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# ATP-binding cassette transporter A1 is significantly involved in the intestinal absorption of $\alpha$ - and $\gamma$ -tocopherol but not in that of retinyl palmitate in mice<sup>1-3</sup>

Emmanuelle Reboul, Doriane Trompier, Myriam Moussa, Alexis Klein, Jean-François Landrier, Giovanna Chimini, and Patrick Borel

## ABSTRACT

**Background:** It has long been assumed that newly absorbed vitamin A and E enter the body only via enterocyte-produced chylomicrons. However, recent results in cell cultures have shown that a fraction of  $\alpha$ -tocopherol is secreted with intestinal HDL.

**Objective:** The aims of this study were to identify this transporter and to assess whether it is significantly implicated in the in vivo intestinal absorption of the 2 main dietary forms of vitamin E (ie,  $\alpha$ - and  $\gamma$ -tocopherol) and in that of retinyl palmitate (vitamin A).

**Design:** Having performed preliminary experiments in the Caco-2 cell model, we compared fasting and postprandial plasma concentrations of vitamins A and E in mice deficient in ATP-binding cassette A1 (ABCA1) transporter and in wild-type mice.

**Results:** A substantial efflux of  $\alpha$ - and  $\gamma$ -tocopherol, but not of retinyl esters, was induced by the presence of apolipoprotein A-I at the basolateral side of Caco-2 monolayers. The efflux of  $\alpha$ - and  $\gamma$ -tocopherol was also impaired by glyburide and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid. The postprandial response of plasma  $\gamma$ -tocopherol was 4-fold lower in ABCA1<sup>-/-</sup> mice ( $P = 0.025$ ) than in wild-type mice, whereas no significant difference was observed for retinyl esters. Fasting plasma  $\alpha$ -tocopherol, but not vitamin A, concentrations were lower in mice bearing the genetic deletion.

**Conclusions:** ABCA1 is the transporter responsible for the in vivo secretion of  $\alpha$ - and  $\gamma$ -tocopherol with intestinal HDL, and this pathway is significantly implicated in the intestinal absorption and plasma status of vitamin E but not of vitamin A. *Am J Clin Nutr* 2009;89:1-8.

## INTRODUCTION

Vitamins A and E are fat-soluble micronutrients that occur in the human diet mainly as retinyl palmitate (preformed vitamin A found in foods from animal origin) and as  $\alpha$ - and  $\gamma$ -tocopherol (the 2 main dietary forms of vitamin E in Western countries). Vitamin A has 2 main functions in the human body: the first is related to vision and is typically mediated via 9-*cis* retinal (1), whereas the second is related to the control of gene expression via *all-trans* and 9-*cis* retinoic acid (2). Vitamin E, although primarily known for its antioxidant properties, can also directly modulate cell signaling, proliferation, and gene expression (3). In the context of the novel areas of nutritional genetics, the identification of the proteins, and thus genes, controlling the intestinal

absorption of micronutrients is considered crucial, because it is potentially instructive for dietary approaches aimed at compensating for genetically determined inadequate status. It has long been assumed that most of the newly absorbed vitamin A and vitamin E is incorporated into chylomicrons (4) as retinyl esters (5) and nonesterified tocopherols, respectively, and is then secreted into the lymph via a pathway dependent on apolipoprotein (apo) B (6). A growing body of evidence suggests that the intestine secretes important amounts of HDL during the postprandial period. This pathway is mainly orchestrated by the ATP-binding cassette A1 (ABCA1) membrane transporter, and it is considered that intestinal ABCA1 contributes to  $\approx 30\%$  of steady state plasma cholesterol concentrations in mice (7). Because the ABCA1 transporter controls HDL formation through the cellular efflux of a broad range of lipids (8-11) and because Oram et al (12) showed that ABCA1 is involved in  $\alpha$ -tocopherol secretion in macrophages, the recent findings in in vitro models showing that a fraction of  $\alpha$ -tocopherol is secreted with intestinal HDL by an ABC transporter (13, 14) and that retinol efflux from intestinal cells is probably facilitated by ABCA1 (15) were not surprising. However, the identification of this ABC transporter and the importance of this pathway in the in vivo intestinal absorption and long-term fasting plasma concentration of the 2 main dietary forms of vitamin E ( $\alpha$ - and  $\gamma$ -tocopherol) and the main

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<sup>2</sup> The work at the Centre d'Immunologie de Marseille Luminy was supported by institutional grants from INSERM and CNRS and specific grants (FLIPPASE/MPCM) from the European Community. DT was supported in part by a fellowship allocated by the Nouvelle Société Française d'Athérosclérose/Pfizer. The protease inhibitor cocktail was a generous gift from F Tosini (Avantage Nutrition, Marseille, France). Caco-2 clone TC-7 cells were a gift from M Rousset (U178 INSERM, Villejuif, France).

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form of vitamin A (retinyl palmitate) have not been assessed yet. Thus, the aims of this study were to determine the role of ABCA1 and of retinyl esters in the basolateral secretion of  $\alpha$ - and  $\gamma$ -tocopherol by using a Caco-2 cell model and ABCA1-deficient mice. To this end, we studied 1) the basolateral efflux of these vitamins in differentiated intestinal cell monolayers in the presence of several molecules assumed to modulate ABCA1-mediated efflux [apo A-I, glyburide, and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS)] and 2) the fasting and postprandial plasma concentrations of vitamins A and E in wild-type and ABCA1-deficient mice.

## MATERIALS AND METHODS

### Chemicals

*R,R,R*- $\alpha$ -Tocopherol ( $\geq 99\%$  pure), *R,R,R*- $\gamma$ -tocopherol ( $\geq 97\%$  pure), and *all-trans*-retinol ( $\geq 99\%$  pure) were purchased from Fluka (Vaulx-en-Velin, France). Tocol, used as an internal standard for vitamin E HPLC analysis, was purchased from Lara Spiral (Couternon, France). *all-trans*-Retinyl palmitate ( $\geq 85\%$  pure) and retinyl acetate (used as an internal standard for vitamin A HPLC analysis), 2-oleoyl-1-palmitoyl-*sn*-glycero-3-phosphocholine (phosphatidylcholine), 1-palmitoyl-*sn*-glycero-3-phosphocholine (lysophosphatidylcholine), monoolein, nonesterified cholesterol, oleic acid, sodium taurocholate, pyrogallol, glyburide, and DIDS were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Purified native apo A-I (purity  $\geq 95\%$ ) was purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). DMEM containing 4.5 g/L glucose and trypsin-EDTA (500 and 200 mg/L, respectively) were purchased from BioWhittaker (Fontenay-sous-Bois, France), fetal bovine serum came from Biomedica (Issy-les-Moulineaux, France), and nonessential amino acids, penicillin/streptomycin, and phosphate-buffered saline were purchased from Invitrogen (Cergy-Pontoise, France). The protease inhibitor cocktail was provided by F Tosini (Avantage Nutrition, Marseille, France). All solvents used were HPLC grade from sodium dodecyl sulfate (Peypin, France).

### Caco-2 TC-7 cell studies

#### Preparation of vitamin-rich micelles

For the delivery of fat-soluble vitamins to cells, either tocopherols or retinol were incorporated into mixed micelles that had a lipid composition similar to those found in vivo (16). Micelles were prepared as previously described (17) to obtain the following final lipid concentrations: 0.04 mmol/L phosphatidylcholine, 0.16 mmol/L lysophosphatidylcholine, 0.3 mmol/L monoolein, 0.1 mmol/L nonesterified cholesterol, 0.5 mmol/L oleic acid, and 5 mmol/L taurocholate. Vitamin concentrations in the micellar solutions were checked before each experiment.

#### Cell culture

Caco-2 clone TC-7 cells (18, 19) were provided by M Rousset (U178 INSERM, Villejuif, France). Cells were cultured in the presence of DMEM supplemented with 20% heat-inactivated fetal bovine serum, 1% nonessential amino acids, and 1% antibiotics (complete medium), as previously described (17). For each experiment, cells were seeded and grown on transwells as

previously described (17) to obtain confluent, differentiated cell monolayers. Twelve hours before each experiment, the medium used in apical and basolateral chambers was a serum free complete medium. During preliminary tests, the integrity of the cell monolayers was checked by measuring *trans*-epithelial electrical resistance before and after the different experiments (including those with ABCA1 inhibitors) by using a voltohmmeter fitted with a chopstick electrode (Millicell ERS; Millipore, Saint-Quentin-en-Yvelines, France).

#### Measurement of the basolateral efflux of vitamins A and E

At the beginning of each experiment, cell monolayers were washed twice with 0.5 mL phosphate-buffered saline. The apical side of the cell monolayers received either *R,R,R*- $\alpha$ -tocopherol (31  $\mu\text{mol/L}$ ), *R,R,R*- $\gamma$ -tocopherol (27  $\mu\text{mol/L}$ ), or retinol (80  $\mu\text{mol/L}$ ) vitamin-rich micelles at the apical side, whereas the other side received the serum free complete medium. A concentration of  $\approx 30$  mmol/L was chosen to have a good cellular amount of newly absorbed vitamin E, as shown in our previous work (17), and to accurately measure the basolateral efflux of vitamin E. Cells were incubated for up to 24 h at 37°C. Aliquots of the basolateral medium were taken at different times and replaced by the same volume of new medium.

#### Effect of apo A-I on the basolateral efflux of vitamins A and E

Cell monolayers received serum free basolateral medium supplemented or not with apo A-I at 5  $\mu\text{g/mL}$  (20). Efflux was measured as described above.

#### Effect of putative chemical inhibitors of ABCA1 (glyburide and DIDS) on the basolateral efflux of vitamin E

Cell monolayers received serum free basolateral medium containing dimethyl sulfoxide (control), 400  $\mu\text{mol/L}$  glyburide [usually used up to 1 mmol/L (8)], or 400  $\mu\text{mol/L}$  DIDS (21). Efflux was measured as described above. All the samples were stored at  $-80^\circ\text{C}$  under nitrogen with 0.5% pyrogallol as a preservative before vitamin extraction and HPLC analysis. Aliquots of cell samples without pyrogallol and supplemented with 20  $\mu\text{L}$  protease inhibitor cocktail were used to assess protein concentrations with a bicinchoninic acid kit (Pierce, Montluçon, France).

### Vitamin bioavailability in wild-type, ABCA1<sup>+/-</sup>, and ABCA1<sup>-/-</sup> mice

All animal studies were approved by the local ethics committee.

#### Preparation of vitamin-rich emulsions

For delivery of either vitamin A or E to mice, oil-in-water vitamin-rich emulsions were prepared as follows. An appropriate volume of *R,R,R*- $\gamma$ -tocopherol or retinyl palmitate stock solution was transferred to microtubes to obtain a final amount of 5 mg in each tube. Stock solution solvent was carefully evaporated under nitrogen. Dried residue was solubilized in 100  $\mu\text{L}$  Isio 4 vegetable oil (Lesieur, Asnières-sur-Seine, France), to which 200  $\mu\text{L}$  of 0.9% NaCl was added. The mixture was vigorously mixed in ice-cold water in a sonication bath (Branson 3510; Branson) for 15 min and used for force-feeding within 10 min.

[AQ1]

[AQ2]

### Animals

ABCA1<sup>-/-</sup> and ABCA<sup>+/-</sup> mice, generated in pure DBA/1acJ background as described previously (22), were bred locally, housed in a temperature-, humidity-, and light-controlled room and maintained on a standard nonpurified diet with water ad libitum. Three to 6-mo-old mice were used for the study. The mice were starved overnight before each experiment. On the day of the experiment, a first blood sample (fasting) was obtained (zero baseline sample) by cutting the extremity of the tail. The mice were then force-fed with either a *R,R,R*- $\gamma$ -tocopherol- or a retinyl palmitate-enriched emulsion. Additional blood samples were taken at regular intervals after force-feeding. Plasma samples were stored at -80°C under nitrogen before lipid and vitamin analysis.

### Analysis of ABCA1 expression in gut samples

Intestinal samples (first 10 cm after the pylorus) were excised from killed age- and sex-matched wild-type and ABCA1<sup>-/-</sup> mice. After being extensively washed in phosphate-buffered saline, the mucosa layer was scraped and solubilized in 1% Triton X-100, 150 mmol/L NaCl, and 50 mmol/L Tris HCl (pH 7.4) for 30 min at 4°C. Homogenates were then centrifuged at 4°C at 15,000  $\times g$  for 15 min, and protein concentration in the cleared postnuclear supernatant fluid were determined. After preclearing (90 min at 4°C) with protein G-Sepharose beads (Amersham Biosciences), lysates were immunoprecipitated (overnight at 4°C) with rat anti-mouse ABCA1 monoclonal antibody (clone 1156) and with protein G-Sepharose beads (90 min at 4°C). Immunoprecipitated complexes were then eluted in loading buffer, and further resolved by 5.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis. After transfer onto a nitrocellulose membrane, the samples were probed with the 891.3 anti-ABCA1 monoclonal antibody (23).

### Vitamin A and E extraction and HPLC analysis

Vitamins A and E were extracted from 500- $\mu$ L aqueous samples by using the following method. Distilled water was added to sample volumes <500  $\mu$ L to reach a final volume of 500  $\mu$ L. Retinyl acetate and tocol, which were used as internal standard for vitamins A and E, respectively, were added to the samples in 500  $\mu$ L ethanol. The mixture was extracted once with 1 volume of hexane for vitamin E and twice with 2 volumes of hexane for vitamin A. The hexane phases obtained after centrifugation (500  $\times g$ , 10 min, 4°C) were pooled if necessary and evaporated to dryness under nitrogen. The dried extracts were dissolved in either 100  $\mu$ L acetonitrile/dichloromethane (50/50, vol:vol) for vitamin A or 100  $\mu$ L methanol for vitamin E. A volume of 5–80  $\mu$ L was used for HPLC analysis. All the analyses were realized by using a 250  $\times$  4.6 nm reversed-phase C<sub>18</sub> 5- $\mu$ m Zorbax column (Interchim, Montluçon, France) maintained at a constant temperature (25°C) and a guard column. Vitamin A was analyzed with a 70% acetonitrile, 20% dichloromethane, and 10% methanol mobile phase (flow rate = 1.8 mL/min), whereas vitamin E was analyzed with a 100% methanol mobile phase (flow rate = 1.5 mL/min). The HPLC system comprised a Dionex separation module (P680 HPLC Pump and ASI-100 Automated Sample Injector; Dionex, Aix-en-Provence, France), a Dionex UVD340U photodiode array detector (vitamin A

detection at 325 nm), and a Jasco fluorimetric detector (Jasco, Nantes, France). For the fluorimetric analysis, vitamin A was detected at 450 nm after light excitation at 325 nm, and tocopherols were detected at 325 nm after light excitation at 292 nm. Both vitamins were identified by retention time compared with pure (>95%) standards. Quantification was performed by using Chromeleon software (version 6.50, SP4 Build 1000) comparing peak area with standard reference curves. Retinyl linoleate, retinyl oleate, and retinyl stearate were identified by retention time and spectral analysis and quantified on the basis of their molecular extinction coefficient ratio compared with retinyl palmitate.

### Lipid analysis of mouse plasma samples

Three to 5- $\mu$ L aliquots of each plasma sample were used for triglyceride, phospholipid, and cholesterol assays with kits from Biomérieux (Marcy l'Etoile, France). For lipid standardization, absorbed vitamin amounts were corrected for total plasma lipids (cholesterol + triglycerides + phospholipids).

### Statistical analysis

Differences between  $\geq 2$  groups of unpaired data underwent the nonparametric Kruskal-Wallis test. The nonparametric Mann-Whitney *U* test was used as a post hoc test when the Kruskal-Wallis test showed significant differences between groups. The Mann-Whitney *U* test was used to test for differences between only 2 groups of unpaired data. *P* values <0.05 were considered significant. All statistical analyses were performed by using Statview software (version 5.0; SAS Institute, Cary, NC).

## RESULTS

### Effect of apo A-I and 2 ABCA1 putative inhibitors on the secretion of vitamins A and E across the basolateral membrane of human intestinal cells

#### Effect of apo A-I

As shown in **Figure 1**, the addition of apo A-I (5  $\mu$ g/mL) to the basolateral medium significantly increased the basolateral secretion of  $\alpha$ - and  $\gamma$ -tocopherol, respectively, by differentiated Caco-2 cells (275.8  $\pm$  4.6 compared with 240.1  $\pm$  4.6 pmol/mg protein for  $\alpha$ -tocopherol and 201.4  $\pm$  2.2 compared with 176.6  $\pm$  4.7 pmol/mg protein for  $\gamma$ -tocopherol after 24 h of incubation). Conversely, apo A-I had no significant effect on the basolateral secretion of retinyl esters (**Figure 2**).

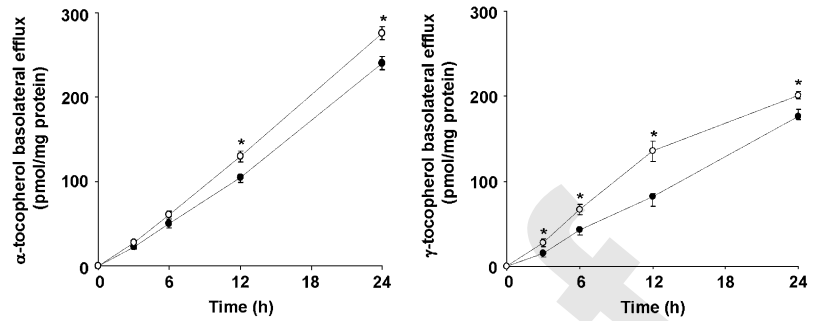
#### Effect of glyburide

This commonly used chemical inhibitor of ABCA1 significantly impaired vitamin E secretion compared with the control (180.9  $\pm$  15.8 compared with 240.1  $\pm$  4.6 pmol/mg protein for  $\alpha$ -tocopherol and 70.5  $\pm$  15.2 compared with 176.6  $\pm$  4.7 pmol/mg protein for  $\gamma$ -tocopherol after 24 h of incubation; **Table 1**).

#### Effect of DIDS

DIDS significantly impaired both  $\alpha$ - and  $\gamma$ -tocopherol secretion (46.3  $\pm$  0.8 compared with 240.1  $\pm$  4.6 pmol/mg protein for  $\alpha$ -tocopherol and 12.4  $\pm$  1.0 compared with 176.6  $\pm$  4.7 pmol/mg protein for  $\gamma$ -tocopherol after 24 h of incubation; **Table 1**).

**FIGURE 1.** Effect of apolipoprotein (apo) A-I on the basolateral efflux of  $\alpha$ - and  $\gamma$ -tocopherol by differentiated Caco-2 TC-7 cell monolayers. Cell monolayers differentiated on filters were treated with either *R,R,R*- $\alpha$ -tocopherol (31  $\mu$ mol/L; left panel) or *R,R,R*- $\gamma$ -tocopherol (27  $\mu$ mol/L; right panel) at the apical side and either fetal bovine serum-free medium (○) or fetal bovine serum-free medium supplemented with 5  $\mu$ g/mL apo A-I (●) at the basolateral side. Values are means  $\pm$  SEMs of 3 assays. \*Significant ( $P < 0.050$ ) difference between the 2 mean values obtained at the same time (Mann-Whitney *U* test).

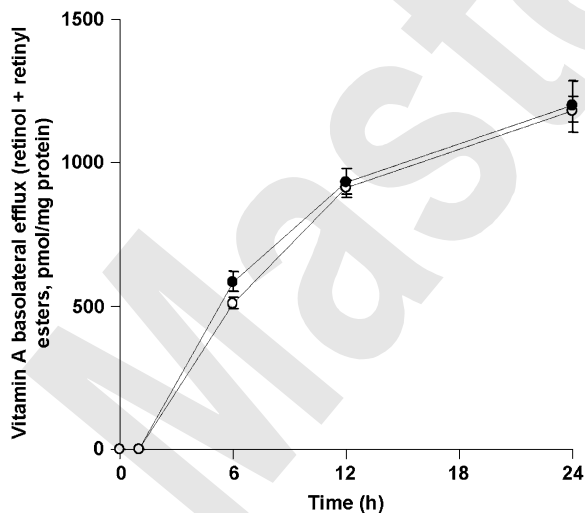


### Comparison of fasting and postprandial plasma lipid concentrations in wild-type, ABCA1<sup>+/-</sup>, ABCA1<sup>-/-</sup> mice

As expected, ABCA1 was detected in the intestine of wild-type mice but was not detected in the intestine of ABCA1<sup>-/-</sup> mice (see inset **Figure 3**). The data presented in **Table 2** show that the fasting plasma cholesterol concentration was significantly lower in ABCA1<sup>-/-</sup> mice than in wild-type mice, with an intermediate result in ABCA1<sup>+/-</sup> mice (0.052  $\pm$  0.002, 0.262  $\pm$  0.001, and 0.372  $\pm$  0.025 g/L for ABCA1<sup>-/-</sup>, ABCA1<sup>+/-</sup>, and wild-type mice, respectively). A comparable result was obtained for fasting plasma phospholipid concentrations (0.189  $\pm$  0.012, 0.622  $\pm$  0.031, and 0.741  $\pm$  0.073 g/L for ABCA1<sup>-/-</sup>, ABCA1<sup>+/-</sup>, and wild-type mice, respectively). Note that these differences remained during all of the postprandial experiment (Table 2).

### Comparison of fasting plasma vitamin A and E concentrations in wild-type, ABCA1<sup>+/-</sup>, and ABCA1<sup>-/-</sup> mice

Fasting plasma samples were obtained before the postprandial experiment. On fasting, vitamin A circulates as nonesterified



**FIGURE 2.** Effect of apolipoprotein (apo) A-I on the basolateral efflux of vitamin A (retinyl esters) by differentiated Caco-2 TC-7 cell monolayers. Cell monolayers differentiated on filters treated with retinol (80  $\mu$ mol/L) at the apical side and either fetal bovine serum-free medium (○) or fetal bovine serum-free medium supplemented with 5  $\mu$ g/mL apo A-I (●) at the basolateral side. Values are means  $\pm$  SEMs of 3 assays. No significant difference between means was observed between means measured at the same time points (Mann-Whitney *U* tests).

retinol (associated with retinol-binding protein), and vitamin E circulates as nonesterified  $\alpha$ - and  $\gamma$ -tocopherol incorporated into lipoproteins. Nevertheless, there was no  $\gamma$ -tocopherol in the fasting plasma of our mice because this form of vitamin E was not present in their diet. ABCA1<sup>-/-</sup> mice ( $n = 5$ ) had significantly lower fasting plasma  $\alpha$ -tocopherol concentrations than did wild-type mice ( $n = 6$ ) (0.62  $\pm$  0.10 compared with 2.11  $\pm$  0.41  $\mu$ mol/L, respectively;  $P = 0.029$ ), although they were provided the same diet from birth. ABCA1<sup>+/-</sup> mice ( $n = 3$ ) had an intermediate result (1.51  $\pm$  0.31  $\mu$ mol/L). Conversely, fasting plasma retinol concentrations were not significantly different between the 3 groups of mice (1.42  $\pm$  0.36, 1.54  $\pm$  0.16, and 1.64  $\pm$  0.21  $\mu$ mol/L for wild-type, ABCA1<sup>+/-</sup>, and ABCA1<sup>-/-</sup> mice, respectively). Note that, as expected, no retinyl esters were detected in the fasting plasma of the mice.

### Comparison of postprandial plasma $\gamma$ -tocopherol concentrations in wild-type, ABCA1<sup>+/-</sup>, and ABCA1<sup>-/-</sup> mice

Postprandial plasma  $\gamma$ -tocopherol responses (expressed as area under the postprandial curves at 0–8 h) after force-feeding the *R,R,R*- $\gamma$ -tocopherol-enriched emulsion were significantly lower in ABCA1<sup>+/-</sup> ( $P = 0.045$ ) and ABCA1<sup>-/-</sup> ( $P = 0.025$ ) mice than in wild-type mice (12.77  $\pm$  0.33, 6.26  $\pm$  0.92, and 3.09  $\pm$  0.91  $\mu$ mol  $\cdot$  h/L for wild-type, ABCA1<sup>+/-</sup>, and ABCA1<sup>-/-</sup> mice, respectively; **Figure 3**). These differences were abolished after lipid adjustment for the  $\gamma$ -tocopherol response (7.56  $\pm$  1.97, 5.09  $\pm$  0.97, and 4.58  $\pm$  1.18  $\mu$ mol  $\cdot$  h/L per gram for wild-type, ABCA1<sup>+/-</sup>, and ABCA1<sup>-/-</sup> mice, respectively; **Figure 4**).

### Comparison of postprandial plasma retinyl ester responses in wild-type, ABCA1<sup>+/-</sup>, and ABCA1<sup>-/-</sup> mice

Postprandial plasma retinyl ester responses (expressed as area under the postprandial curve at 0–8 h) after force-feeding with the retinyl palmitate-enriched emulsion were not significantly different in wild-type, ABCA1<sup>+/-</sup>, and ABCA1<sup>-/-</sup> mice (23.27  $\pm$  7.17, 29.47  $\pm$  11.67, and 17.43  $\pm$  6.37  $\mu$ mol  $\cdot$  h/L, respectively; **Figure 5**).

## DISCUSSION

To study the involvement of ABCA1 in the secretion of vitamin E ( $\alpha$ - and  $\gamma$ -tocopherol) and vitamin A (retinyl palmitate) at the basolateral side of the enterocyte, we used the Caco-2 TC-7 cell model in a first set of experiments. This human intestinal cell

**TABLE 1**

Effect of glyburide and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS)—chemical inhibitors of ATP-binding cassette A1—on the basolateral efflux of  $\alpha$ -tocopherol and  $\gamma$ -tocopherol in Caco-2 cells<sup>1</sup>

Time	$\alpha$ -Tocopherol in basolateral medium			$\gamma$ -Tocopherol in basolateral medium			
	Control	Glyburide	DIDS	Control	Glyburide	DIDS	
		<i>pmol/mg protein</i>				<i>pmol/mg protein</i>	
3 h	22.5 ± 1.9	22.2 ± 7.2	16.4 ± 1.6 <sup>2</sup>	15.2 ± 0.7	8.2 ± 0.7 <sup>2</sup>	5.0 ± 0.5 <sup>2</sup>	
6 h	50.5 ± 4.4	38.3 ± 5.5 <sup>2</sup>	19.4 ± 1.1 <sup>2</sup>	43.4 ± 0.1	23.2 ± 3.6 <sup>2</sup>	6.3 ± 0.7 <sup>2</sup>	
12 h	104.9 ± 1.9	98.0 ± 1.9	28.7 ± 2.3 <sup>2</sup>	82.0 ± 2.2	41.8 ± 6.9 <sup>2</sup>	6.7 ± 1.1 <sup>2</sup>	
24 h	240.1 ± 4.6	180.9 ± 15.8 <sup>2</sup>	46.3 ± 0.8 <sup>2</sup>	176.6 ± 4.7	70.5 ± 15.2 <sup>2</sup>	12.4 ± 1.0 <sup>2</sup>	

<sup>1</sup> All values are mean ± SEM of 3 assays. The apical side was treated with fetal bovine serum-free medium containing either *R,R,R*- $\alpha$ -tocopherol-rich mixed micelles (31  $\mu$ mol/L) or *R,R,R*- $\gamma$ -tocopherol-rich mixed micelles (27  $\mu$ mol/L). The basolateral side was treated with fetal bovine serum-free medium containing dimethyl sulfoxide (control), 400  $\mu$ mol/L glyburide, or 400  $\mu$ mol/L DIDS.

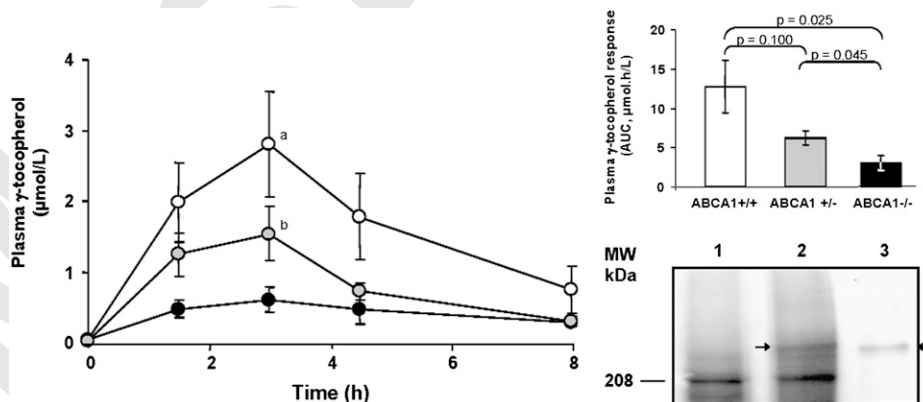
<sup>2</sup> Significantly different from control,  $P < 0.05$  (Kruskal-Wallis test followed by a post hoc Mann-Whitney  $U$  test).

line, a widely used and accepted model to study micronutrient absorption (13, 17, 24, 25), expresses ABCA1 at its basolateral side (26–29). A first experiment was performed with apo A-I, which specifically triggers ABCA1-mediated lipid effluxes (20, 30). The results showed that apo A-I can promote the effluxes of both  $\alpha$ - and  $\gamma$ -tocopherol, but it has no effect on retinyl ester efflux, which suggests that ABCA1 may be involved in vitamin E secretion only. To confirm this result on vitamin E, we then measured the inhibitory effect of glyburide, a commonly used inhibitor of ABC transporters (8). The results obtained with  $\alpha$ -tocopherol agreed with the data of Anwar et al (13, 14) and therefore validated our new results on  $\gamma$ -tocopherol. Nevertheless, because glyburide can inhibit a broad range of ABC transporters (31), these results, as well as those of Anwar et al, were not sufficient to identify the transporter. To further support the involvement of an ATP-dependent transporter, we measured the effect of DIDS (21). Again, the basolateral efflux of both  $\alpha$ - and  $\gamma$ -tocopherol was significantly impaired, strengthening the hypothesis of the involvement of an ABC transporter.

To obtain definitive evidence on the involvement of ABCA1, we next decided to validate our results in vivo using ABCA1-

deficient mice. Because these mice were not specifically deficient for ABCA1 in the intestine, and to discriminate between the effects of intestinal and nonintestinal ABCA1, we compared the postprandial plasma responses of  $\gamma$ -tocopherol and retinyl esters between ABCA1-deficient and wild-type mice after oral loads of these vitamins. The choice of  $\gamma$ -tocopherol was dictated by the following reasons. First,  $\alpha$ - and  $\gamma$ -tocopherol were equally efficient at monitoring absorption because there is no intestinal discrimination between these forms with regard to absorption (32). Second, mice bred in-house had very low concentrations of circulating  $\gamma$ -tocopherol. Third, hepatic  $\alpha$ -tocopherol-transfer protein resecreted  $\gamma$ -tocopherol less efficiently than  $\alpha$ -tocopherol in VLDL. Taken together, these observations allow the use of postprandial plasma  $\gamma$ -tocopherol as a natural tracer for newly absorbed vitamin E. Similarly, no plasma retinyl esters were observed in fasting blood samples in mice, which makes these molecules equivalent to a tracer in terms of measuring newly absorbed vitamin A. Our results provide evidence of a significantly lower postprandial plasma  $\gamma$ -tocopherol response in ABCA1<sup>-/-</sup> mice than in wild-type mice and an intermediate response in ABCA1<sup>+/-</sup> mice. This finding provides convincing

[AQ6]



**FIGURE 3.** Postprandial plasma  $\gamma$ -tocopherol responses in wild-type ( $\circ$ ;  $n = 6$ ), ABCA1<sup>+/-</sup> ( $\bullet$ ;  $n = 5$ ), and ABCA1<sup>-/-</sup> ( $\bullet$ ;  $n = 6$ ) mice and expression of ABCA1 in mouse duodenum. Mice were force-fed *R,R,R*- $\gamma$ -tocopherol-rich emulsions. Values are means ± SEMs. Kruskal-Wallis test followed by a post hoc Mann-Whitney  $U$  test: <sup>a</sup>significant difference between wild-type and ABCA1<sup>-/-</sup> mice; <sup>b</sup>significant difference between ABCA1<sup>+/-</sup> and ABCA1<sup>-/-</sup> mice. Upper inset: postprandial  $\gamma$ -tocopherol responses (areas under the curves at 0–8 h);  $P$  values represent differences between groups (Kruskal-Wallis test followed by a post hoc Mann-Whitney  $U$  test). Lower inset: expression of ABCA1 in mouse duodenum. Lysates of duodenal mucosa from ABCA1<sup>-/-</sup> (lane 1) and ABCA1<sup>+/-</sup> (lane 2) mice were immunoprecipitated with an anti-ABCA1 monoclonal antibody (clone 1156) before fractionation on sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotting onto nitrocellulose membrane. ABCA1 was further detected with the 891.3 anti-ABCA1 monoclonal antibody. The total lysate of HeLa cells expressing ABCA1 is shown as a control of migration (lane 3).

**TABLE 2**Plasma lipid variables for wild-type, ABCA1<sup>+/-</sup>, and ABCA1<sup>-/-</sup> mice before feeding (0 h) and during the postprandial experiment<sup>1</sup>

Time	Plasma triacylglycerols			Plasma nonesterified cholesterol			Plasma phospholipids		
	Wild type	ABCA1 <sup>+/-</sup>	ABCA1 <sup>-/-</sup>	Wild type	ABCA1 <sup>+/-</sup>	ABCA1 <sup>-/-</sup>	Wild type	ABCA1 <sup>+/-</sup>	ABCA1 <sup>-/-</sup>
	g/L			g/L			g/L		
0 h	0.449 ± 0.049	0.554 ± 0.006	0.335 ± 0.032	0.372 ± 0.025 <sup>2</sup>	0.262 ± 0.001	0.052 ± 0.002 <sup>2</sup>	0.741 ± 0.073 <sup>2</sup>	0.622 ± 0.031 <sup>2</sup>	0.189 ± 0.012
1.5 h	0.791 ± 0.074	0.415 ± 0.085	0.481 ± 0.039	0.369 ± 0.026 <sup>2</sup>	0.203 ± 0.021	0.075 ± 0.007 <sup>2</sup>	0.917 ± 0.065 <sup>2</sup>	0.505 ± 0.053 <sup>2</sup>	0.233 ± 0.016
3 h	0.501 ± 0.045	0.318 ± 0.069	0.410 ± 0.037	0.322 ± 0.025 <sup>2</sup>	0.288 ± 0.025	0.086 ± 0.011 <sup>2</sup>	0.739 ± 0.078 <sup>2</sup>	0.585 ± 0.041 <sup>2</sup>	0.241 ± 0.020
4.5 h	0.533 ± 0.039	0.267 ± 0.046	0.359 ± 0.054	0.323 ± 0.019 <sup>2</sup>	0.252 ± 0.039	0.075 ± 0.009 <sup>2</sup>	0.742 ± 0.050 <sup>2</sup>	0.447 ± 0.031 <sup>2</sup>	0.203 ± 0.020
8 h	0.331 ± 0.024	0.243 ± 0.034	0.273 ± 0.029	0.315 ± 0.030 <sup>2</sup>	0.267 ± 0.070	0.073 ± 0.008 <sup>2</sup>	0.689 ± 0.040 <sup>2</sup>	0.541 ± 0.071 <sup>2</sup>	0.183 ± 0.013
12 h	1.157 ± 0.189	0.770 ± 0.341	0.178 ± 0.028	0.380 ± 0.006 <sup>2</sup>	0.278 ± 0.137	0.067 ± 0.013 <sup>2</sup>	0.986 ± 0.060 <sup>2</sup>	0.780 ± 0.379 <sup>2</sup>	0.202 ± 0.026

<sup>1</sup> All values are mean ± SEM; *n* = 6 for wild-type mice, 5 for ABCA1<sup>+/-</sup> mice, and 6 for ABCA1<sup>-/-</sup> mice. Mice were force-fed an *R,R,R*- $\gamma$ -tocopherol-rich emulsion. ABCA1, ATP-binding cassette A1. No significant differences were observed between areas under the curves (Kruskal-Wallis test).

<sup>2</sup> Significantly different from ABCA1<sup>+/-</sup> mice (within group), *P* < 0.05 (Kruskal-Wallis test followed by a post hoc Mann-Whitney *U* test).

evidence that ABCA1 is involved in the intestinal absorption of  $\gamma$ -tocopherol and likely in that of vitamin E in general. Note that the flattening of the differences between groups, observed after correction for plasma lipid concentrations, is logical because ABCA1<sup>-/-</sup> mice have low HDL-cholesterol concentrations (33) and secrete less lipids than do wild-type mice (Table 2), which leads to increased  $\gamma$ -tocopherol/lipids ratios. Concerning vitamin A, our results indicated equivalent postprandial plasma retinyl ester responses in the 3 groups of mice, which suggested that ABCA1 is not involved in the secretion of retinyl esters or at least to less of an extent than is  $\gamma$ -tocopherol. This observation agreed with the lack of effect of apo A-I on retinyl ester secretion by Caco-2 cells, which confirms that the chylomicron pathway is not affected in the absence of the ABCA1 transporter and adds further evidence that the apo A-I pathway contributes to the intestinal absorption of vitamin E.

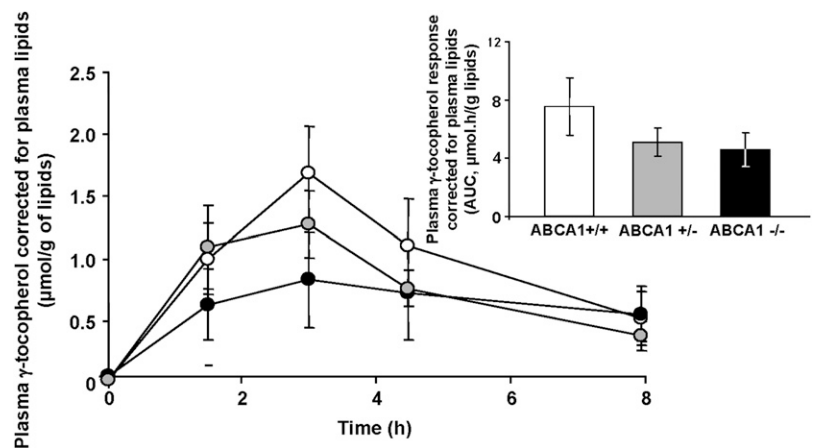
The fasting plasma concentrations of vitamins A and E observed in the ABCA1-deficient mice, as compared with the concentrations in wild-type mice, confirmed the postprandial observations and indicated that ABCA1 participates in the long-term plasma status of vitamin E, either by its involvement in the efflux of vitamin E out of the intestinal cell or by its involvement in the concentration of plasma HDL.

Tangier disease is an autosomal recessive genetic disorder resulting from the loss of ABCA1 function and leads to a severe HDL deficiency (34). Despite the paucity of data on the vitamin A and E status of patients with Tangier disease, 2 independent

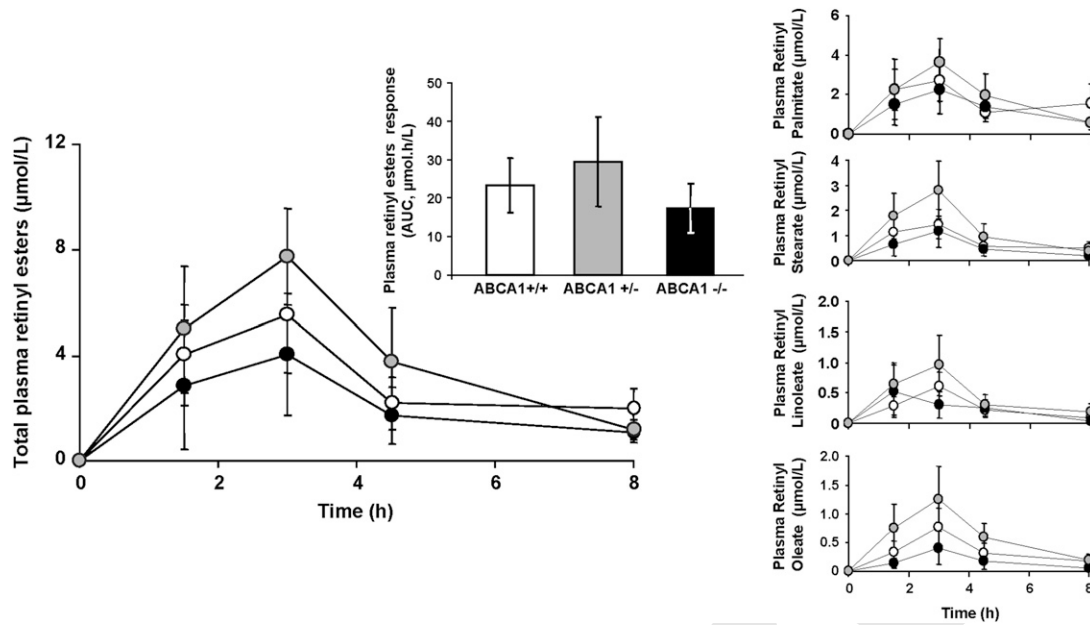
studies have reported vitamin E deficiency (35) and chronic oxidative stress (36) in these patients. The oxidative stress was partially reverted by selenite and  $\alpha$ -tocopherol supplementation, which suggests the correction of a low vitamin E status in these patients. These observations are consistent with our finding of a significantly lower intestinal absorption of vitamin E and lower fasting  $\alpha$ -tocopherol concentrations in ABCA1<sup>-/-</sup> mice. Before definitive conclusions on the effect of ABCA1 on vitamin E status are reached, a careful and broad analysis should be carried out in a sufficient number of patients with Tangier disease and corroborated by correlative studies of ABCA1 single nucleotide polymorphisms known to have a phenotypic effect on lipid metabolism (36).

In summary, our results establish for the first time that ABCA1 is involved in the in vivo intestinal absorption of  $\gamma$ -tocopherol and is thus likely involved in the secretion of vitamin E with intestinal HDL in general. Conversely, ABCA1 is not significantly implicated in the secretion of retinyl esters. Our results also suggest that ABCA1 plays a role in the status of plasma vitamin E. Several physiologic consequences of our findings are conceivable. Previous studies showed that several genes involved in lipid metabolism were also involved in the status of plasma vitamin E (37, 38), and several nonsynonymous single nucleotide polymorphisms in the ABCA1 gene have a phenotypic effect on cholesterol absorption (39). Thus, it is possible that the high interindividual variability in tocopherol absorption (40) could be due, in part, to interindividual variations in ABCA1 expression

**FIGURE 4.** Postprandial plasma  $\gamma$ -tocopherol responses corrected for plasma total lipids (cholesterol plus phospholipids plus triacylglycerols) in wild-type (○; *n* = 6), ABCA1<sup>+/-</sup> (●; *n* = 5), and ABCA1<sup>-/-</sup> (●; *n* = 6) mice force-fed *R,R,R*- $\gamma$ -tocopherol-rich emulsions. Values are means ± SEMs. Inset: areas under the curves (for details, see Figure 3 legend). No significant differences were observed between areas under the curves (Kruskal-Wallis test).







**FIGURE 5.** Postprandial plasma retinyl ester responses in wild-type (○; n = 5), ABCA1<sup>+/-</sup> (◐; n = 5), and ABCA1<sup>-/-</sup> (●; n = 3) mice force-fed retinyl palmitate-rich emulsions. Values are means ± SEMs. Inset: areas under the curves (for details, see Figure 3 legend).

or efficiency. Moreover ezetimibe, an inhibitor of cholesterol intestinal absorption, has been shown to decrease both the absorption of carotenoids and the expression of ABCA1 in Caco-2 cells (41), which raises questions about the long-term consequences of the use of such drugs on vitamin E status. Finally, additional studies are necessary to determine whether vitamin E has a different fate in the body depending on whether it is transported by the chylomicron-dependent pathway or by the intestinal HDL pathway.

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[AQ1] Is Peypin a city or the company name? Both are needed.

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[AQ6] Review revision for accuracy.

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