Apolipoprotein E regulates dietary cholesterol absorption and biliary cholesterol excretion: Studies in C57BL/6 apolipoprotein E knockout mice

Ephraim Sehayek*, Sarah Shefer†, Lien B. Nguyen†, Jennie G. Ono*, Martin Merkel*, and Jan L. Breslow*‡

*Laboratory of Biochemical Genetics and Metabolism, The Rockefeller University, 1230 York Avenue, New York, NY 10021; and †Department of Medicine and Liver Center, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, NJ 07103

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The present study examined the role of apolipoprotein E (apoE) in the regulation of dietary cholesterol absorption and biliary cholesterol excretion. Increasing dietary cholesterol from 0.02% to 0.5% in C57BL/6 wild-type mice decreased the percentage of dietary cholesterol that is absorbed by 25%, and this decrease was associated with a 2-fold increase in gallbladder biliary cholesterol concentration. In contrast, increasing dietary cholesterol from 0.02% to 0.5% in C57BL/6 apoE knockout mice produced no significant suppression of the percentage dietary cholesterol absorption and increased gallbladder biliary cholesterol concentration only 16%. Whereas in wild-type mice, the increase in dietary cholesterol increased the hepatic excretion of biliary cholesterol 4-fold, there was only a 2-fold increase in apoE knockout mice. On both the low- and the high-cholesterol diets, whole liver and isolated hepatocyte cholesterol content was higher in the apoE knockout mice. These results suggest that, in response to dietary cholesterol, apoE may play a critical role in decreasing the percentage absorption of dietary cholesterol and increasing biliary cholesterol excretion. These observations suggest a mechanism whereby the absence of apoE contributes to the propensity for tissue cholesterol deposition and accelerated atherogenesis in apoE knockout mice.

The absorption of dietary cholesterol from the intestine represents the first step that allows dietary cholesterol to exert its metabolic effects. Within the intestinal lumen, dietary cholesterol is presented to the brush border of mucosal enterocytes in a micellarized form that is partially taken up, partially esterified, and secreted into lymph in the form of chylomicrons (1). In the plasma compartment, dietary cholesterol, in the form of chylomicron remnants, is cleared from the circulation mainly by the liver through the interaction of apolipoprotein E (apoE) with liver receptors (2). The uptake of dietary cholesterol by liver parenchymal cells initiates regulatory events that control hepatic cholesterol content. For example, the influx of cholesterol into the liver suppresses the biosynthesis and stimulates the esterification and storage of cholesterol. In addition, the liver is provided with a unique capacity to handle cholesterol loads, primarily through excretion into the bile, by conversion to bile acids, and secretion of lipoproteins (3). Thus, intestinal cholesterol absorption and biliary cholesterol excretion represent key steps in the metabolism of dietary cholesterol.

In our laboratory, we are using induced mutant mice to study the regulation of dietary cholesterol absorption. In a previous study, we showed that increasing dietary cholesterol from 0.02% to 0.5% in C57BL/6 mice decreased the percentage of dietary cholesterol, which is absorbed by 25%, and that this decrease strongly correlated with the 2- to 3-fold increase observed in gallbladder biliary cholesterol concentration. We then showed, using transgenic mice, that liver-specific overexpression of SRB1 increased biliary cholesterol concentration and decreased dietary cholesterol absorption. From these observations, we have postulated that the stimulation of biliary cholesterol excretion saturates the micellar compartment with biliary cholesterol, which in turn decreases the transfer of dietary cholesterol into micelles in the intestinal lumen and thereby decreases the percentage absorption of dietary cholesterol (4).

In view of the importance of apoE in delivering dietary cholesterol to the liver, and because there is some precedent in humans that apoE may play a role in the regulation of dietary cholesterol absorption (5), we examined the regulation of dietary cholesterol absorption and biliary cholesterol excretion in apoE knockout mice (6). We found that, in apoE knockout mice, dietary cholesterol fails to suppress the percentage absorption of dietary cholesterol, and inefficiently stimulates cholesterol excretion into the bile. These studies suggest that, in response to dietary cholesterol, apoE knockout mice have a defect in suppressing the percentage dietary cholesterol absorption and in stimulating biliary cholesterol excretion.

Experimental Procedures

Animals and Diets. Wild-type (WT) and apoE knockout C57BL/6 males were purchased from The Jackson Laboratory. Animals were housed in a humidity- and temperature-controlled room with a 12-h dark-light cycle at the Laboratory Animal Research Center at The Rockefeller University and fed for 3 wk with Picolab Rodent Chow 20 (5053) pellet containing 0.02% (wt/wt) cholesterol or the same diet fortified with cholesterol to a final cholesterol content of 0.5% (wt/wt) (TD 97275; Harlan Teklad, Madison, WI). The 0.02% and 0.5% cholesterol diets correspond to human cholesterol intake of approximately 40 mg and 1,000 mg/1,000 kcal, respectively.

Cholesterol Absorption Measurements. Cholesterol absorption was determined in WT and apoE knockout animals aged 10–13 wk by using a modified form of the "dual isotope single meal feeding" method as described (4). Briefly, after 3 wk of feeding either the 0.02% or the 0.5% cholesterol diet, animals were placed in metabolic cages, fasted for 4–6 h, and toward the end of the light cycle administered an intragastric bolus of olive oil containing [^{14}C]cholesterol (DuPont) and β-[^{3}H]sitostanol (American Radiolabeled Chemicals, St. Louis). Feces then were collected for 24 h, dried, homogenized, and lipids were extracted

Abbreviations: apoE, apolipoprotein E; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; WT, wild type.

‡To whom reprint requests should be addressed. E-mail: breslow@rockvax.rockefeller.edu.

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and counted for [^{14}C] and [^{3}H]. The percentage absorption of dietary cholesterol was calculated by using the formula: % cholesterol absorption = {1 − [fecal(^{14}C dpm/^{3}H dpm)]/[administered(^{14}C dpm/^{3}H dpm)]} × 100.

Gallbladder Bile Aspiration and Bile Collection. Mice were fasted for 6 h, weighed, and anesthetized. For gallbladder bile aspiration, the abdominal cavity was exposed through a ventral incision and the gallbladder bile was aspirated. For bile collection, the common bile duct was ligated in proximity to the duodenum, the gallbladder was nicked and cannulated with a polyethylene P-10 tube (Becton Dickinson), and bile was collected for 1 h. The bile volume was estimated by measuring the weight of the bile and assuming a bile fluid-specific gravity of 1. The gallbladder bile and collected bile were stored at 4°C, and their cholesterol, bile acid, and phospholipid contents were analyzed within 14 days using enzymatic methods (Sigma Diagnostics commercial kits 352 and 450-A for cholesterol and bile acids, respectively, and Wako Commercial GmbH kit for phospholipids). The output of the biliary lipid constituents was calculated by multiplying the collected bile volume by the concentrations of the biliary lipid constituents and expressed as μg/g body weight/h. Plasma cholesterol levels were determined enzymatically in EDTA containing blood samples using a Sigma kit, as described above for biliary cholesterol. The percent cholesterol saturation of the gallbladder bile was calculated by using the critical tables published by Carey (7).

Isolation of Hepatocytes. Mice were fasted and anesthetized, and the abdominal cavity was exposed as described above. The portal vein was cannulated with a 24-gauge polyethylene i.v. catheter (Becton Dickinson). The inferior vena cava was nicked and the liver was perfused for 3–5 min with oxygenated (95% O_2/5% CO_2) calcium/magnesium-free Hank's buffer. This process was followed by an additional 20 min of perfusion with the same buffer but now containing 0.03 g % type 1 collagenase (Worthington). The collagenase-treated liver was harvested, transferred into ice-cold Williams medium E (GIBCO/BRL), minced with a razor, and gently homogenized using a plastic Pasteur pipette while on ice. The volume of the homogenate was adjusted to 25 ml with ice-cold Williams medium, mixed with 24 ml of pH 2.2 $NaCO_3$ Hank's buffered Percoll (Sigma), and centrifuged at 76 × *g* for 10 min at 4°C. The hepatocyte pellet was resuspended and washed twice with ice-cold PBS (pH 7.4) by centrifugation at 300 × *g* for 10 min at 4°C. Under these conditions, the hepatocyte pellet contains a negligible number of Kupffer and endothelial cells as judged by horseradish peroxidase immunostaining with antimacrophage and antiendothelial cell antibodies.

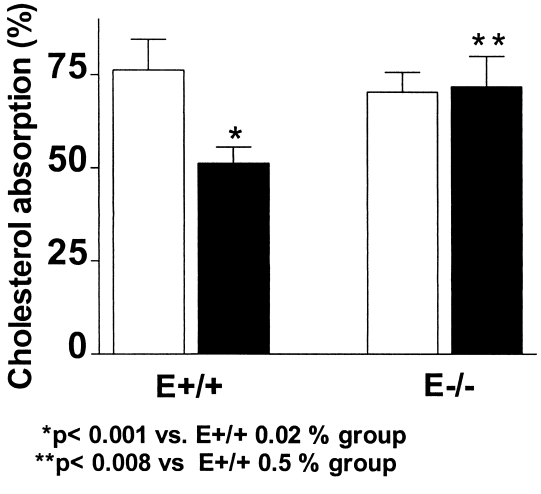

Fig. 1. Effect of dietary cholesterol on cholesterol absorption in WT and apoE knockout mice. WT (E+/+) and apoE knockout (E−/−) C57BL/6 males were fed for 3 wk with either dietary 0.02% (open bars) or 0.5% wt/wt cholesterol (filled bars). Mice received a gastric bolus of 100 μl of olive oil containing 1.67 μCi [^{14}C]cholesterol and 0.67 μCi [^{3}H]β-sitostanol and were placed in metabolic cages, and feces were collected for 24 h. The ratio of [^{14}C]/[^{3}H] labels in the fecal lipid extracts was determined and % absorption was calculated as detailed in *Experimental Procedures*. Data presented are the mean ± SD of five animals in each group. *, $P < 0.001$ vs. E+/+ 0.02% group; **, $P < 0.008$ vs. E+/+ 0.5% group.

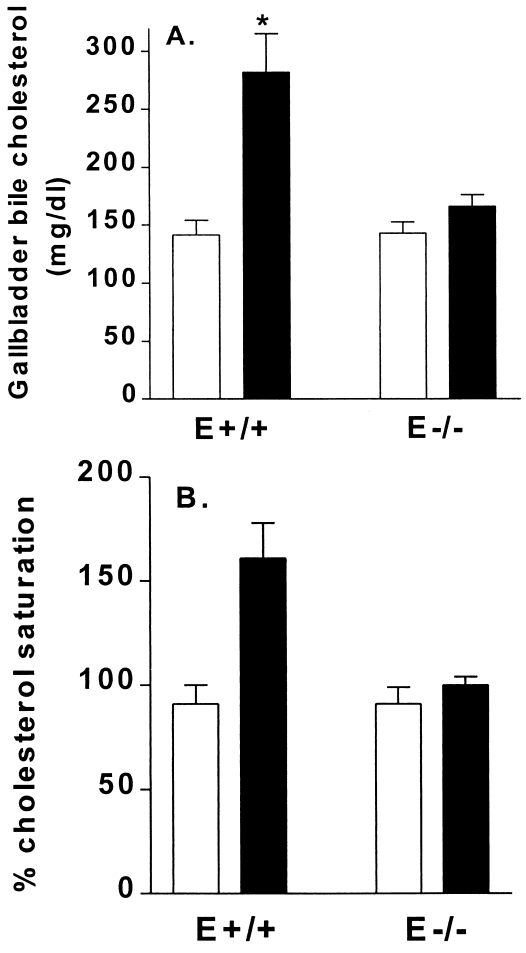

Fig. 2. Effect of dietary cholesterol on gallbladder bile concentrations (*A*) and percent saturation (*B*) of cholesterol in WT and apoE knockout mice: WT (E+/+) and apoE knockout (E−/−) mice were fed for 3 wk with 0.02% (open bars) and 0.5% cholesterol (filled bars). After 3 wk, the animals were fasted, the peritoneal cavity was exposed, and gallbladder bile was aspirated. Biliary cholesterol concentrations of cholesterol were measured and percent saturation was calculated as described in *Experimental Procedures*. Data displayed are the mean ± SD of five animals in each group.

Measurements of Liver Cholesterol Content, Activities of Cholesterol 7α-Hydroxylase and 3-Hydroxy-3-Methylglutaryl CoA Reductase (HMGR). Animals were fasted for 6 h and then anesthetized. The abdominal cavity was exposed, the liver was perfused with ice-cold saline and harvested, and the total and free cholesterol content were determined by gas-liquid chromatography as described (4). The content of cholesterol ester was calculated by subtracting the value for free cholesterol from the total cholesterol content after KOH hydrolysis. The cholesterol content of

Table 1. Effect of dietary cholesterol on plasma cholesterol levels, bile flow, and biliary lipids output in WT and apoE knockout mice

Animal group (*n*)	Plasma cholesterol, mg/dl	Biliary cholesterol	Biliary phospholipids, μg/g body weight/h	Biliary bile acids	Bile flow*
E+/+ 0.02% (10)	66 ± 12	0.73 ± 0.14	15.6 ± 2.9	63.7 ± 18.4	3.01 ± 0.88
E+/+ 0.5% (10)	67 ± 12	3.30 ± 0.81‡	26.6 ± 5.5‡	107.3 ± 44.5§	5.09 ± 1.06†
E−/− 0.02% (14)	403 ± 178	0.82 ± 0.15	20.0 ± 8.3	66.8 ± 24.9	3.22 ± 0.96
E−/− 0.5% (14)	788 ± 256	1.70 ± 0.47†	22.6 ± 4.9	76.3 ± 25.9	4.68 ± 1.42§

*μl bile/g body weight/h.
†$P < 0.0003$; ‡$P < 0.0001$; §$P < 0.02$, compared to 0.02% group of the same genotype.

isolated hepatocytes was measured by gas-liquid chromatography as above and expressed as ng cholesterol/μg hepatocyte protein. Upon liver harvesting, tissue samples were frozen immediately in liquid nitrogen and used for the measurements of cholesterol 7α-hydroxylase and HMGR activities, as described (4).

Statistical Analysis. The differences in percentage cholesterol absorption, gallbladder bile composition, biliary lipids output, liver, and isolated hepatocytes cholesterol content and the activities of 7α hydroxylase and HMGR were analyzed by using unpaired Student's *t* test.

Results

To study the role of apoE in the regulation of dietary cholesterol absorption, we fed WT and apoE knockout mice diets containing 0.02% (wt/wt) and 0.5% (wt/wt) cholesterol. WT mice efficiently suppress the percentage dietary cholesterol absorption when going from 0.02% to 0.5% cholesterol diets (Fig. 1). However, apoE knockout mice do not suppress the percentage dietary cholesterol absorption. Thus, whereas WT and apoE knockout mice absorb cholesterol at the same rate on the 0.02% cholesterol diet, the apoE knockout mice absorb cholesterol at a higher rate than WT on the 0.5% cholesterol diet.

We determined the lipid composition of the gallbladder bile in 0.02% and 0.5% cholesterol-fed WT and apoE knockout. Whereas in WT animals the increase in cholesterol intake from 0.02% to 0.5% resulted in a 2-fold increase in biliary cholesterol concentrations (142 ± 12 mg/dl to 282 ± 34 mg/dl, $P < 0.011$, respectively) it did not change in the apoE knockouts (143 ± 10 mg/dl to 166 ± 10 mg/dl, $P = 0.427$, respectively) (Fig. 2*A*). No significant changes in gallbladder bile concentration of phospholipids or bile acids were found in response to dietary cholesterol in either WT or apoE knockout mice (data not shown). To examine how the changes in cholesterol concentrations affected the capacity of the bile to solubilize cholesterol, we calculated the percent cholesterol saturation of the gallbladder bile in the different groups. As shown in Fig. 2*B*, whereas in WT going from 0.02% to 0.5% cholesterol in the diet resulted in percent saturation that exceed by 61% the maximal capacity of the gallbladder bile to solubilize cholesterol (91 ± 9% vs. 161 ± 17% in 0.02% and 0.5% cholesterol-fed WT animals, $P < 0.0001$, respectively), in the apoE knockouts, the percent saturation remained within the limits of their maximal capacity (91 ± 8% vs. 100 ± 4% in 0.02% and 0.5% cholesterol-fed apoE knockout animals, $P < 0.03$, respectively).

To extend the observations on gallbladder bile composition to biliary lipid excretion, we cannulated the biliary tract of WT and apoE knockout mice fed 0.02% and 0.5% cholesterol diets and measured the bile flow and output of biliary cholesterol, phospholipids, and bile acids. On the 0.02% cholesterol diet, WT and apoE knockout mice had similar biliary cholesterol output (0.73 ± 0.14 vs. 0.82 ± 0.15 μg/g body weight/h, $P = 0.15$, respectively). However, on the 0.5% cholesterol diet, WT and apoE knockout mice had different biliary cholesterol output (3.30 ± 0.81 vs. 1.70 ± 0.47 μg/g body weight/h, $P < 0.0007$, respectively). Thus, in response to dietary cholesterol challenge, WT mice increase biliary cholesterol excretion 4.5-fold, whereas apoE knockout mice increase only 2-fold (Table 1). In addition, in response to dietary cholesterol challenge, WT mice significantly increased the excretion of biliary phospholipids and bile acids, but this does not occur in the apoE knockout mice (Table 1).

Next, we measured the liver cholesterol content of WT and apoE knockout mice on the 0.02% and 0.5% cholesterol diets. After carefully flushing out the livers, the apoE knockout mice had greater amounts of liver cholesterol than WT mice both on 0.02% and 0.5% cholesterol diets (Fig. 3*A*). In addition, isolated hepatocytes from apoE knockout mice also had greater amounts of cholesterol (Fig. 3*B*). These results indicate unequivocally that, under a wide range of dietary cholesterol intake, hepatocytes in apoE knockout mice have increased cholesterol content.

The apparent blockage of biliary cholesterol excretion in response to dietary cholesterol prompted us to study other effects of dietary cholesterol. WT and apoE knockout mice displayed similar levels of HMGR and cholesterol-7α hydroxylase activities on the 0.02% cholesterol diet, and both activities suppressed and stimulated, respectively, to the same degree when fed the 0.5% cholesterol diet (Fig. 4).

The response of plasma cholesterol to the increase in dietary cholesterol differed between WT and apoE knockout mice. Dietary cholesterol challenge of WT mice did not result in an increase of plasma cholesterol levels (66 ± 12 and 67 ± 12 mg/dl), whereas there was a 2-fold increase in apoE knockout mice (403 ± 178 to 788 ± 256 mg/dl) (Table 1). The particles that accumulate in the plasma of apoE knockout mice are largely intestinal in origin. Therefore, the increase in plasma cholesterol in response to dietary cholesterol is probably just because of very slow clearance of these particles by the liver in the apoE knockout mice.

Discussion

The present study was designed to examine the role of apoE in the regulation of dietary cholesterol absorption and biliary cholesterol excretion. We could show that apoE knockout mice have an impaired capacity for dietary cholesterol to suppress the percentage absorption of dietary cholesterol, increase the concentration of cholesterol in the gallbladder bile, and stimulate the excretion of cholesterol into the biliary compartment.

The role of apoE in the regulation of cholesterol absorption has been addressed by Woollett *et al.* (8). In this study, similar to our results, the authors found no differences in cholesterol absorption rates in low cholesterol-fed WT and apoE knockouts. It is therefore important to note that our findings on the role of apoE in the regulation of cholesterol absorption emerged only

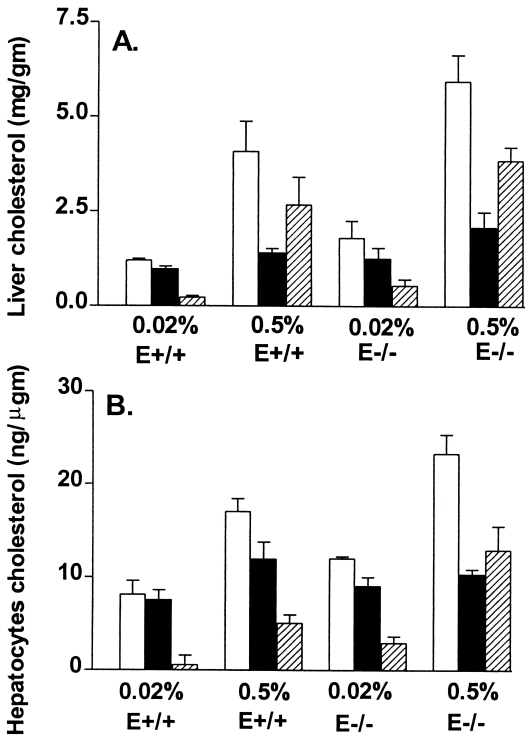

Fig. 3. Effect of dietary cholesterol on liver (*A*) and hepatocytes (*B*) cholesterol content in WT (E+/+) and apoE knockout (E−/−) mice. Animals were fed for 3 wk with either 0.02% or 0.5% cholesterol and fasted for 4–6 h. (*A*) For liver cholesterol measurements, livers were perfused with PBS and harvested and, (*B*) for hepatocyte cholesterol measurements, livers were perfused with collagenase and hepatocytes were isolated, as described in *Experimental Procedures*. Liver and hepatocytes lipids were extracted, and total cholesterol (open bars), free cholesterol (filled bars), and cholesterol ester (hatched bars) contents were measured as described in *Experimental Procedures*. Data displayed are the mean ± SD of five animals in each group.

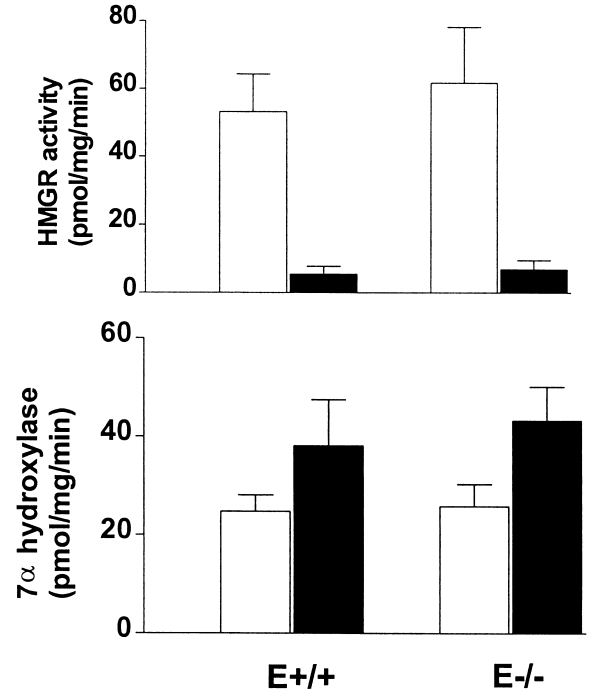

Fig. 4. Effect of dietary cholesterol on liver HMGR and cholesterol 7α hydroxylase activities in WT and apoE knockout mice: WT (E+/+) and apoE knockout (E−/−) animals were fed with either 0.02% (open bars) or 0.5% cholesterol (filled bars), livers were harvested, and HMGR and cholesterol 7α hydroxylase activities were measured as described in *Experimental Procedures*. Data displayed are the mean ± SD of five animals in each group.

after the apoE knockouts were challenged with high-cholesterol diet.

In a previous study on the regulation of dietary cholesterol absorption, we found that increasing dietary cholesterol from 0.02% to 0.5% in C57BL/6 mice decreased the percentage of dietary cholesterol, which is absorbed by 25%, and that this decrease strongly correlated with the 2- to 3-fold increase observed in gallbladder biliary cholesterol concentration. We then showed, using transgenic mice, that liver-specific overexpression of SRB1 increased biliary cholesterol concentration and decreased dietary cholesterol absorption. (4). This study led us to hypothesize that the increase in gallbladder bile cholesterol concentration, brought about by dietary cholesterol, saturates the micellar compartment with biliary cholesterol, which in turn decreases the transfer of dietary cholesterol from the oil phase into the micellar phase within the intestinal lumen, and thereby decreases the percentage absorption of dietary cholesterol. In the present study, by showing that apoE knockout mice fail to: (*i*) suppress dietary cholesterol absorption, (*ii*) increase gallbladder cholesterol concentration, and (*iii*) exceed the capacity of their gallbladder bile to solubilize cholesterol in response to dietary cholesterol, we provide further support for our hypothesis.

The present study also shows in WT mice that dietary cholesterol increases biliary excretion of cholesterol, phospholipid, and bile acids, as well as increasing bile flow. In apoE knockout mice, dietary cholesterol does not increase the biliary excretion of phospholipid and bile acids. The apoE knockout mice showed only a 2-fold increase in biliary cholesterol excretion, compared with 4.5-fold in WT mice. We are not aware of previous studies that have carefully addressed the relationship between dietary cholesterol and biliary lipid excretion and bile flow. Our observation of impaired capacity of dietary cholesterol to stimulate excretion into the bile in apoE knockout mice suggests a direct role for apoE in this process.

ApoE plays a well-known pivotal role in determining the liver uptake and disposal of dietary cholesterol in the form of chylomicron remnants. This is best exemplified by the increased plasma cholesterol levels and impaired clearance of chylomicron remnants in the apoE knockout mice (6). However, we found at both low and high dietary cholesterol intake that apoE knockout mice have increased whole liver and hepatocyte cholesterol content (Fig. 3). Thus, decreased liver cholesterol was not responsible for the decreased excretion of biliary cholesterol in dietary cholesterol challenged apoE knockout mice. In addition, it is also noteworthy that our present study does not resolve the question on what causes the increase in cholesterol content in apoE knockouts and that these findings deserve additional studies.

The absence of apoE could alter the trafficking of dietary cholesterol in hepatocytes. This could be a global problem resulting in dietary cholesterol sequestered in an inactive pool. However, we have shown in apoE knockout mice that dietary cholesterol normally suppresses HMGR activity and stimulates cholesterol 7α hydroxylase activity (Fig. 4). Therefore, apoE must be involved in trafficking cholesterol to specific pools necessary for the presentation of cholesterol to the canalicular membrane. This probably occurs at the level of the plasma membrane or in endocytic vesicles. In macrophages, it has been shown that the type of presenting lipoprotein particle can determine the intracellular fate of the delivered cholesterol and

a similar process could occur in hepatocytes (9, 10). In addition, *in vivo* studies in rats have shown a preferential use of HDL cholesterol for bile acid formation and biliary cholesterol excretion (11). Furthermore, SRB1 overexpression has been shown to increase liver cholesterol content and gallbladder bile cholesterol concentration (4, 12, 13). Therefore, lipoprotein particles that preferentially interact with SRB1 might favor this pathway. Finally, it is also possible, although unlikely, that apoE plays a direct intracellular role in the movement of cholesterol to the canalicular membrane.

There are two observations in our data set that remain unexplained. On a low-cholesterol diet, WT and apoE knockout mice have equivalent levels of hepatic HMGR and cholesterol 7α hydroxylase activities, yet the apoE knockout mice have higher levels of cholesterol. It may be that, on the low-cholesterol diet, the increase is in a cholesterol pool that is relatively unavailable for metabolic regulation of these two enzymes, just as we have shown that on the high-cholesterol diet in the apoE knockout mouse there is probably a pool unavailable for biliary cholesterol excretion. Second, increasing dietary cholesterol in the apoE knockout mouse stimulated cholesterol 7α hydroxylase activity but did not significantly increase biliary bile acid secretion, whereas normal mice showed a 68% increase in biliary bile acid secretion. It is conceivable that apoE might influence alternate pathways of bile acid production or the bile acid pool in such a way to prevent a measurable increase in biliary bile acid secretion.

Feeding apoE knockout mice a high-fat, high-cholesterol diet results in accelerated atherosclerosis and cholesterol deposition in soft tissue with xanthoma formation (14, 15) and deposition of cholesterol crystals in the central nerve system (16). In the present study, we show that, in response to dietary cholesterol, apoE knockout mice are defective in decreasing the percentage absorption of dietary cholesterol and stimulating cholesterol excretion into the bile. These results provide further insight into possible mechanisms that can promote tissue cholesterol deposition in these animals.

There is some precedent in humans that apoE may play a role in the regulation of dietary cholesterol absorption. Mietinnen (5) showed the percentage dietary cholesterol absorption decreased in the order E4, E3, E2. He also showed that fecal excretion of cholesterol tended to be higher in E2 compared with E3 or E4 subjects (17). There has been no careful study in individuals of different apoE genotypes of percentage dietary cholesterol absorption and either gallbladder biliary cholesterol concentration or biliary cholesterol excretion on different cholesterol intakes as we have done in the apoE knockout mouse.

Of course, we are aware that dietary cholesterol absorption can be regulated at many different levels besides biliary cholesterol concentration. There is evidence for intestinal mucasal mechanisms, as revealed by the selective absorption of cholesterol in preference to plant sterols and the increased absorption of both in the disorder β-sitosterolemia. In addition, Hauser (18, 19) has shown that specific proteins derived from enterocyte brush border membranes promote the uptake of cholesterol by vesicles and more recently that SR-BI might be involved in this process. Also implicated in the regulation of dietary cholesterol absorption are pancreatic enzymes, CEL, and phospholipase A2 (20, 21), the enterocyte ACAT that esterifies dietary cholesterol (22), apoA-IV (23, 24), and different bile acid species that are the result of the classical and alternative bile acid biosynthetic pathways (25, 26). The actual *in vivo* role for many of these processes is under active evaluation in a number of laboratories.

In summary, in the present study, we used the mouse model to show that apoE plays a critical role in the regulation of dietary cholesterol absorption and biliary cholesterol excretion. Our results clearly show that the absence of apoE results in increased hepatocyte cholesterol content that is associated with inadequate stimulation of cholesterol excretion into the bile and impaired suppression of dietary cholesterol absorption rates in response to high cholesterol intake. If similar mechanisms operate in humans, then the elucidation of the molecular events that underlay the excretion of cholesterol into the bile may lead to new insights into the understanding of the whole body cholesterol homeostasis, the regulation of dietary cholesterol absorption, plasma lipoprotein responsiveness, and possibly new approaches to the prevention of atherosclerotic cardiovascular diseases.

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