desired concentrations of TC, OA:TC, and [^{3}H]α-tocopherol. For solubilization with Tween-40, stock solutions (12–800 mg of Tween-40 per milliliter of acetone) were prepared, and 20 μl of these stocks was added to 14 μCi of [^{3}H]α-tocopherol and dried under nitrogen. SFM (14 ml) was then added to obtain [^{3}H]α-tocopherol concentration of 1 μCi/ml and different required concentrations of Tween-40.

Cell culture

Caco-2 cells obtained from the American Type Culture Collection (Rockville, MD) were cultured in DMEM containing high glucose, L-glutamine, 10% FBS, and a 1% antibiotic-antimycotic mixture. For experiments, half of the cells from a 70–80% confluent, 75 cm^2 flask were seeded onto polycarbonate micropore inserts (Transwells®; six-well plate, 24 mm diameter, 3 μm pore size; Corning Costar Corp., Corning, NY), and medium was changed every 48 h for 21 days. This treatment is known to induce the differentiation of Caco-2 cells into enterocyte-like cells and the secretion of apoB (28, 29, 32, 33). Experiments were conducted to study vitamin E uptake and secretion using either the pulse-labeling and/or the pulse-chase protocol. In the pulse-labeling protocol, differentiated Caco-2 cells were supplemented on the apical side with SFM containing either TC or OA:TC with Tween-40-solubilized [^{3}H]α-tocopherol. The basolateral side received SFM only. For pulse-chase experiments, differentiated Caco-2 cells were initially treated with SFM containing Tween-40-solubilized [^{3}H]α-tocopherol for 24 h. Cells were washed, and the apical side received SFM containing TC, OA:TC, OA:TC plus MTP inhibitor (1 μM), OA:TC plus brefeldin A, or OA:TC plus monensin. SFM was added to the basolateral side and used for various analyses after incubation. To study the effect of HDL or glyburide, cells were subjected to the pulse-chase protocol. During the chase, either glyburide (1 mM)

or HDL (100 μg) was added to the basolateral SFM, whereas the apical side received SFM containing TC.

Subcellular fractionation

Differentiated Caco-2 cells were pulsed with Tween-40-solubilized [^{3}H]α-tocopherol for various time periods. At each indicated time point, the cells were washed with excess PBS to remove extracellular (unbound) [^{3}H]α-tocopherol. The cells were then treated with ice-cold homogenization buffer (250 mM sucrose, 1 mM EDTA, and 20 mM HEPES-KOH, pH 7.4) and incubated at 4°C for 15 min. For pulse-chase studies, the cells were pulsed with Tween-40-solubilized [^{3}H]α-tocopherol for 6 h and chased with OA:TC for 18 h or for the indicated times. After the incubation, cells were scraped and collected and a protease inhibitor cocktail (Sigma) was added. Cells were then homogenized using a syringe with a 25G needle (10 strokes). To obtain the nuclear pellet (OptiPrep Application Sheet S7), cell homogenates were centrifuged (500 *g*, 10 min, 4°C; Beckman GS-15R centrifuge). The postnuclear supernatant was then centrifuged (3,000 *g*, 10 min, 4°C) to get the heavy mitochondrial pellet. To isolate the microsomal fraction, the postmitochondrial supernatant was then centrifuged at 100,000 *g* for 1 h at 4°C using a Beckman tabletop ultracentrifuge TLA-110 rotor. The remaining supernatant was designated the cytosolic fraction. To separate membrane and lumenal proteins (34–36), the microsomal pellet was resuspended in 200 μl of sodium carbonate (0.1 M) containing 0.025% deoxycholate for 30 min with gentle shaking and centrifuged (1 h, TLA-110 rotor, 100,000 *g*). The membrane pellet and the supernatant containing lumenal proteins were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with polyclonal anti-calnexin antibody (Stressgen; 1:2,000 dilution) or anti-KDEL antibody (Stressgen; 1:2,000 dilution) that recognizes BiP (35).

Density gradient ultracentrifugation

Two types of density gradients were used to characterize the secreted lipoproteins. Sequential density gradient ultracentrifugation was performed to isolate large chylomicrons, small chylomicrons, and CM_{VLDL} (for nomenclature, see 37) from the basolateral cell culture medium based on the methods used to isolate these particles from lymph and plasma (38–40), as described previously for cell culture medium (28, 29, 33). To the conditioned medium (2 ml) was added 2 ml of 1.006 g/ml density solution containing KBr (0.57 g/ml) to obtain a final density of 1.10 g/ml. The medium was then overlaid with 3 ml each of 1.063 g/ml and 1.019 g/ml density solutions, and 2 ml of 1.006 g/ml density solution, and subjected to sequential ultracentrifugation. Large chylomicrons ($S_f > 400$) were obtained by collecting the top 1 ml after ultracentrifugation (SW41 rotor, 33 min, 40,000 rpm, 15°C). The tubes were again overlaid with 1 ml of 1.006 g/ml density solution and ultracentrifuged (SW41 rotor, 3.5 h, 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, 17.5 h, 40,000 rpm, 15°C). The first 1.0 ml fraction represented CM_{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. A second, denser gradient (1.08–1.34 g/ml) was used to separate high density lipoproteins and characterize Tween-solubilized and secreted vitamin E. To vitamin E (1 μCi/ml) solubilized in Tween-40 (29 μg/ml) or conditioned medium (4 ml) was added 1.792 g of KBr to obtain a final density of 1.34 g/ml. The medium was then overlaid with 2 ml each of 1.24 g/ml, 1.15 g/ml, and 1.063 g/ml density solutions followed by 1 ml of 1.019 g/ml and 1.006 g/ml density solutions and subjected to ultracentrifugation (SW41, 17.5 h, 40,000 rpm, 15°C). The gradient was fractionated into 1 ml fractions. Aliquots from each fraction were then used to measure radioactivity or apoA-I.

Other methods

ApoB was quantified by enzyme-linked immunoassay as described previously (41, 42). Total [^{3}H]α-tocopherol was extracted from cell monolayers using isopropanol (2 ml/well) 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 (43) using BSA as a standard. The presence of apoB and apoA-I in different density gradient fractions was determined using ELISA (41, 42). Statistical significance was evaluated using Student's *t*-test. All quantitative data are presented as means ± SD.

RESULTS

Uptake of vitamin E by Caco-2 cells

To study vitamin E transport across the intestinal epithelial cells, an initial hurdle was to find a suitable means of delivery. Previously, researchers have used a combination of bile acids and fatty acids to mediate vitamin E uptake (15). Recently, During et al. (44) showed that a nonionic detergent, Tween-40, facilitated carotenoid uptake by intestinal cells. To find a suitable agent for vitamin E delivery to cells, we compared various agents to solubilize radiolabeled vitamin E (**Fig. 1**). [^{3}H]α-tocopherol solubilization (Fig. 1A) was low in SFM and medium containing TC and modest in OA:TC. The amount of vitamin E solubilized by Tween was significantly higher than that solubilized by OA:TC ($P < 0.0001$). In fact, all of the vitamin E was solubilized in Tween-40. Next, we studied uptake by adding solubilized vitamin E to the apical side of differentiated Caco-2 cells. Vitamin E was barely detectable in cells supplemented with [^{3}H]α-tocopherol in medium or TC (Fig. 1B). Significant amounts of [^{3}H]α-tocopherol were taken up when provided with OA:TC. However, the greatest amounts were in cells that received Tween-40-solubilized [^{3}H]α-tocopherol. Although these data indicated that the amount of vitamin E taken up by cells was higher when vitamin E was solubilized with Tween-40, we considered the possibility that the higher uptake might be secondary to a larger amount of vitamin E added to the cells. Thus, we calculated the percentage of vitamin E taken up by the cells relative to the amounts added to the cells (Fig. 1C). The uptake was 1.7 ± 0.5%, 3.7 ± 0.1%, 23.5 ± 0.5%, and 46.7 ± 2.6% when vitamin E was provided with medium, TC, OA:TC, and Tween-40 ($P < 0.001$, compared with OA:TC), respectively. These studies indicate that Tween-40 is not only a better solubilization agent but also a better vehicle for the delivery of vitamin E to Caco-2 cells.

Because we achieved the greatest cellular uptake using Tween-40, we reasoned that cellular uptake of radiolabeled vitamin E can be enhanced further by increasing its concentration. As shown in Fig. 1D, all of the concentra-

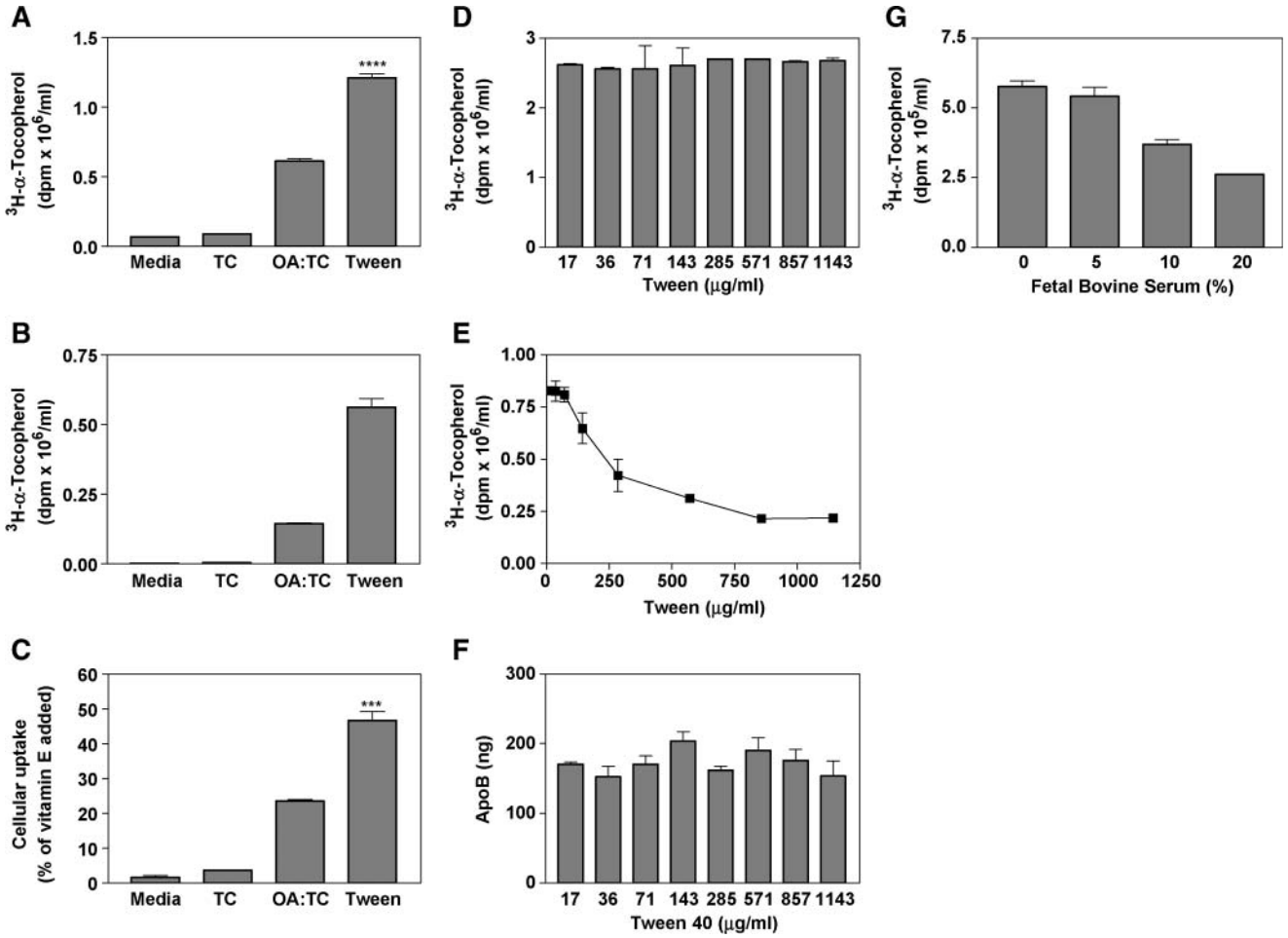

Fig. 1. Uptake of vitamin E by Caco-2 cells. A: Solubilization. [^{3}H]α-tocopherol (1 μCi/ml) was evaporated under nitrogen and solubilized in serum-free medium (SFM; Media), SFM + 0.5 mM taurocholate (TC), SFM + 1.6:0.5 mM oleic acid (OA):TC (OA:TC), or SFM + 29 μg/ml Tween-40 (Tween) by vortexing. The amount of vitamin E solubilized was measured by quantifying the amount of radioactivity present in the medium. Amounts solubilized with Tween 40 were significantly greater than in other conditions (**** $P < 0.0001$). B: Cellular uptake. Vitamin E solubilized using various agents as described for A was added (2 ml) to the apical side of differentiated Caco-2 cells. After 18 h, cells were washed with ice-cold PBS, and lipids were extracted in isopropanol and counted. C: Percentage cellular uptake. The amount of radioactivity taken up was calculated as the percentage of the amount of vitamin E added to the cells. Tween-solubilized vitamin E uptake was significantly greater compared with other treatments (*** $P < 0.001$). D: Solubilization. Vitamin E was solubilized in 2 ml of SFM containing different amounts of Tween-40. An aliquot was used for scintillation counting to determine the amounts of vitamin E solubilized. E: Cellular uptake. Solubilized vitamin E in 2 ml of medium (1 μCi/ml) was added to the apical side of differentiated Caco-2 cells. The basolateral side received 2 ml of SFM. After 18 h, cells were washed with ice-cold PBS, and lipids were extracted in isopropanol and counted. F: Effect of Tween-40 on apolipoprotein B (apoB) secretion. Cells were incubated with increasing concentrations of Tween-40 for 18 h, and total apoB secreted in the basolateral medium was measured by ELISA (41, 42). G: Effect of serum on cellular uptake. Differentiated Caco-2 cells were incubated for 18 h with Tween-solubilized vitamin E in medium containing increasing amounts of fetal bovine serum. After 18 h, cells were washed with ice-cold PBS, and lipids were extracted in isopropanol and counted. All data are presented as means ± SD.

tions tested achieved relatively the same degree of solubilization. This is consistent with the observation that low amounts of Tween-40 (Fig. 1A) solubilize 100% of the radiolabeled vitamin E. Surprisingly, increasing concentrations of Tween-40 decreased the cellular uptake of radiolabeled vitamin E (Fig. 1E). Suspecting that the decrease in uptake might be attributable to cellular toxicity, we measured total amounts of apoB secreted by cells. ApoB secretion was not affected by different concentrations of Tween-40 (Fig. 1F), ruling out cellular toxicity. These studies indicate that higher concentrations of Tween-40 inhibit the cellular uptake of vitamin E. In subsequent experiments, we used Tween-40 at a concentration of 29 μg/ml.

All of the experiments described above were done using SFM. To determine whether medium containing FBS might increase the cellular uptake of radiolabeled vitamin E, differentiated Caco-2 cells were treated apically with radiolabeled solubilized vitamin E in medium containing 0–20% FBS. After 18 h of incubation, cellular vitamin E levels were determined. Increasing concentrations of FBS in medium decreased the cellular uptake of radiolabeled

vitamin E (Fig. 1G). With 20% FBS, there was a 45% decrease in cellular uptake relative to SFM. It is possible that lipoproteins in FBS, such as LDL and HDL, sequester vitamin E and prevent its uptake. Hence, we supplied Tween-40-solubilized vitamin E to Caco-2 cells in SFM in all subsequent experiments.

Intracellular trafficking of vitamin E

To study intracellular trafficking of vitamin E, we measured the time-dependent accumulation of vitamin E in cells and various subcellular compartments. The cellular uptake of vitamin E by Caco-2 cells was initially linear with time and later reached saturation (**Fig. 2A**). Differential centrifugation revealed that vitamin E mainly accumulated in microsomes (~45–50%) in a time-dependent manner (Fig. 2B). To study the intracellular distribution of vitamin E during lipoprotein assembly and secretion, Caco-2 cells were pulse-labeled with vitamin E, washed, and incubated with TC (Fig. 2C) or OA:TC (Fig. 2D). Under both conditions, the majority of the vitamin E was in the microsomal fractions ($P < 0.0001$, compared with cytosol). The microsomal fraction is known to contain the endoplasmic reticulum, Golgi, lysosomes, and peroxisomes. No further attempts were made to separate these fractions. Small amounts of vitamin E were also present in the cytosol and heavy mitochondria. Very low background levels were found in the nucleus. These studies show that cells take up vitamin E very rapidly and target it to microsomes.

Within microsomes, vitamin E could be either in the membranes or in the lumen. To determine the localization of vitamin E, microsomes were treated with sodium carbonate and deoxycholate (34–36) and the membranes and lumenal fractions were separated by ultracentrifugation. The separation of these fractions was assessed by Western blot detection (**Fig. 3A**) of calnexin, a 91 kDa membrane-bound protein, and BiP, a 78 kDa protein known to be present in the lumen of the endoplasmic reticulum (35). The cellular homogenate contained calnexin and BiP (Fig. 3A, column H). After separation, the membrane fraction was enriched in calnexin and was

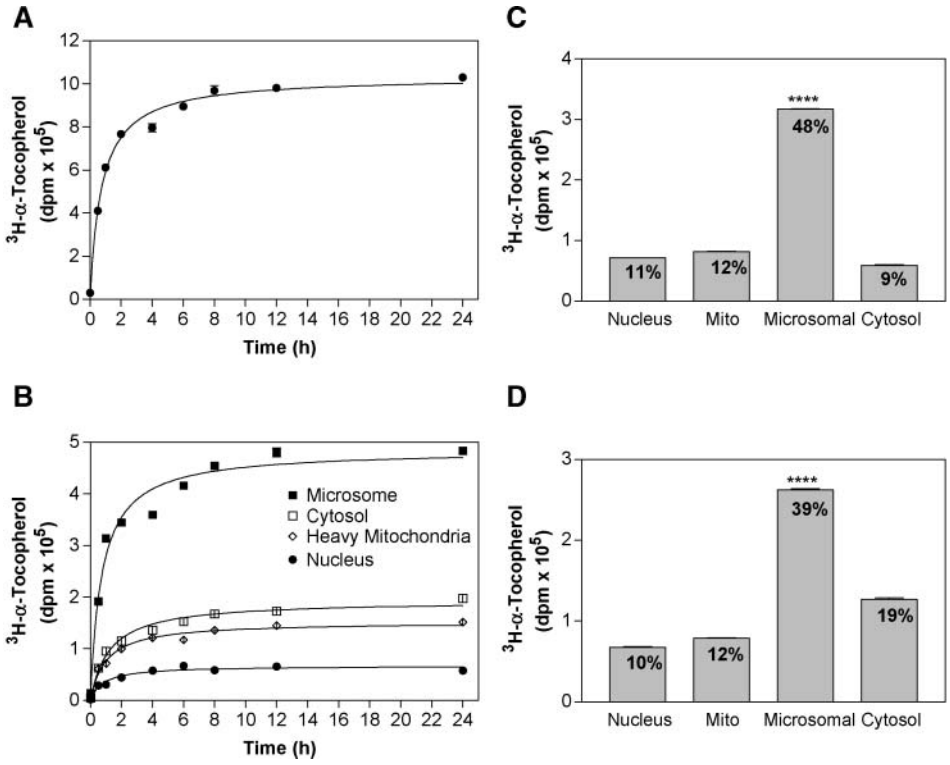

Fig. 2. Subcellular localization of vitamin E. A: Differentiated Caco-2 cells were incubated with 1 μCi/ml Tween-solubilized vitamin E for the indicated times. At each time point, cells were isolated and homogenized, and radioactivity was measured. B: Homogenates were subjected to differential centrifugation to separate various organelles, as described in Materials and Methods, and radioactivity present in different subcellular compartments was plotted against time. C: Differentiated Caco-2 cells were incubated with 1 μCi/ml Tween-solubilized vitamin E for 6 h, washed, and then treated with TC for 18 h. The cells were then isolated, homogenized, and subjected to differential centrifugation as described. Aliquots were used to determine radioactivity. Amounts in microsomes were significantly greater than in other organelles (**** $P < 0.0001$). The numbers inside the bars represent the percentage of radioactivity recovered in each fraction starting from homogenates. Mito, mitochondria. D: Differentiated Caco-2 cells were incubated with 1 μCi/ml Tween-solubilized vitamin E for 6 h and then treated with OA:TC for 18 h. The cells were then isolated, homogenized, and subjected to differential centrifugation. Aliquots were used to determine radioactivity and percentage recovery. Again, vitamin E levels in microsomes were significantly greater (**** $P < 0.0001$). All data are presented as means ± SD.

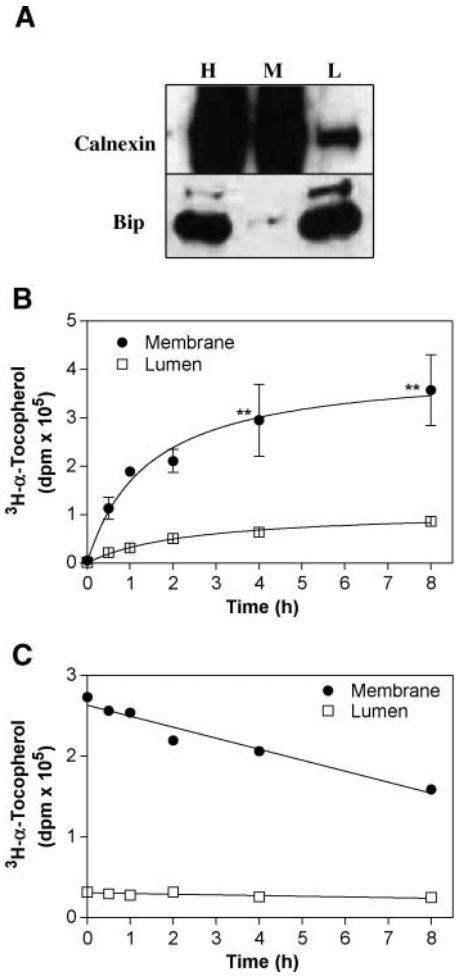

Fig. 3. Distribution of vitamin E in microsomal membrane and lumenal fractions. A: Separation of membrane and lumenal proteins. Cells were homogenized, and microsomal fractions (H) were obtained by differential ultracentrifugation. Microsomes were treated with sodium carbonate/deoxycholate and centrifuged to separate membrane (M) and lumenal (L) proteins. Proteins (10 μg/lane) were applied to SDS-PAGE gels, subjected to electrophoresis, transferred to nitrocellulose membranes, and reacted with specific antibodies. B: Distribution within microsomes. Cells were labeled for 0–8 h, and microsomes were isolated at the indicated times, treated with sodium carbonate/deoxycholate for 30 min, and subjected to ultracentrifugation to separate the membranes from the lumenal contents. Aliquots of the resuspended membrane and lumen were then counted in triplicate. Membranes had greater amounts of vitamin E at all time points (** $P < 0.01$). Data are presented as means ± SD. C: Removal from microsomes. Caco-2 cells were incubated for 6 h with vitamin E, washed, and then supplemented with medium containing OA:TC for 0–8 h. At each time point, cells were homogenized and subjected to differential ultracentrifugation, and microsomal pellets were treated with sodium carbonate/deoxycholate and subjected to ultracentrifugation to precipitate membranes from the lumenal contents. Aliquots of the lumenal contents and resuspended membranes were counted.

devoid of BiP (Fig. 3A, column M), indicating no contamination of lumenal proteins with membrane proteins. The lumenal fraction contained BiP (Fig. 3A, column L) and a small amount of calnexin, probably representing some contamination from membrane fractions. These studies indicate that microsomal membranes were successfully separated from their lumenal contents.

Next, the distribution of vitamin E in membrane and lumenal fractions was determined (Fig. 3B). Vitamin E was mainly present in membranes (Fig. 3B). For example, at 4 and 8 h, membranes contained ~6-fold higher amounts of vitamin E than the lumenal contents ($P < 0.01$). Low amounts of vitamin were in the lumen, most likely representing contamination of the membrane fraction. These data suggest that cells take up vitamin E and store it mainly in the microsomal membranes.

We then determined the effect of OA:TC, which is known to induce chylomicron assembly and secretion (28, 29, 33), on the fate of microsomal membrane-associated vitamin E. Caco-2 cells were pulse-labeled for 6 h and chased with OA:TC for the indicated times. At each time point, vitamin E distribution in the membrane and lumenal fractions was determined (Fig. 3C). We observed a time-dependent decrease in vitamin E present in the microsomal membranes, with no apparent increase in the microsomal lumen. These data suggest that the microsomal membrane is the major reservoir of vitamin E in Caco-2 cells and that removal from this depot is probably the rate-limiting step during secretion.

Secretion of vitamin E

We used continuous pulse and pulse-chase protocols to study the secretion of vitamin E (**Fig. 4**). For continuous pulse-labeling, differentiated Caco-2 cells were treated apically with Tween-40-solubilized vitamin E in the presence and absence of OA. After 18 h, the basolateral medium was subjected to density gradient ultracentrifugation to determine the secretion of vitamin E with different lipoproteins. As a control, we also measured the distribution of vitamin E in various density gradient fractions (Fig. 4A). Tween-40-solubilized vitamin E given to cells was mainly present in the bottom high-density (~1.1 g/ml) fractions. Similarly, vitamin E secreted by TC-treated cells was mainly in the bottom fractions, corresponding to intestinal high density lipoprotein (I-HDL) of 1.08–1.10 g/ml (Fig. 4B). In contrast, vitamin E secreted by OA:TC-treated cells was in two distinct peaks (Fig. 4B). One peak was in fractions 1 and 2, which correspond to large and small chylomicrons, and the other peak was in the bottom with I-HDL. Second, we performed secretion studies under a pulse-chase protocol. TC-treated cells secreted vitamin E mainly with I-HDL (Fig. 4C). In contrast, vitamin E secreted by OA:TC-treated Caco-2 cells was distributed in two lipoproteins: chylomicrons and I-HDL (Fig. 4C). All of the vitamin E secreted by TC-treated cells was associated with I-HDL. In contrast, OA:TC-treated cells secreted 40% and 60% of vitamin E with chylomicrons and I-HDL, respectively. To identify apolipoproteins present in different fractions, we also measured apoB and apoA-I in these fractions. TC-treated cells secreted very small amounts of apoB, whereas cells treated with OA:TC secreted significantly higher amounts of apoB (Fig. 4D) in fractions 1–5, as observed previously (28, 29, 33). Under both conditions, the majority of apoA-I was in the bottom

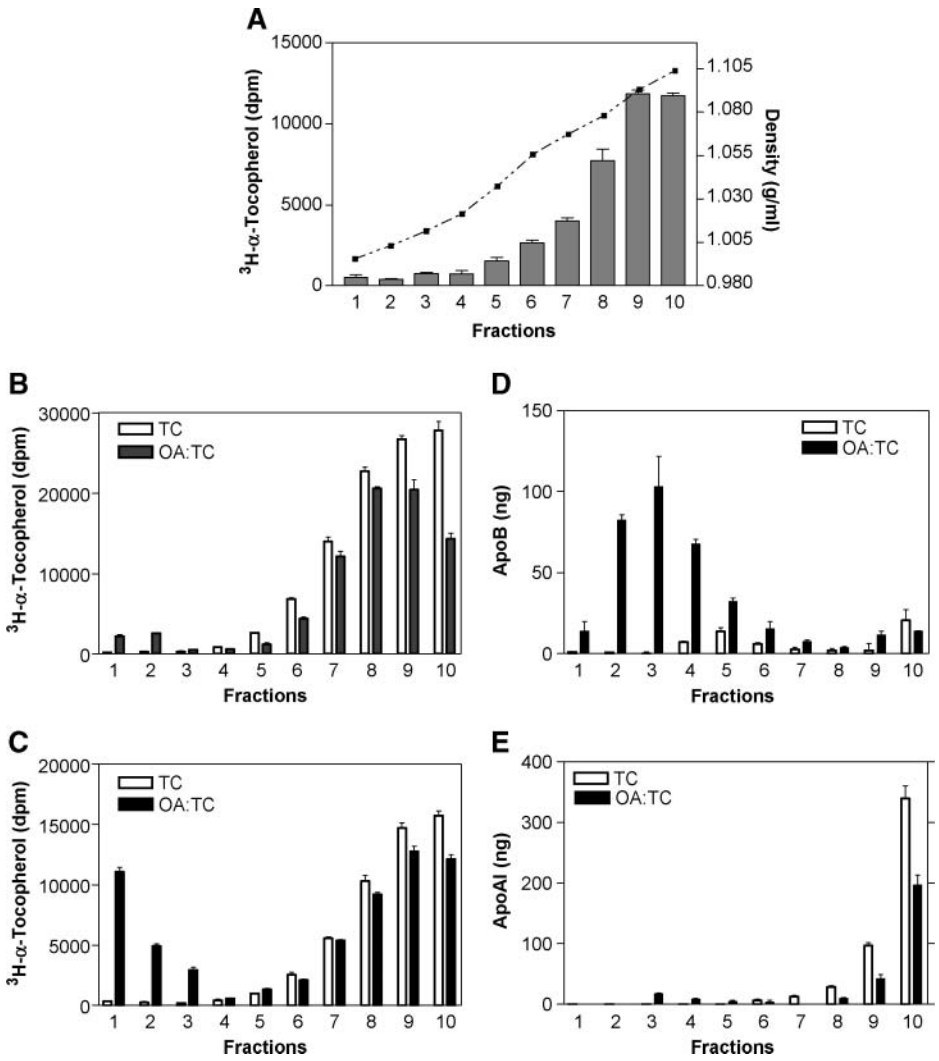

Fig. 4. Secretion of vitamin E by Caco-2 cells. A: Floatation properties of vitamin E. Vitamin E (1 μCi/ml) solubilized in Tween-40 (29 μg/ml) was subjected to density gradient ultracentrifugation as described in Materials and Methods. Fractions were collected from the top, and aliquots were used for radioactivity measurements. B: Secretion during continuous pulse. Differentiated Caco-2 cells were treated apically with 2 ml of SFM containing radiolabeled vitamin E (1 μCi/ml) solubilized in Tween-40 (29 μg/ml) and TC (0.5 mM) or OA:TC (1.6:0.5 mM). After 18 h, basolateral medium was collected and subjected to density gradient ultracentrifugation. Aliquots from each fraction were counted. C: Secretion during pulse-chase. Differentiated Caco-2 cells were supplemented apically with SFM containing radiolabeled vitamin E (1 μCi/ml) in Tween-40 (29 μg/ml) for 24 h. Cells were washed with SFM and treated apically with SFM containing either TC (0.5 mM) or OA:TC (1.6:0.5 mM) for 18 h. Basolateral medium was subjected to density gradient ultracentrifugation, and amounts of radioactivity were measured. D: ApoB secretion. ELISA was used to measure apoB in different fractions obtained in C. E: ApoA-I secretion. ELISA was used to measure apoA-I present in different fractions. All data are presented as means ± SD.

fractions (Fig. 4E). These studies indicate that Caco-2 cells secrete vitamin E with I-HDL in the absence of OA and secrete it with I-HDL and apoB-containing lipoproteins after OA supplementation.

In Fig. 4, supplemented tocopherol and tocopherol secreted with I-HDL were in similar fractions. To distinguish between supplemented and secreted vitamin E, we used a different density gradient (**Fig. 5**). Tween-40-solubilized vitamin E was mainly present in ~1.1 g/ml density fractions (Fig. 5A, fractions 4 and 5). In contrast, the majority of the vitamin E secreted by TC-treated cells was in the bottom dense fractions (Fig. 5A, fractions 9–11). We also measured the distribution of apoA-I in these fractions (Fig. 5B). ApoA-I was present in all of the fractions, signifying its association with a broad range of secreted lipoproteins. However, secreted vitamin E was only associated with a subset of lipoproteins that correspond to small, dense HDL particles. These studies show that secreted vitamin E has different floatation properties than Tween-40-solubilized vitamin E and that vitamin E is most likely secreted with small, dense apoA-I-containing lipoproteins.

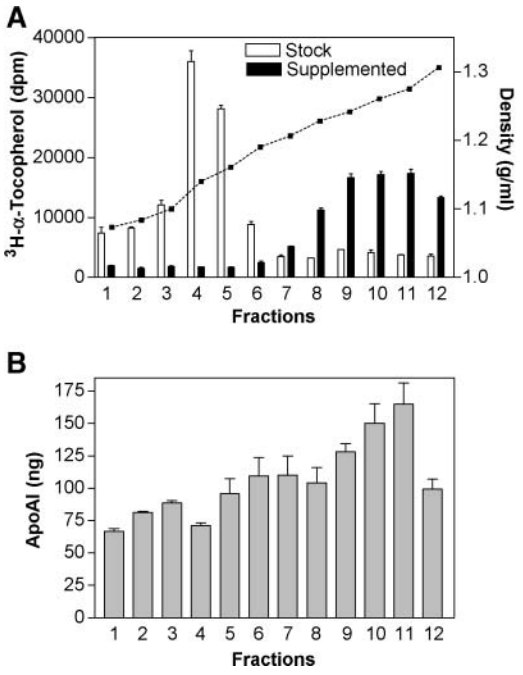

Fig. 5. Characterization of supplemented and secreted vitamin E. To vitamin E (1 μCi/ml) solubilized in Tween-40 (29 μg/ml) or conditioned medium (4 ml) was added 1.792 g of KBr to obtain a final density of 1.34 g/ml. The medium was then overlaid with 2 ml each of 1.24 g/ml, 1.15 g/ml, and 1.063 g/ml density solutions, followed by 1 ml of 1.019 g/ml and 1.006 g/ml density solutions, and subjected to ultracentrifugation (SW41, 17.5 h, 40,000 rpm, 15°C). The gradient was fractionated into 1 ml fractions. Aliquots from each fraction were then used to measure radioactivity (A) or apoA-I (B). All data are presented as means ± SD.

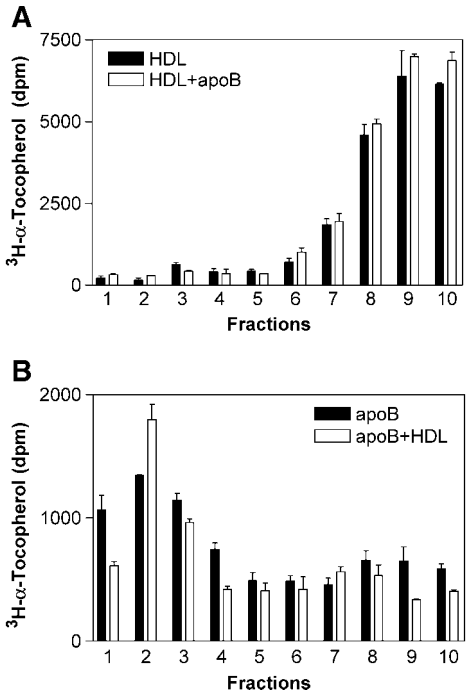

Fig. 6. No extracellular transfer of vitamin E was seen between lipoproteins. To study the possible transfer of vitamin E between lipoproteins, Caco-2 cells were pulsed-labeled with vitamin E for 6 h and then treated with OA:TC for 18 h. In parallel, cells were treated similarly except for the omission of radiolabeled vitamin E. The basolateral medium was collected and subjected to density gradient ultracentrifugation. Three 4 ml fractions were collected from the labeled and unlabeled samples. The top and bottom 4 ml fractions corresponding to chylomicrons and intestinal high density lipoprotein (I-HDL), respectively, were used for mixing experiments. A: I-HDL (2 ml) was mixed with unlabeled chylomicrons (2 ml), incubated at 37°C for 2 h, and subjected to density gradient ultracentrifugation. B: Similarly, 2 ml of vitamin E-containing chylomicrons was mixed with unlabeled I-HDL, incubated, and centrifuged. Radioactivity in different fractions was measured. All data are presented as means ± SD.

In liver-derived cells, vitamin E has been shown to be secreted independent of apoB-lipoproteins, and its association with VLDL has been suggested to occur extracellularly. Thus, we considered the possibility that vitamin E found associated with chylomicrons might be attributable to extracellular transfer. To study extracellular transfer of vitamin E between I-HDL and chylomicrons, we incubated labeled and unlabeled lipoproteins and subjected them to density gradient ultracentrifugation. Incubation of I-HDL containing radiolabeled vitamin E with unlabeled chylomicrons did not result in any measurable transfer of vitamin E to chylomicrons (**Fig. 6A**). Similarly, there was no measurable transfer of vitamin E from chylomicrons to I-HDL (Fig. 6B). From these data, we conclude that there is no measurable transfer of vitamin E among lipoproteins after secretion under these conditions.

Role of chylomicron assembly and the efflux pathway in vitamin E secretion

We hypothesize that the presence of vitamin E in two different lipoprotein fractions might represent secretion by two independent mechanisms: intracellular chylomicron assembly and efflux to I-HDL. To test this hypothesis, we used MTP antagonists and efflux inhibitors (**Fig. 7**). MTP is a required chaperone for the intracellular assembly of apoB-lipoproteins (12, 13), whereas ABCA1 is critical for cholesterol efflux to HDL (45, 46). ABCA1 has been shown to be expressed on the basolateral side of Caco-2 cells (33, 47, 48). Differentiated Caco-2 cells were radiolabeled with vitamin E and then treated with OA:TC or OA:TC plus the MTP inhibitor BMS197636. The MTP antagonist specifically inhibited the secretion of vitamin E with apoB-lipoproteins (fractions 1–3) without affecting its secretion with I-HDL (Fig. 7A). To gain some insight into the mechanisms of vitamin E secretion with I-HDL, Caco-2 cells were pulse-labeled with vitamin E and then chased by providing TC on the apical side (Fig. 7B). The basolateral side received medium supplemented with or without glyburide, an antagonist of ABC transporters (33, 47, 49). TC-treated cells mainly secreted vitamin E with I-HDL, in agreement with Fig. 4C. No vitamin E was in the chylomicron fractions because cells were not treated with OA. Glyburide inhibited vitamin E secreted with I-HDL. These data suggest that efflux may play a role in the transport of vitamin E with I-HDL, whereas MTP activity is required for vitamin E secretion with chylomicrons.

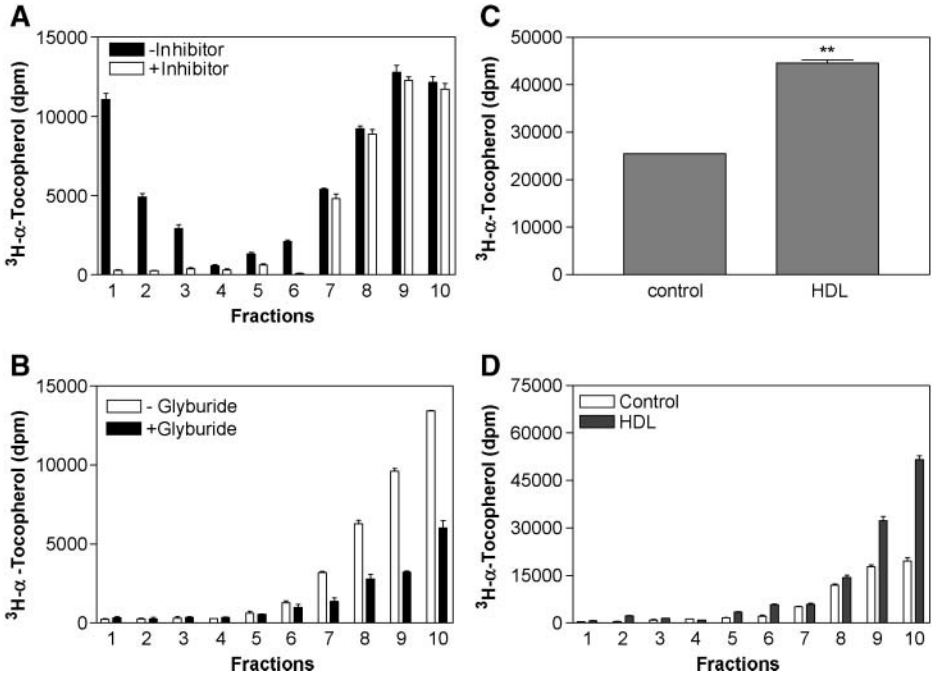

Fig. 7. Role of microsomal triglyceride transfer protein (MTP) and efflux in vitamin E secretion. A: Differentiated Caco-2 cells were incubated with radiolabeled vitamin E (1 μCi/ml) and Tween-40 (29 μg/ml) for 24 h. Cells were washed and supplemented apically with SFM containing OA:TC (1.6:0.5 mM) either with (+Inhibitor) or without (−Inhibitor) 1 μM BMS197636 for 18 h. Basolateral medium was collected and subjected to density gradient ultracentrifugation. B: Differentiated Caco-2 cells were pulse-labeled with Tween-solubilized vitamin E. After 24 h, cells were washed and supplemented with SFM containing TC on the apical side. The basolateral side received SFM with or without glyburide (1 mM). After 18 h, conditioned medium from the basolateral side was collected and subjected to ultracentrifugation, and radioactivity was measured in each fraction. C: Differentiated Caco-2 cells were incubated with radiolabeled vitamin E (1 μCi/ml) and Tween-40 (29 μg/ml) for 24 h. Cells were washed and supplemented apically with SFM containing TC (0.5 mM). The basolateral side received SFM with or without 100 μg of human plasma HDL. After 18 h, basolateral medium was collected and counted for total radioactivity secreted (** $P < 0.01$). D: The remaining basolateral medium was then subjected to density gradient ultracentrifugation. Radioactivity in different fractions was measured. All data are presented as means ± SD.

To further substantiate the role of efflux, we determined whether exogenous HDL would act as an acceptor of vitamin E. Caco-2 cells were pulse-labeled and treated with TC. During the treatment, the basolateral medium was supplemented with or without plasma HDL (Fig. 7C, D). Incubation with exogenous plasma HDL increased total vitamin E secretion by 75% compared with the control ($P < 0.01$) (Fig. 7C). Further analysis by density gradient ultracentrifugation revealed that the increase was mainly in I-HDL (Fig. 7D). These results indicate that HDL can facilitate vitamin E efflux from Caco-2 cells.

Involvement of secretory pathways in vitamin E secretion with chylomicrons

Brefeldin A and monensin are known to interfere with the transport of secretory vesicles from the endoplasmic reticulum to the Golgi and from the Golgi to the plasma membrane, respectively (50). Brefeldin A decreased apoB secretion in a dose-dependent manner (**Fig. 8A**). Next, we studied the effect of brefeldin A on the secretion of vitamin E with chylomicrons (Fig. 8B, fractions 1–4) and I-HDL (Fig. 8B, fractions 5–10). Brefeldin A inhibited vitamin E secretion with chylomicrons in a concentration-dependent manner, whereas its secretion with I-HDL was not affected by this treatment (Fig. 8B). Similar studies were also performed with monensin. Again, monensin inhibited apoB secretion in a dose-dependent manner (Fig. 8C) and only inhibited vitamin E secretion with chylomicrons (Fig. 8D). These data suggest that secretion of vitamin E with apoB-lipoproteins is sensitive to interruptions in the secretory pathways, whereas the HDL pathway is resistant to these treatments.

DISCUSSION

Two independent pathways for the transport of vitamin E by Caco-2 cells

It is generally believed that dietary vitamin E is transported across the intestinal cells with chylomicrons. Our studies show that differentiated Caco-2 cells use two independent pathways to transport vitamin E. One involves the intracellular incorporation of vitamin E with apoB-lipoproteins and secretion, and the other involves extracellular efflux (**Fig. 9**). MTP antagonists (Fig. 7), brefeldin A, and monensin (Fig. 8) inhibit the apoB

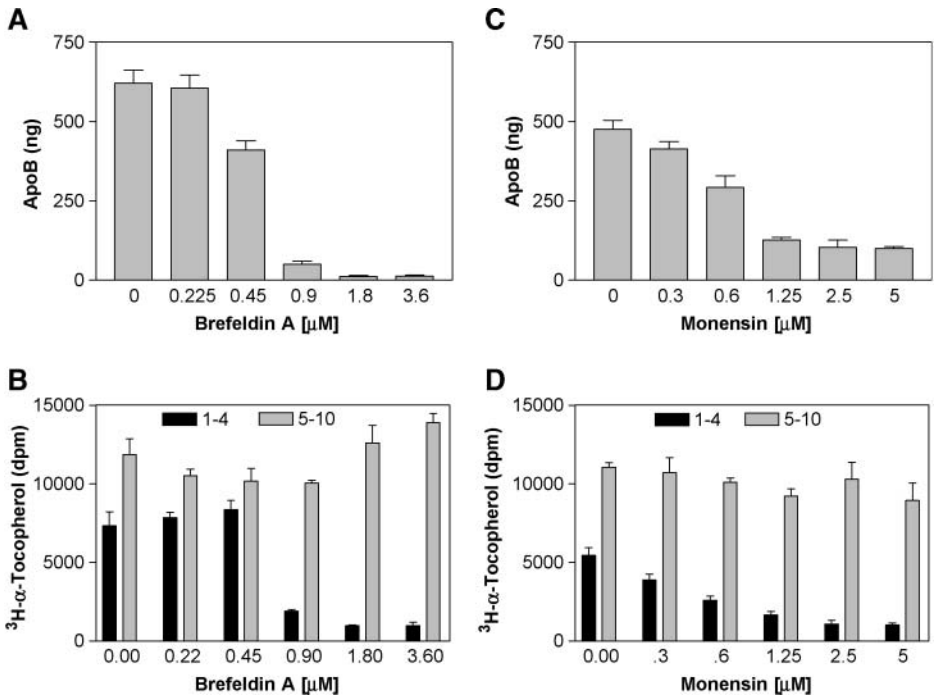

Fig. 8. Effects of brefeldin A and monensin on vitamin E secretion by Caco-2 cells. Differentiated Caco-2 cells were labeled with Tween-solubilized vitamin E for 24 h. Cells were washed and supplemented with SFM containing OA:TC and increasing concentrations of brefeldin A (A, B) or monensin (C, D) on the apical side. The basolateral side received SFM. After 18 h, conditioned medium from the basolateral side was collected. ELISA was used to determine apoB in the conditioned basolateral medium. Medium was also subjected to density gradient ultracentrifugation. Fractions 1–4 (apoB-lipoproteins) and 5–10 (I-HDL) were pooled, and radioactivity was measured. All data are presented as means ± SD.

pathway, whereas OA (Fig. 4) induces vitamin E secretion with apoB-lipoproteins. All of these treatments have no effect on the efflux pathway. In contrast, efflux is increased by exogenous HDL and inhibited by glyburide (Fig. 7). Thus, secretion of vitamin E by Caco-2 cells involves two independent pathways that can be differentially induced by agonists and obliterated by specific antagonists.

The mechanism of the secretion of vitamin E by Caco-2 cells appears to be very different from that observed in hepatoma cell lines. In liver-derived cells, the vitamin E secretion pathway is independent of the intracellular assembly of VLDL. A cytoplasmic αTTP has been suggested to deliver vitamin E to ABCA1 for efflux by hepatic cells (17, 18, 20, 22). Vitamin E is then transferred to apoB-lipoproteins extracellularly by unidentified mechanisms.

It is known that chylomicron assembly is critical for the absorption because of the absence of vitamin E in the plasma of abetalipoproteinemia subjects (5). The vitamin E deficiency in these patients was successfully treated by oral supplementation of the vitamin, which resulted in increased plasma and tissue levels of α-tocopherol, and this treatment was effective in preventing and ameliorating the neurologic abnormalities associated with vitamin E deficiency in these subjects (5, 51). However, the absorption routes of vitamin E in these patients have not been elucidated (5). Two possible mechanisms, synthesis of I-HDL and incorporation of vitamin E into these particles or direct absorption into the portal stream, have been suggested (5). Based on the data presented here, we propose that vitamin E might be absorbed via the I-HDL pathway when given as large oral doses. It should be noted that the intestine is the major organ synthesizing apoA-I (52) and could contribute to I-HDL assembly. In fact, our recent studies indicate that intestine contributes ~30% of plasma HDL in mice (31, 53). This pathway might contribute to the absorption of vitamin E in the absence of the chylomicron pathway in abetalipoproteinemia patients receiving large doses of the vitamin.

Recent studies indicate that cholesterol transport by Caco-2 cells as well as primary rat enterocytes involves two distinct apoB-lipoproteins and I-HDL pathways (26). These pathways have been shown to be independently regulated and to complement each other (31). In contrast, triglycerides and retinyl esters are mainly transported via apoB-lipoproteins (29). Thus, fat and fat-soluble vitamins use multiple mechanisms for their transport across the intestinal epithelial cells.

Microsomal membranes are the central sites of cellular vitamin E accumulation

Our studies show that the vitamin E taken up by Caco-2 cells is rapidly transported to the microsomal membranes. It remains to be determined which organelle in the microsomal fraction is the major site of vitamin E accumulation in Caco-2 cells. During secretion, vitamin E decreased in the membrane fraction (Fig. 3C). However, we

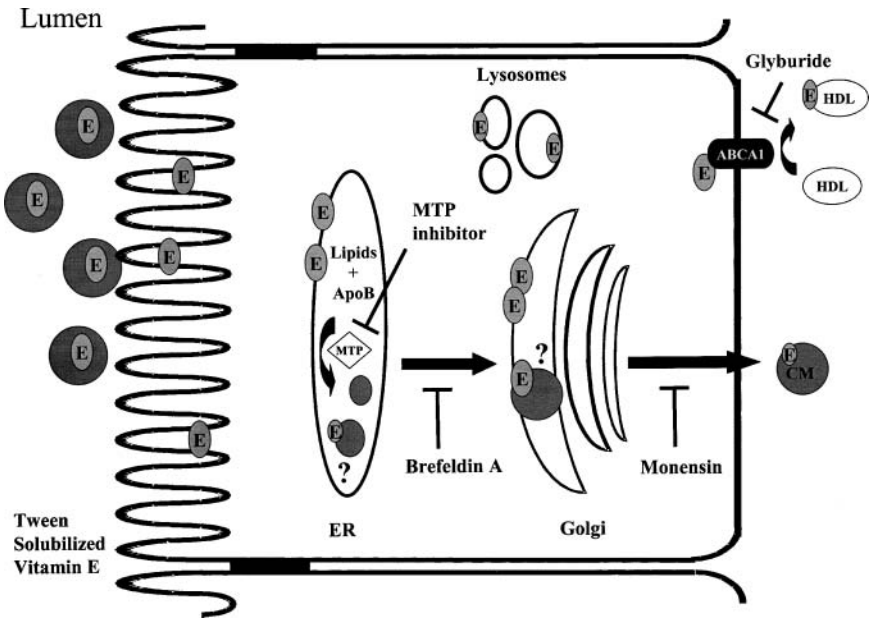

Fig. 9. Schematic diagram showing the transport of vitamin E across the differentiated Caco-2 cells. Tween-solubilized vitamin E is supplemented toward the apical side. This results in the uptake of vitamin E by differentiated Caco-2 cells. After uptake, vitamin E rapidly associates with microsomes, where it can associate with the major microsomal components, namely the endoplasmic reticulum (ER), the Golgi apparatus, and lysosomes. Induction of lipoprotein synthesis leads to the secretion of vitamin E with chylomicrons (CM), and this secretion is inhibited by MTP inhibitor and the secretory inhibitors brefeldin A and monensin. The efflux pathway for vitamin E secretion is independent of lipoprotein synthesis and can be inhibited or increased by glyburide or exogenous HDL, respectively. Question marks denote possible sites for vitamin E incorporation into chylomicrons.

were unable to establish any increase in other compartments concomitant with this decrease. We interpret these data to suggest that the release of vitamin E from the microsomal membrane is the rate-limiting step and that other steps during the secretion are very rapid.

Brefeldin A and monensin inhibited the mobilization of vitamin E from microsomal membranes and secretion with apoB-lipoproteins, suggesting that intracellular vesicular trafficking is critical for this process. In contrast, the I-HDL pathway was resistant to these treatments, indicating that this pathway does not involve intracellular vesicular transport. In the liver, αTTP is known to transfer vitamin E from lysosomes to plasma membranes (22). However, αTTP is not expressed in Caco-2 cells and enterocytes. It is possible that these cells may express another protein with the same function that might be induced after exposure to high doses of vitamin E.

Vitamin E uptake by intestinal cells

Tocopherols have been delivered to bovine aortic endothelial cells and U937 cells using ethanol (54, 55) and to Caco-2 cells using TC and OA (15). Arita et al. (18) used tocopheryl acetate for cellular delivery of vitamin E to rat hepatoma cells, whereas lipoproteins were used by Qian et al. (22). We observed that Tween-40 is a better agent for the solubilization and delivery of vitamin E to Caco-2 cells (Fig. 1), in agreement with other studies (56). The enhanced delivery of vitamin E to cells by Tween-40 is also in agreement with animal studies describing the superior uptake of vitamin E in the presence of polysorbate 80 (also known as Tween-80 or Aquasol E) than from oily solutions (4). In fact, polysorbate 80-solubilized vitamin E delivery is the method of choice for vitamin E administration in total prenatal nutrition and under other conditions of fat-soluble vitamin malabsorption (4). These studies indicate that emulsifiers, such as Tween-40 and Tween-80, are better vehicles for vitamin E solubilization and delivery to intestinal cells. Tweens (polyoxyethylene sorbitan esters) are nontoxic surfactants that are used extensively in the emulsification and solubilization of drugs and fat-soluble vitamins (for reviews, see 56, 57). Tweens are believed to alter permeability reversibly and to allow the entry of various substances that are ordinarily unable to enter intact cells (58).

Our studies also demonstrate that careful standardization is required to obtain optimal uptake, as increasing concentrations of Tween-40 decreased the cellular uptake of vitamin E (Fig. 1). The decreased uptake was not attributable to cellular toxicity, because Tween-40 had no effect on the apoB secretion, and is in agreement with studies showing no cellular toxicity (56). The inhibition of vitamin E uptake by higher concentrations of Tween-40 may be related to the formation of Tween-40 micelles. In micelles, vitamin E may partition into the core, limiting its availability for cellular uptake. If this is true, the solubilization

of vitamin E with Tween-40 monomers may be more useful for cellular delivery in vivo and in cell cultures. Similar mechanisms may explain the inhibitory effect of serum on vitamin E uptake (Fig. 1G).

In summary, our studies indicate that an appropriate solubilizing and delivery reagent is required for the cellular uptake of vitamin E. After uptake, Caco-2 cells store vitamin E in the microsomal membranes, and release from these membranes is perhaps the rate-limiting step in the secretion of vitamin E. This is the first report describing the incorporation and secretion of vitamin E along with apoB-containing lipoproteins by intestinal epithelial cells in culture. The secretion of vitamin E with the apoB pathway involves an intracellular association with these lipoproteins and is dependent on intracellular vesicular trafficking. This secretion is induced by high concentrations of OA and inhibited by MTP antagonists, brefeldin A, and monensin. The appearance of vitamin E with I-HDL is enhanced by HDL and inhibited by glyburide and most likely represents extracellular efflux. This pathway might contribute to vitamin E absorption in patients with abetalipoproteinemia who receive large oral doses of vitamin E.

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