Evidence for multiple complementary pathways for efficient cholesterol absorption in mice

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Abstract Apolipoprotein B (apoB)-dependent and apoB-independent pathways for cholesterol transport have been described in cultured cells. Here, we show that the apoB-independent pathway involves apoA-I-containing high density lipoproteins (HDLs). Cholesterol secretion by the HDLs, but not by the apoB pathway, was significantly reduced in primary enterocytes isolated from chow- and cholesterol-fed apoA-I$^{-/-}$ mice. These enterocytes were capable of cholesterol efflux when apoA-I was provided extracellularly. In apoA-I$^{-/-}$ mice, the absorption of a bolus of cholesterol was similar in control and apoA-I$^{-/-}$ mice fed chow or high-cholesterol diet. However, short-term studies revealed that cholesterol absorption was occurring over longer lengths of the intestine, and cholesterol but not triglyceride transport to the plasma and liver in chow- and cholesterol-fed apoA-I$^{-/-}$ mice was significantly reduced. These studies indicate that in apoA-I deficiency, there is a delay in cholesterol absorption, but cholesterol is eventually absorbed because of the compensatory apoB pathway. Nonetheless, long-term studies involving multiple feedings showed significant reduction in cholesterol absorption after 4 days. We propose that multiple compensatory mechanisms ensure efficient cholesterol absorption in mice.—Iqbal, J., and M. M. Hussain. **Evidence for multiple complementary pathways for efficient cholesterol absorption in mice.** ***J. Lipid Res.*** **2005.** 46: **1491–1501.**

Cholesterol absorption is defined as the transport of cholesterol from intestinal lumen to plasma (1, 2). There are three major steps in this process. The first step occurs in the lumen of the intestine and involves emulsification and hydrolysis of dietary fat and cholesteryl esters by bile acids and pancreatic enzymes. Genetic ablation of cholesteryl ester hydrolase (3) and pancreatic triglyceride lipase (4) has been shown to decrease cholesterol absorption, emphasizing their role in the hydrolysis of dietary fat in the intestinal lumen. The second step is the uptake of free cholesterol by enterocytes. Several candidate proteins that can facilitate cholesterol uptake by enterocytes have been identified. Current emerging evidence suggests that Niemann-Pick C1-like 1 (NPC1L1) might play a key role in this process. NPC1L1 gene deletion reduces cholesterol absorption in mice (5, 6). Furthermore, ezetimibe, a cholesterol absorption inhibitor, was shown to be ineffective in the absence of NPC1L1. The third step is the assembly of cholesterol and cholesteryl esters with lipoproteins in the enterocytes. The importance of apolipoprotein B (apoB) lipoproteins, mainly chylomicrons, in this process has been appreciated for a long time (7). Recently, we have provided evidence for multiple, independently regulated pathways for cholesterol transport across the intestinal epithelial cells (8). These pathways were broadly classified into apoB-dependent and apoB-independent pathways. However, it is not known whether such mechanisms play a role in cholesterol absorption in vivo. To test the hypothesis that multiple pathways contribute to efficient cholesterol absorption in vivo, we used apoA-I knockout mice as a model system.

ApoA-I is the major protein present in plasma high density lipoproteins (HDLs), and its deficiency in mice and humans is associated with low levels of plasma cholesterol (9–11). The major function of apoA-I is in the reverse cholesterol transport from peripheral tissues to the liver. In this process, apoA-I picks up free cholesterol from peripheral tissues by interacting with a plasma membrane resident ATP binding cassette protein A1 (ABCA1) and forms a nascent HDL (12–14). The free cholesterol in HDL is then esterified by lecithin cholesterol acyltransferase, and apoA-I acts as a cofactor for this enzyme (15). Furthermore, by interacting with scavenger receptor class B type I, it helps deliver cholesteryl esters from lipoproteins to the liver and steroidogenic tissues by selective uptake (13, 16, 17). There is evidence to suggest that elevated levels of HDL and apoA-I are antiatherogenic and have been correlated with decreased risk for cardiovascular disease. ApoA-I gene knockout increases and transgenic overexpression decreases atherosclerosis in mice (9–11, 18–22).

Among all the tissues studied, intestines produce the most apoA-I (23). Yet, its function in the intestinal lipid

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Abbreviations: ABCA1, ATP binding cassette protein A1; apoB, apolipoprotein B; HDL, high density lipoprotein; OA, oleic acid; TC, taurocholate.

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metabolism has not been defined. Cholesterol absorption studies in apoA-I knockout mice have generally suggested that it plays no role in cholesterol absorption (10, 24, 25). In these studies, absorption of a bolus of cholesterol over 24–72 h was monitored (26–29). We hypothesized that no differences were observed in these studies because there are multiple complementary pathways for cholesterol transport and a defect in one pathway may not necessarily affect total absorption of a bolus of cholesterol over a long period. Thus, we used different approaches to evaluate the role of different pathways in cholesterol absorption. First, cholesterol secretion with different lipoproteins was studied in enterocytes isolated from wild-type and apoA-I knockout mice. Second, acute experiments were performed in animals to study differences in cholesterol absorption in these animals. Third, changes in cholesterol absorption across the length of the intestine were documented. Fourth, chronic experiments were conducted to stress the system and to record differences in cholesterol absorption in these mice. Using these approaches, we provide evidence for the presence of complementary pathways that constitute a remarkably efficient system for cholesterol absorption.

MATERIALS AND METHODS

Materials

[1,2-^{3}H]cholesterol and [4-^{14}C]cholesterol were from NEN Life Science Products, and [5,6-^{3}H]sitostanol was from American Radiolabeled Chemicals. Oleic acid (OA), sodium cholic acid, sodium deoxycholic acid, taurocholate (TC), and monoacylglycerol were from Sigma, whereas phosphatidylcholine was from Avanti Polar Lipids. Other chemicals and solvents were from Fisher Scientific.

Animals and diets

C57BL/6J apoA-I knockout inbred mice (B6.129P2-*Apoa1*^{tm1Unc}) were from the Jackson Laboratory. For controls, C57BL/6J mice were used. Mice had free access to water and rodent chow containing trace cholesterol (<0.02%). Male 10–12-week-old mice were used in this study. To study the effects of high-cholesterol diet, animals were provided with a high-fat, high-cholesterol (0.2%), and no-cholic-acid Western diet (Tekland 88137) for 14 days (30). Food was removed the night before the experiments. The institutional animal and use committee approved these experiments.

Isolation of primary enterocytes

Primary enterocytes were isolated from control and knockout mice by the method of Weiser (31), as described before (8). Briefly, overnight-fasted mice were anesthetized, and small intestines were used for enterocyte isolation. Contents from the intestinal lumen were removed, washed with 117 mM NaCl, 5.4 mM KCl, 0.96 mM NaH_2PO_4, 26.19 mM $NaHCO_3$, and 5.5 mM glucose, and then filled with 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 (buffer A). Intestines were then bathed in oxygenated saline at 37°C for 10 min. The buffer was discarded, and intestinal lumen were refilled with buffer A containing 1.5 mM EDTA and 0.5 mM dithiothreitol and incubated in 0.9% sodium chloride solution at 37°C for 10 min. Lumenal contents were collected and centrifuged at 1,500 rpm for 5 min. All buffers were adjusted to pH 7.4, gassed with 95% O_2/5% CO_2 for 20 min, and maintained at 37°C prior to use.

Secretion of cholesterol by primary enterocytes

Enterocytes were resuspended in 4 ml of DMEM containing 1 μCi/ml of [^{3}H]cholesterol and incubated at 37°C in a cell culture incubator with 5% CO_2. Cells were gassed for 1 min with 95% O_2/5% CO_2 at regular intervals of 15 min. After 1 h, enterocytes were centrifuged (1,500 rpm for 5 min), washed twice with DMEM, and incubated with micelles containing 0.14 mM sodium cholate, 0.15 mM sodium deoxycholate, 0.17 mM phosphatidylcholine, 1.2 mM OA, and 0.19 mM monopalmitoylglycerol at 37°C with regular gassing. After 2 h, enterocytes were centrifuged (3,000 rpm, 5 min), supernatants were subjected to density gradient ultracentrifugation, fractions were collected, and radioactivity was measured in a Beckman liquid scintillation counter. Cell pellets were incubated overnight at 4°C with 1 ml of isopropanol to isolate total lipids. After lipid extraction, 1 ml of 0.1 N NaOH was added to dissolve proteins. Protein was measured by the Bradford method (32), using Coomassie reagent (Pierce Chemical Company; Rockford, IL).

Studies with Caco-2 cells

Secretion of cholesterol by Caco-2 cells was studied as described earlier (8). Briefly, differentiated Caco-2 cells in transwells were pulse labeled with 1 μCi/ml [^{3}H]cholesterol in DMEM + 20% FBS on the apical side for 17 h. Cells were washed and incubated with 2 ml of DMEM containing 20% FBS and OA-TC (1.6: 0.5 mM) or TC (0.5 mM) on the apical side and 2 ml of DMEM ± 1% BSA on the basolateral side. Basolateral conditioned media (1.6 ml) were used for ultracentrifugation and measurements of apolipoproteins and radiolabeled cholesterol.

Cholesterol absorption studies

The absorption of a single bolus of cholesterol by chow- or cholesterol-fed mice (three per group) was determined by the fecal dual-isotope ratio method, using [^{3}H]sitostanol as a nonabsorbable reference sterol (26–29). ApoA-I$^{-/-}$ and control mice (10–12-week-old, male and female, three per group) were placed in individual cages that had wire mesh floors. Animals were fasted for 6 h and gavaged with 0.1 μCi of [^{14}C]cholesterol and 0.2 μCi of [^{3}H]sitostanol in 15 μl of olive oil. Subsequently, animals had free access to water and chow pellets. Feces were collected over a period of 48 h and used for cholesterol absorption studies using the dual-isotope ratio method (26–29).

For long-term cholesterol absorption studies, a mixture of labeled (0.1 μCi) and unlabeled cholesterol (0.2 mg) and labeled sitostanol (0.2 μCi) in 15 μl of olive oil was fed to each mouse three times a day, at 11 AM, 2 PM, and 5 PM for 5 days. Feces were collected every 24 h, and isotope ratio was determined (26–29).

Short-term triglyceride or cholesterol absorption

For short-term triglyceride or cholesterol absorption studies (4, 5, 33), age-matched male mice (three per group) on either chow or high-cholesterol diet for 2 weeks were fasted overnight and gavaged with either 0.1 μCi of [^{3}H]triolein or 1 μCi of [^{3}H]cholesterol with 0.1 mg of unlabeled cholesterol in 15 μl of olive oil. After 2 h, plasma, livers, and 2 cm segments of small intestines were collected. Radioactivity in 100 μl of plasma was determined after the addition of 5 ml of the scintillation cocktail. Tissue samples (~0.1 g) were rinsed twice in phosphate-buffered saline, blot dried, transferred to a glass vial, and incubated with 1 ml of OptiSolv for 48 h. After complete digestion of the tissue, 5 ml of scintillation cocktail was added and used for counting.

Density gradient ultracentrifugation

Two types of density gradient ultracentrifugations were performed. One ultracentrifugation involves the separation of lipoproteins between d<1.006 and 1.12 g/ml. This procedure sepa-

rates large, triglyceride-rich apoB-containing lipoproteins (34–36) and has been used for cell culture media (8, 37–39). The second ultracentrifugation was designed to separate HDLs. Conditioned media (2 ml) in SW 41 rotor ultracentrifuge tubes were adjusted to a density of 1.30 g/ml and then overlaid with 2 ml each of 1.24, 1.21, 1.15, and 1.063 g/ml, and 1 ml each of 1.019 and 1.006 g/ml density solutions and subjected to ultracentrifugation (40,000 rpm, 15°C, 17 h), and 1 ml fractions were collected from the top.

Other methods

Density was measured using a refractometer (Fisher Scientific, Medford, MA). ApoB and apoA-I were quantified using a sandwich ELISA (40, 41). Plasma total cholesterol (Infinity™ Cholesterol, TR13421) and triglycerides (Infinity™ Triglyceride, TR22421) were measured using commercial kits (Thermo Trace, Melbourne, Australia). Cholesterol and triglycerides in HDL were measured after the precipitation of apoB lipoproteins with sodium phosphotungstate and magnesium chloride (42).

RESULTS

Presence of cholesterol and apoA-I in HDLs secreted by Caco-2 cells

We have previously shown that Caco-2 cells secrete cholesterol in two types of lipoproteins (8). To characterize apolipoproteins present in these two lipoprotein fractions, Caco-2 cells were radiolabeled with cholesterol and chased in the presence of TC and OA to promote chylomicron secretion (**Fig. 1**, OA:TC) and in the absence (Fig. 1, TC) of OA. In the absence of OA, the majority of cholesterol was in the bottom fraction, corresponding to a density of 1.12 g/ml (Fig. 1A). However, cholesterol secreted by OA:TC-treated Caco-2 cells was in two well-separated lipoprotein fractions. Quantification of apolipoproteins revealed that apoB (Fig. 1B) was mainly in the top

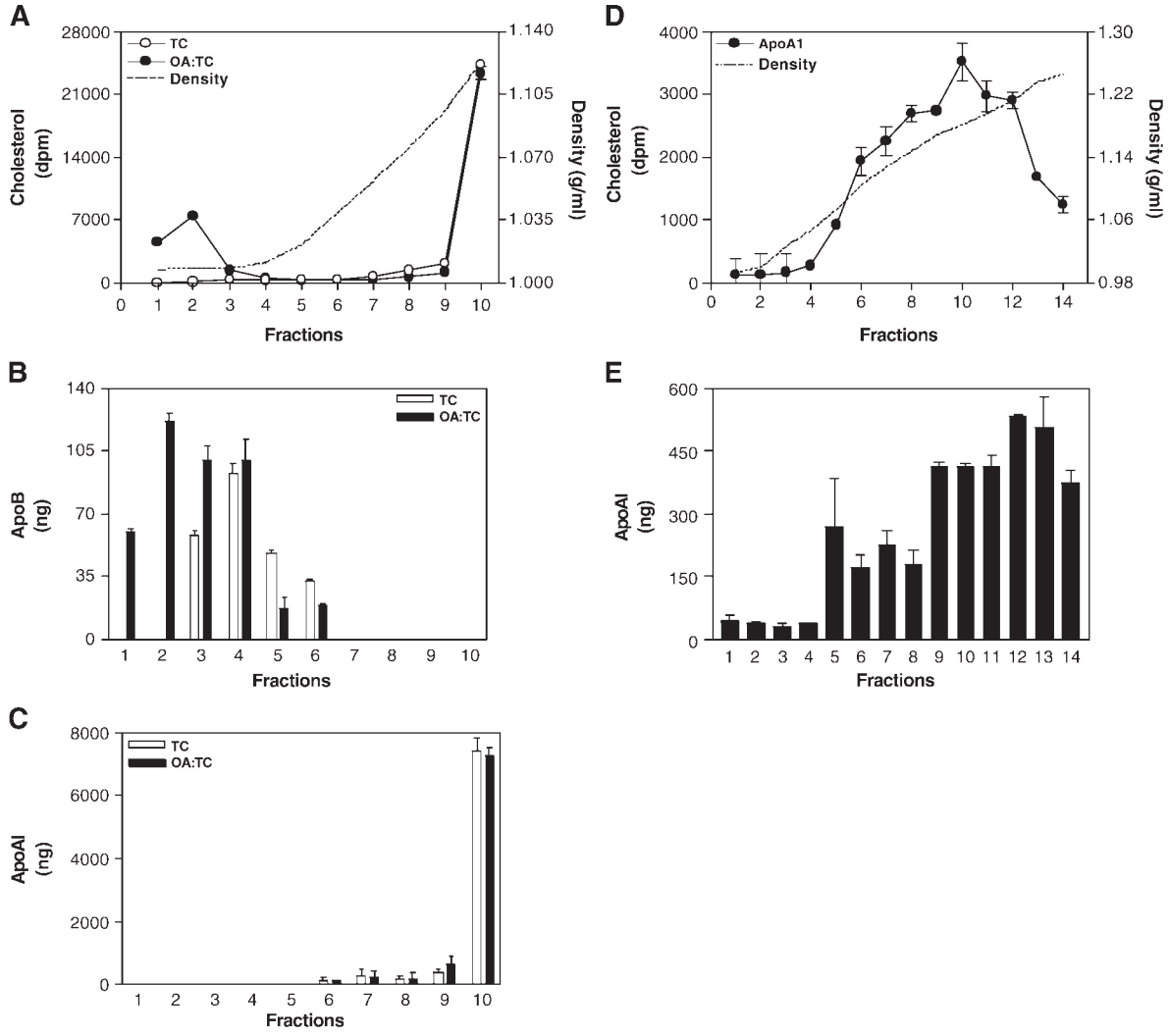

Fig. 1. Characterization of cholesterol secreted by differentiated Caco-2 cells. Caco-2 cells differentiated in transwells were pulse labeled with cholesterol for 17 h and then chased in the presence of oleic acid and taurocholate (OA:TC) and absence (TC) of OA on the apical side for 24 h. The basolateral side received media containing 1% BSA. Conditioned media (1.6 ml) were subjected to density gradient ultracentrifugation to separate apolipoprotein B (apoB) lipoproteins as described in Materials and Methods. Fractions were collected from the top and used in triplicate to measure density (A), cholesterol (A), apoB (B), and apoA-I (C). The basolateral media were also used to separate high density lipoproteins (HDLs) as described in Materials and Methods. Fractions (1 ml each) were collected from the top and used to measure cholesterol and density (D), and apoA-I (E). Error bars indicate standard deviation.

fractions, in agreement with our earlier studies (37, 38, 43). In contrast, apoA-I was in the bottom fractions, in both the presence and the absence of OA (Fig. 1C). We further characterized the bottom fractions using a density gradient designed to separate HDL fractions. The majority of cholesterol was present in fractions 6 to 12, corresponding to densities of 1.14–1.22 g/ml (Fig. 1D). This peak was enriched in apoA-I (Fig. 1E). These studies showed that cholesterol and apoA-I were present in HDL and suggested that apoA-I may play a role in apoB-independent cholesterol secretion.

Role of apoA-I in cholesterol transport across the intestinal cells

To determine the role of apoA-I in cholesterol secretion, enterocytes were isolated from chow-fed apoA-I knockout and control mice, incubated with radiolabeled cholesterol for 1 h, and then chased in the presence of OA for 2 h (**Fig. 2**). The amount of radiolabeled cholesterol secreted by apoA-I$^{-/-}$ enterocytes was ~20% less ($P < 0.02$) compared with controls (Fig. 2A). Cholesterol secreted by control enterocytes was distributed in two separate fractions, corresponding to apoB lipoproteins (fractions 1 and 2) and HDLs (Fig. 2B). Similar analysis with apoA-I$^{-/-}$ enterocytes revealed that cholesterol secretion in apoB lipoproteins was not significantly different from controls. Secretion of cholesterol in HDLs (Fig. 2B, fractions 9 and 10), on the other hand, was significantly reduced (40% decrease) in apoA-I$^{-/-}$ enterocytes.

Next, similar studies were performed in enterocytes isolated from animals fed a high-cholesterol diet for 2 weeks. The amount of cholesterol secreted by apoA-I$^{-/-}$ enterocytes was decreased by 25% (Fig. 2C) and was in two fractions (Fig. 2D). The apoB lipoproteins were in fractions 1 and 2. The HDL cholesterol was distributed broadly (Fig. 2D), compared with the standard chow-fed mice (Fig. 2B). There were no significant differences in the secretion of cholesterol in apoB lipoproteins in wild-type and knockout mice (Fig. 2D, fractions 1 and 2). But, cholesterol secretion in the HDL was significantly reduced in apoA-I$^{-/-}$ mice (Fig. 2D). Thus, apoA-I deficiency results in decreased cholesterol secretion by the HDL pathway and has no effect on the apoB pathway.

Cholesterol efflux from normal and knockout mice enterocytes

ApoA-I is a known extracellular cholesterol efflux acceptor. ApoA-I has also been shown to stabilize ABCA1 (44, 45). We reasoned that the absence of apoA-I might have decreased the extracellular efflux pathway by affecting ABCA1 activity. To test this hypothesis, isolated enterocytes were pulse labeled with cholesterol and then chased in the presence of human apoA-I for 2 h (**Fig. 3**). In the absence of added apoA-I, the amount of cholesterol secreted by apoA-I$^{-/-}$ enterocytes was significantly lower (Fig. 3A), compared with controls, as seen in Fig. 2. Addition of extracellular apoA-I resulted in significant increases (2- to 3-fold) in the amount of cholesterol effluxed by both

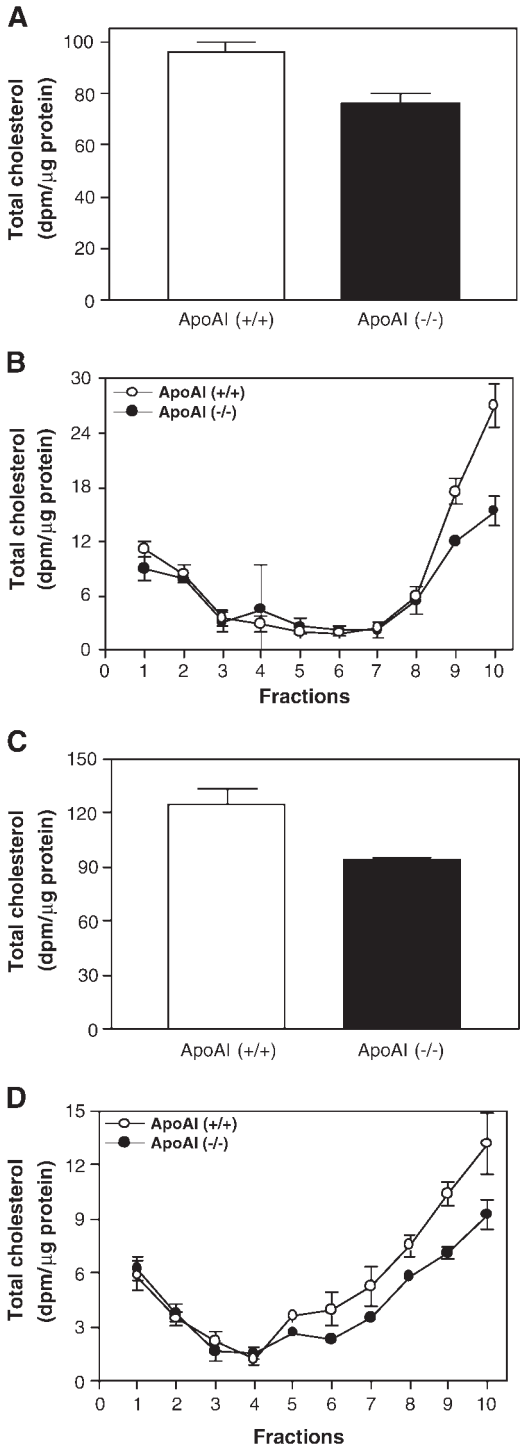

Fig. 2. Effect of apoA-I ablation on cholesterol transport by enterocytes. Enterocytes were isolated from wild-type and apoA-I knockout mice (three per group, 10–12-week-old males) fed either chow (A, B) or high-cholesterol (C, D) diet, pulsed for 1 h with 1 μCi/ml of [^{3}H]cholesterol, washed, and chased for 2 h in the presence of 1.2 mM OA-containing micelles. The conditioned media were used to measure secreted cholesterol (A, C) and to separate various lipoproteins. Fractions were collected from the top and used to measure cholesterol in triplicate (B, D). Differences in wild-type and knockout enterocytes were evaluated by Student's *t*-test. Error bars indicate standard deviation.

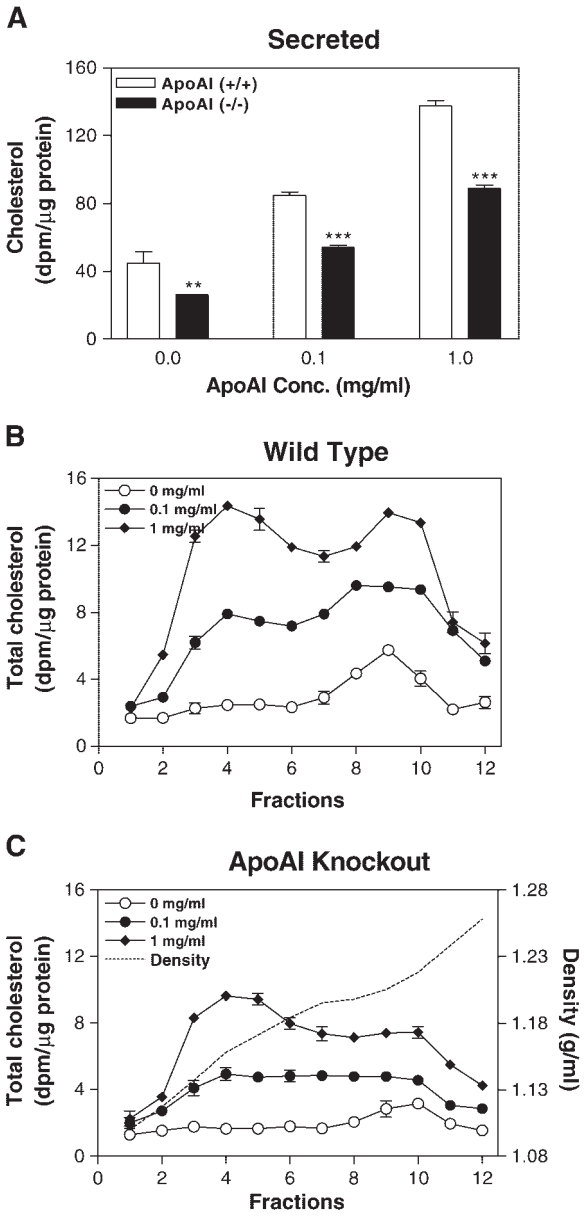

Fig. 3. Efflux of cholesterol in the presence of purified apoA-I. Enterocytes from three male wild-type and apoA-I knockout mice were pulsed for 1 h with 1 μCi/ml of [^{3}H]cholesterol, washed, and divided into three different tubes. Cells were chased for 2 h in the presence of 0, 0.1, and 1 mg/ml of purified human apoA-I. No OA was given during this chase. A: Secreted radiolabeled cholesterol was measured. ** $P < 0.01$; *** $P < 0.001$. The conditioned media were then subjected to ultracentrifugation to separate HDL subclasses. B, C: Distribution of cholesterol secreted by wild-type and knockout mice, respectively. Error bars indicate standard deviation.

types of enterocytes. At all the concentrations of apoA-I, the amounts of cholesterol effluxed by knockout enterocytes were significantly lower. The amount of cholesterol effluxed in the presence of 0.1 mg/ml of apoA-I by apoA-I$^{-/-}$ enterocytes was similar to that effluxed by wild-type enterocytes in the absence of apoA-I supplementation. Similarly, the amount of cholesterol effluxed by apoA-I-deficient enterocytes after the addition of 1 mg/ml apoA-I was similar to that effluxed by wild-type enterocytes supplemented with 0.1 mg/ml apoA-I. These studies indicate that both types of enterocytes could efflux cholesterol in the presence of extracellular free apoA-I. Thus, decreased cholesterol secretion in apoA-I$^{-/-}$ enterocytes is probably due to apoA-I deficiency and not to the complete loss of efflux activity.

In the absence of purified apoA-I, cholesterol secreted by wild-type enterocytes was in fractions 8–10, corresponding to densities of 1.19–1.21 g/ml (Fig. 3B). Addition of 0.1 mg/ml of human apoA-I resulted in a significant increase in this peak. In addition, another peak (fractions 3–6, 1.15–1.17 g/ml) was evident. Higher amounts of apoA-I (1 mg/ml) increased cholesterol in both of these peaks, but the amounts of cholesterol were higher in the 1.15–1.17 g/ml peak. These studies may indicate that the early particle secreted by primary enterocytes has a density of 1.19–1.21 g/ml and that in the presence of secreted apoA-I, other particles that float at a density of 1.15–1.17 g/ml are formed.

ApoA-I$^{-/-}$ enterocytes secreted (Fig. 3C) small amounts of cholesterol in a peak (fractions 8–10) similar to wild type (compare Fig. 3C with Fig. 3B). Addition of increasing amounts of purified apoA-I resulted in increased amounts of cholesterol in this peak, as well as the emergence of an additional peak in fractions 3–6 similar to that observed with control enterocytes. We interpret these results to indicate that apoA-I$^{-/-}$ enterocytes can efflux cholesterol as the controls. However, the extent of this process was reduced in the absence of endogenous apoA-I.

Absorption of a single bolus of cholesterol

Next, we determined whether apoA-I ablation affects cholesterol absorption in vivo. Cholesterol absorption studies are usually performed by studying the changes in fecal dual-isotope ratio 48 h after the gavage of a bolus of radiolabeled cholesterol and sitostanol (26–29). We performed similar studies in chow-fed animals (**Table 1**). In control and knockout male and female mice, we did not find a significant difference in cholesterol absorption. Similarly, significant differences were not observed in cholesterol absorption between wild-type and apoA-I knockout mice fed a high-cholesterol diet. Furthermore, there were no significant gender differences with respect to percent of cholesterol absorption. These data are in agreement with published studies (10, 24, 25). Thus, apoA-I knockout mice absorbed a bolus of radiolabeled cholesterol over a period of 48 h as efficiently as did controls.

Short-term cholesterol absorption studies

Enterocyte studies (Figs. 2, 3) demonstrated decreased cholesterol secretion in apoA-I$^{-/-}$ enterocytes. However, these mice efficiently absorbed a bolus of cholesterol (Table 1). To resolve the discrepancy, we performed short-term cholesterol absorption studies in chow- and cholesterol-fed wild-type and apoA-I$^{-/-}$ mice (**Table 2** and **Fig. 4**) and long-term absorption studies (**Fig. 5**). For short-term studies, mice were given a bolus of radiolabeled cholesterol (Table 2). After 2 h, plasma, liver, and intestine were

TABLE 1. Absorption of a single bolus of cholesterol in normal and apoA-I$^{-/-}$ mice

	Normal Chow-fed Animals			Cholesterol-fed Animals		
	+/+	−/−	*P*	+/+	−/−	*P*
Males						
Sitostanol (dpm × 10^{-5})	6.17 ± 0.78	6.25 ± 0.25	NS	5.58 ± 0.50	5.27 ± 0.70	NS
Cholesterol (dpm × 10^{-5})	1.66 ± 0.22	1.50 ± 0.18	NS	1.65 ± 0.11	1.25 ± 0.23	NS
Ratio	0.270 ± 0.02	0.240 ± 0.02	NS	0.296 ± 0.03	0.237 ± 0.03	NS
Cholesterol absorption (%)	60.3 ± 2.4	64.6 ± 2.7	NS	54.7 ± 4.6	63.7 ± 4.6	NS
Recovery in feces (%)						
Sitostanol	84.4 ± 3.1	85.5 ± 4.6	NS	79.6 ± 7.1	75.2 ± 10.0	NS
Cholesterol	33.5 + 2.0	30.2 ± 1.9	NS	36.0 ± 2.5	27.2 ± 5.1	NS
Females						
Sitostanol (dpm × 10^{-5})	5.09 ± 0.33	4.76 ± 0.21	NS	4.97 ± 0.20	4.87 ± 0.20	NS
Cholesterol (dpm × 10^{-5})	1.08 ± 0.28	1.04 ± 0.81	NS	1.31 ± 0.13	1.13 ± 0.12	NS
Ratio	0.212 ± 0.02	0.218 ± 0.01	NS	0.264 ± 0.04	0.232 ± 0.02	NS
Cholesterol Absorption (%)	63.2 ± 2.3	62.2 ± 1.4	NS	59.3 ± 5.8	64.5 ± 2.6	NS
Recovery in feces (%)						
Sitostanol	82.4 ± 5.4	77.1 ± 3.5	NS	75.1 ± 3.1	73.6 ± 3.0	NS
Cholesterol	30.2 ± 0.8	29.2 ± 2.3	NS	30.2 ± 3.0	26.2 ± 2.9	NS

Normal and apoA-I knockout mice were placed in individual cages that had wire mesh floors. Mice (10–12-week-old, three per group) were fasted for 6 h and then gavaged with a mixture of 0.2 μCi of [^{3}H]sitostanol and 0.1 μCi of [^{14}C]cholesterol in 15 μl of olive oil. Feces were collected for 48 h and processed as described in Materials and Methods. Apo, apolipoprotein. NS, not significant.

collected, and radiolabeled cholesterol levels were measured. Cholesterol counts in apoA-I$^{-/-}$ plasma were 45% less than in controls, mainly as a result of a reduction (64%) in HDL, because diminution (14%) in apoB lipoprotein cholesterol was not significantly different. These studies indicate that apoA-I deficiency significantly reduces the transport of dietary cholesterol in plasma HDL but not in plasma apoB lipoproteins. Comparison of the amounts of cholesterol revealed that livers from knockout mice accumulated 26% less cholesterol, compared with controls (Table 2). In contrast, the amount of cholesterol in the knockout intestines was not decreased.

Next, studies were performed in high-cholesterol (0.2%)-fed mice (Table 2). Cholesterol counts in apoA-I$^{-/-}$ plasma were 62% lower than in controls. Again the major reduction (82%) was in HDL. No significant differences in apoB lipoprotein cholesterol levels were observed. Tissue uptake studies revealed increased accumulation of cholesterol in the apoA-I$^{-/-}$ intestines. In contrast, cholesterol levels in the livers of knockout mice were significantly lower (38% reduction). These short-term studies suggest that apoA-I deficiency decreases cholesterol transport to the plasma and liver but does not decrease cholesterol uptake by the intestinal cells.

We also studied the effects of apoA-I ablation on triglyceride absorption (Table 2). No significant differences in radiolabeled triglycerides in the plasma, HDL, and apoB lipoproteins were found in these mice. Tissue uptake studies also revealed no significant differences in the liver and intestine. Thus, apoA-I ablation has no significant effect

TABLE 2. Short-term cholesterol absorption studies

	Chow-fed Animals				Cholesterol-fed Animals			
	+/+	−/−	Change	*P*	+/+	−/−	Change	*P*
			%				%	
Cholesterol (dpm × 10^{-3})								
Plasma	3.68 ± 0.37	2.03 ± 0.36	−45	<0.01	11.6 ± 1.87	4.4 ± 1.01	−62	<0.005
HDL	2.27 ± 0.26	0.82 ± 0.06	−64	<0.001	7.8 ± 0.85	1.4 ± 0.37	−82	<0.0005
ApoB lipoproteins	1.41 ± 0.22	1.21 ± 0.38	−14	NS	3.8 ± 1.03	3.0 ± 0.67	−21	NS
Liver	52.5 ± 4.28	38.9 ± 1.77	−26	<0.01	77.5 ± 10.16	48.4 ± 5.70	−38	<0.02
Intestine	250.5 ± 29.4	271.1 ± 15.5	+8	NS	644.0 ± 30.0	718.2 ± 19.1	+11	<0.03
Triglycerides (dpm × 10^{-3})								
Plasma	2.84 ± 0.22	2.55 ± 0.13	−10	NS				
HDL	1.97 ± 0.06	1.82 ± 0.09	−8	NS				
ApoB lipoproteins	0.87 ± 0.02	0.73 ± 0.11	−16	NS				
Liver	21.1 ± 2.91	20.5 ± 1.34	−3	NS				
Intestine	11.4 ± 0.05	10.8 ± 0.46	−5	NS				

Mice (three per group) were fed either 1 μCi of [^{3}H]cholesterol and 0.1 mg of unlabeled cholesterol or 0.1 μCi [^{3}H-]triolein in 15 μl of olive oil. After 2 h, plasma, liver, and intestine were collected. Plasma was used for radioactivity measurements. Intestines were flushed with saline and cut into 2 cm pieces, incubated with OptiSolve, and used for radioactivity determinations in the presence of scintillation cocktail. Similarly, liver pieces were washed with PBS, blot dried, digested, and used for radioactivity measurements. HDL, high density lipoprotein.

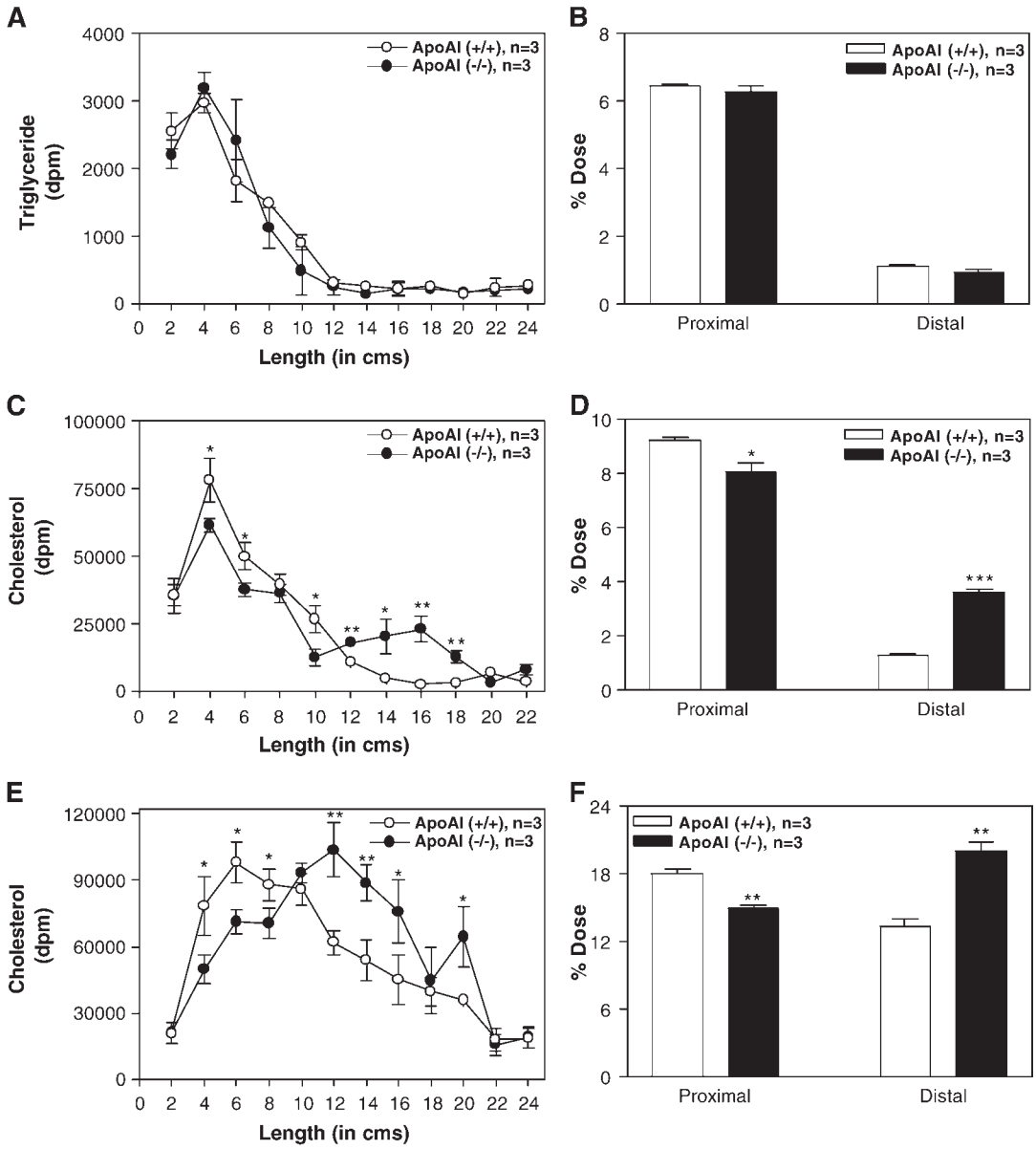

Fig. 4. Distribution of triglyceride and cholesterol along the length of the small intestine during short-term absorption studies. Control and apoA-I$^{-/-}$ mice were fasted overnight and gavaged with either 0.1 μCi of [^{3}H]triolein (A, B) or 1 μCi of [^{3}H]cholesterol (C, D) and 0.1 mg of unlabeled cholesterol in 15 μl of olive oil. Control and knockout mice were also fed a high-cholesterol diet for 2 weeks, fasted overnight, and fed with radiolabeled cholesterol in olive oil (E, F). Two hours later, the small intestine was collected from the base of the stomach and cut into 2 cm segments. Each segment was digested with 1 ml of OptiSolv, mixed with 5 ml of liquid scintillation cocktail, and counted (A, C, E). Counts from the proximal and distal 10 cm were integrated and plotted (B, D, F) to determine regional differences in the amounts of triglyceride or cholesterol present in the intestine. The data are presented as the percent of fed dose present in different regions of the intestine. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Error bars indicate standard deviation.

on triglyceride absorption, and its effect is specific to cholesterol absorption.

Involvement of distal segments of intestine in cholesterol absorption in the apoA-I knockout mice

The short-term studies showed that the amount of cholesterol in the intestines of these mice was not reduced. Yet, these mice had less cholesterol in their plasma and livers. To understand the reasons for the tissue-specific differences, we quantified radiolabeled lipids present in 2 cm segments of the small intestine after 2 h gavage (Fig. 4). Maximum amounts of triglycerides were in the second segment, followed by a gradual decrease (Fig. 4A). There were no differences in the amounts of triglycerides in the proximal and distal segments of the intestine in control and apoA-I$^{-/-}$ mice (Fig. 4B). In control mice, similar to triglycerides, the majority of cholesterol was in the proximal 10 cm of the small intestine (Fig. 4C). Again, the maximum amount of cholesterol was in the second segment, followed by a gradual decrease in the subsequent segments. There were very few counts beyond 12 cm. In con-

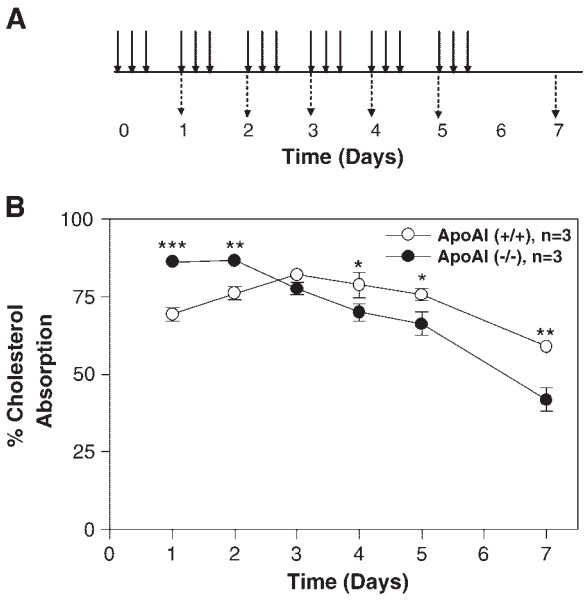

Fig. 5. Long-term cholesterol absorption studies. Three 14-week-old male wild-type and apoA-I$^{-/-}$ mice were gavaged with a mixture of labeled (0.1 μCi) and unlabeled (0.2 mg) cholesterol and labeled sitostanol (0.2 μCi) in 15 μl of olive oil three times a day for 5 days (A, solid arrows). Feces were collected at indicated times (A, broken arrows) and used to measure cholesterol absorption (B) by the dual-isotope ratio method. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Error bars indicate standard deviation.

trast, cholesterol was present in 18 cm of the small intestine in the knockout mice. Integration of the counts in the proximal part of the intestine revealed slightly lower amounts of cholesterol in knockout mice (Fig. 4D, proximal). On the other hand, integration of counts in 10–22 cm segments revealed that knockout animals contained 3-fold higher amounts of cholesterol (Fig. 4D, distal). These studies demonstrate that in wild-type mice, cholesterol absorption occurs mainly in the proximal 10 cm of the small intestine. In apoA-I$^{-/-}$ mice, cholesterol absorption continues further into the distal portions of the intestine.

Short-term cholesterol absorption studies were also performed in control and apoA-I$^{-/-}$ mice fed a high-cholesterol diet (Fig. 4E). In cholesterol-fed control mice, the cholesterol accumulation was now extended to the middle portions of the small intestine. The maximum amounts of cholesterol were in the 4–10 cm region, followed by a broad descending shoulder extending to 20 cm. In apoA-I$^{-/-}$ mice, the cholesterol counts were in the 4–20 cm region. The amount of cholesterol in the proximal 10 cm was lower in apoA-I$^{-/-}$ mice, but was significantly higher (51% increase) in the distal 10 cm compared with wild-type mice (Fig. 4F). These studies indicate that the majority of cholesterol uptake in cholesterol-fed control animals occurs in the central portion of the small intestine and is extended to 20 cm in apoA-I$^{-/-}$ mice.

Long-term cholesterol absorption studies

So far, we noted that a bolus of cholesterol was efficiently absorbed (Table 1), and yet there was less cholesterol absorption in short-term studies (Table 2). We reasoned that compensatory mechanisms involving secretion with apoB lipoproteins might complete the absorption of a bolus of cholesterol in 48 h, and tested the possibility that multiple feedings of radiolabeled cholesterol over a longer period might show a difference in cholesterol absorption between control and knockout mice (Fig. 5). Animals were fed three times a day with unlabeled cholesterol, along with radiolabeled cholesterol and sitostanol for 5 days, and feces were collected every day (Fig. 5A). Isotopic ratio analysis revealed that apoA-I knockout mice actually absorbed more cholesterol than did the wild-type in the first two days (Fig. 5B). Subsequently, cholesterol absorption in these mice decreased (11–29%) and remained significantly lower for 4–7 days. In control mice, cholesterol absorption increased slightly for the first 3 days and then declined slightly. These data show that cholesterol absorption is significantly reduced after 4 days in apoA-I knockout mice under chronic conditions.

DISCUSSION

Previously, we provided evidence for multiple, independently regulated pathways of cholesterol transport across the intestinal epithelial cells in culture (8). Here, we show for the first time that there are multiple compensatory pathways that constitute a high-capacity system for efficient cholesterol absorption in vivo. Ablation of apoA-I decreases cholesterol secretion with HDL but has no effect on its secretion with apoB lipoproteins. In short-term studies, we observed decreased absorption, but a bolus of cholesterol was completely absorbed within 48 h by the knockout mice. We interpret these studies to suggest that there is an initial delay in the absorption of cholesterol and that a bolus of cholesterol is eventually absorbed, most likely by the complementary apoB pathway. This would explain why there is no difference in liver cholesterol metabolism in other studies (24). Even when a high-cholesterol diet is fed, there is no significant difference in the absorption of a single bolus of cholesterol, perhaps because these feeding conditions are not sufficient to stress the system. This is supported by the fact that absorption of a single bolus of cholesterol is as good or even better (24) in cholesterol-fed apoA-I$^{-/-}$ mice, compared with controls (Table 1). We did, however, observe reduced cholesterol absorption in long-term studies involving multiple feedings of radiolabeled cholesterol with higher amounts of unlabeled cholesterol. These studies were designed to stress the system to amplify subtle differences so that they can be quantified and are not physiologic.

Short-term cholesterol absorption studies revealed two major differences between wild-type and apoA-I knockout mice. First, the amount of cholesterol transported in 2 h to the plasma and liver was significantly lower in apoA-I$^{-/-}$ mice compared with controls (Table 2). This indicated that apoA-I deficiency resulted in significant decrease in the early phases of cholesterol absorption. Similar decreased

cholesterol transport during short-term studies has been observed in NPC1L1 and pancreatic triglyceride lipase-deficient mice (4–6).

Second, significant differences were also observed in the amounts of cholesterol present along the length of the small intestine (Fig. 4). In the chow-fed control mice, all of the cholesterol was in the proximal 10 cm. This is in agreement with the general understanding that cholesterol absorption mainly occurs in the first part of the jejunum (46). In contrast, cholesterol was present over 20 cm of the small intestine in apoA-I knockout animals. Similarly, in cholesterol-fed control and knockout animals, there was a separation with regard to the maximum cholesterol counts present along the length of the intestine. These studies indicated that in the absence of apoA-I, cholesterol absorption was not completed in the proximal small intestine but was extended to the distal portions. Involvement of distal segments of the intestine in the absorption of triglycerides has been observed in pancreatic triglyceride lipase-deficient mice (4). The differences are more likely because apoA-I deficiency results in decreased cholesterol absorption in the proximal intestine as well as increased traveling of the cholesterol to the distal parts.

Despite the two major differences discussed above, a bolus of cholesterol was efficiently absorbed by apoA-I knockout mice (Table 1). We propose that, in the absence of apoA-I, apoB lipoproteins might complete the absorption of a bolus of cholesterol in the distal parts. It is also possible that the HDL pathway may compensate partially in the absence of the apoB pathway. Illingworth et al. (47) have observed that cholesterol absorption was 45% and 30% in control and abetalipoproteinemia patients, respectively, indicating significant reduction but not a complete absence of cholesterol absorption. Recently, evidence has been presented in an abstract that ACAT2 and ABCA1 may play an additive role in cholesterol absorption (48). In this study, the absence of either ACAT2 or ABCA1 resulted in a 23% or 48% reduction in cholesterol absorption, respectively. However, a combined deficiency of these two proteins resulted in a 79% reduction in cholesterol absorption. Thus, the apoB and high density lipoprotein pathways are perhaps complimentary and most likely constitute a high capacity system for efficient cholesterol absorption in mice. In chicken, the HDL-pathway has been suggested to be the major pathway for cholesterol transport (49).

One reason for the decreased cholesterol absorption in short-term studies in apoA-I$^{-/-}$ mice could be reduced amounts of bile acids in the intestinal lumen of these animals. Jolley, Dietschy, and Turley (24) have shown that wild-type and apoA-I knockout mice contain the same amounts of bile acids and have concluded that bile acid synthesis and secretion is not altered in apoA-I-deficient mice despite low plasma HDL cholesterol levels. Furthermore, we did not find any differences (Fig. 4A) in triglyceride absorption, which is also dependent on bile acids. Thus, decreased cholesterol absorption in knockout mice is probably not due to bile acid deficiency.

How does apoA-I play a role in cholesterol secretion? There are possibly two mechanisms. It is known that apoA-I is secreted independent of lipids and that the lipid-poor apoA-I can accept cholesterol from ABCA1. We and others have shown that ABCA1 plays a role in apoB-independent cholesterol transport across the intestinal epithelial cells (8, 50, 51). Thus, it is possible that secreted apoA-I mediates cholesterol efflux after binding to ABCA1. Some evidence for this pathway was obtained when free apoA-I was supplied to enterocytes (Fig. 3). Free apoA-I increased cholesterol secretion by intestinal cells isolated from control and knockout mice. The second mechanism may involve intracellular assembly and secretion of HDL. Glickman and associates, using mesenteric lymph cannulation, provided significant evidence in favor of the synthesis and secretion of HDL by intestinal cells (52–55). They showed that apoA-I in the lymph HDL was secreted independent of apoB chylomicrons.

In summary, we have provided evidence that cholesterol secretion by the small intestine via the apoB-independent pathway involves apoA-I. ApoA-I deficiency results in decreased cholesterol secretion by primary enterocytes. Furthermore, cholesterol absorption is significantly delayed in apoA-I-deficient mice. In apoA-I deficiency, cholesterol transport across the proximal intestine is decreased, and increased amounts of cholesterol travel to distal ends of the intestine. In the distal region, over a longer period of time, apoB-dependent pathways may complete the absorption of cholesterol. Thus, the apoB- and apoA-I-dependent pathways may complement each other to maximize cholesterol absorption by the intestine.

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