Role of intestinal sterol transporters Abcg5, Abcg8, and Npc1l1 in cholesterol absorption in mice: gender and age effects

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Because elevated plasma cholesterol levels are an important risk factor for cardiovascular diseases, National Cholesterol Education Program Adult Treatment Panel III guidelines have proposed to treat high-risk patients to a LDL-cholesterol goal of <100 mg/dl (9). The cholesterol carried in LDL is derived principally from de novo synthesis and absorption from the diet (25). In the Finnish population, there is a significant and positive correlation between the level of plasma LDL-cholesterol and the efficiency of intestinal cholesterol absorption (14). Therefore, understanding the genetic regulation of cholesterol absorption may lead to novel approaches to the treatment of cardiovascular diseases that affect millions of people in Westernized societies. Thus great efforts have been made to search for molecular, genetic, biochemical, and physical-chemical determinants of intestinal cholesterol absorption (16). Recent studies show that ATP-binding cassette (ABC) transporters ABCG5 and ABCG8 may work together as an apical sterol export pump promoting active efflux of cholesterol and plant sterols from the enterocyte back into the intestinal lumen for excretion (20, 38–41). Also, the newly identified Niemann-Pick C1-like 1 (NPC1L1) protein may play a critical role in the ezetimibe-sensitive cholesterol absorption pathway and might induce active influx of cholesterol from the intestinal lumen into the enterocyte (1, 3). These findings strongly support the notion that cholesterol absorption is a multistep process that is regulated by multiple genes at the enterocyte level (16, 33). Therefore, it is imperative to investigate molecular mechanisms underlying the dominant rate-limiting step/factor in cholesterol absorption.

In addition, it has been found that there are gender differences in intestinal cholesterol absorption efficiency in animals and humans, and the efficiency of cholesterol absorption increases with age (12, 26, 27, 30). More recently, we (30, 36) reported that high doses of estrogen and aging greatly increase hepatic outputs of biliary lipids and cholesterol content of bile as well as biliary bile salt pool size and hydrophobicity index in mice, consistent with the results from human studies (2, 5, 11, 28). These alterations in biliary lipid outputs explain, in part, how gender and age exert a major influence on the efficiency of intestinal cholesterol absorption. Hitherto, little is known whether gender and aging, two independent factors, have an effect on intestinal cholesterol absorption via the newly identified intestinal sterol transporters Abcg5, Abcg8, and Npc1l1. In the present study, we investigated whether estrogen may influence intestinal cholesterol absorption by regulating gene expression levels of the intestinal sterol transporters through an estrogen receptor (ER) pathway and whether aging per se may augment intestinal cholesterol absorption by regulating expression levels of the intestinal sterol transporter genes. To further explore whether there are any differences in intestinal cholesterol absorption regulated by estrogen and aging between the higher cholesterol-absorbing C57L mice and the lower cholesterol-absorbing AKR mice, we studied these two unique mouse models.

Materials and Methods

Chemicals. Medium-chain triglyceride was purchased from Mead Johnson (Evansville, IN), and Intralipid [20%, (wt/vol)] was from Pharmacia (Clayton, NC). ICI 182,780 [Fulvestrant, 7α-[9-(4,4,5,5,5-penta fluoropentylsulphinyl)nonyl]estr-1,3,5-(10)-triene-3,17β-diol] was purchased from AstraZeneca Pharmaceuticals (Wilmington, DE).

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17β-estradiol (E2)-releasing pellets were purchased from Innovative Research of America (Sarasota, FL). Radioisotope [5,6-3H]sitosanol was from American Radiolabeled Chemicals (St. Louis, MO).

Animals and diets. Inbred AKR/J and C57L/J mice of both genders (3 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were maintained in a temperature-controlled room (22 ± 1°C) with a 12-h day cycle (6:00 AM-6:00 PM). Mice were fed with normal rodent chow (Harlan Teklad F6 Rodent Diet 8664, Madison, WI) containing trace (<0.02%) amounts of cholesterol. All procedures were in accordance with current National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of Harvard University.

To exclude possible interindividual differences in endogenous estrogen concentration, at 4 wk of age, female mice were ovarioectomized and males were orchidectomized, and, at 8 wk of age, the mice were randomly implanted subcutaneously with E2-releasing pellets (36). To test the hypothesis that ERs stimulated by E2 play an important regulatory role in intestinal cholesterol absorption, we determined cholesterol absorption efficiency in the gonadectomized mice that were implanted with E2-releasing pellets at 0, 3, or 6 μg/day. Additional groups of mice that were implanted with E2-releasing pellets at 6 μg/day were treated with antiestrogenic ICI 182,780 at a dose of 125 μg/day (5 mg/kg) by subcutaneous injection daily. In addition, both male and female AKR and C57L mice with intact gonads were used as controls.

In general, the total life expectancy of C57L mice is ~80 wk and that of AKR mice is ~60 wk (7, 42). On the basis of measurements of physical growth and sexual maturation, the different age groups of mice were considered to be in the following stages of maturation: 8 wk, young adult; 36 wk, older adult; and 50 wk, aged (7, 42). To explore whether aging per se influences gene expression of intestinal sterol transporters, we examined cholesterol absorption efficiency and intestinal Abcg5, Abcg8, and Npc1l1 mRNA levels in AKR and C57L mice of both genders (8 yr old), 36 (older adult), and 50 (aged) wk of age.

Cholesterol balance analysis. Under chow diet conditions, the gonadectomized AKR and C57L mice of both genders (n = 4 mice/group) treated with various doses of E2 (0, 3, or 6 μg/day) or E2 (6 μg/day) plus ICI 182,780 (125 μg/day) as well as male and female AKR and C57L mice with intact gonads (n = 5 per group) at different ages (8, 36, or 50 wk old) were housed in individual metabolic cages with wire mesh bottoms. All experimental animals were allowed to adapt to the environment for 2 wk. When body weight, food ingestion, and fecal excretion were constant, i.e., an apparent metabolic steady state, food intake was measured and feces were collected daily for 7 continuous days for the balance study. At the end of the study, animals were anesthetized with pentobarbital sodium. After cholecystectomy was performed, the common bile duct was cannulated with a polyethylene-10 catheter and hepatic bile was collected for the first hour of biliary secretion. Bile cholesterol and cholesterol content in the diet were measured by HPLC. Fecal neutral steroids were saponified and extracted as well as being measured by HPLC. Percent cholesterol absorption was calculated according to previously established methods (31).

Measurement of small intestinal transit time. Intestinal transit time was performed in the gonadectomized AKR and C57L mice of both genders (n = 4 mice/group) treated with various doses of E2 (0 or 6 μg/day) according to published methods (30, 33). Nonabsorbable [3H]sitosanol was used as a reference marker. In brief, 2 μCi [3H]sitosanol dissolved in 100 μl of medium-chain triglyceride was instilled into the small intestine via a duodenal catheter. Exactly 30 min after being instilled, the mice were anesthetized with pentobarbital sodium. The intestinal transit time was evaluated by geometric mean for 2 wk. When body weight, food ingestion, and fecal excretion were constant, the intestines of female AKR and C57L mice display ~1.5-fold higher expression levels of Erα and Erβ mRNAs compared with those in males of the same strains (data not shown). Most notably, expression levels of Erα are ~10-fold higher than those of Erβ. The relative mRNA levels for Erα are similar between mice with intact ovaries and mice receiving E2 at 3 μg/day, both being slightly higher than the E2-deficient mice. Furthermore, expression levels of Erα are significantly (P < 0.01) increased in mice treated with E2 at 6 μg/day. However, the E2 effects on Erα expression are completely blocked by ICI 182,780. Compared with mice with intact ovaries, expression levels of Erβ are slightly (P = not significant) up-regulated by E2 at 6 μg/day, but not by E2 at 0 or 3 μg/day, or E2 (6 μg/day) plus antiestrogenic ICI 182,780 at 125 μg/day.

Quantitative real-time PCR assay. The fresh small intestines were collected, flushed with ice-cold saline solution, and cut into three segments with length ratios of 1:3:2 (duodenum to jejunum to ileum). In the middle of each intestinal segment, 1.5 cm of the duodenal, jejunal, and ileal tissues were cut out, and the tissues from four mice per group were pooled. Total RNA was extracted from the intestine using RNeasy Midi (Qiagen, Valencia, CA). Reverse transcription reaction was performed using the SuperScript II First-Strand Synthesis System (Invitrogen, Carlsbad, CA) with 5 μg total RNA and random hexamers to generate cDNA. Primers and probes for mouse Erα, Erβ, Abcg5, and Abcg8 have been described elsewhere (4, 36), which were designed by Primer Express Software (Applied Biosystems, Foster City, CA) based on sequence data available from GenBank. The following primers and probes were used for mouse Npc1l1 (AY437866): forward 5'-CCACAGACCCGTGGAACTGT-3', reverse 5'-GGTCCTGATGGAAAAGCCTTT-3', and probe 5'-CCACAGACCCGTGGAACTGT-3'.

**Fig. 1.** Relative mRNA levels of the intestinal estrogen receptor (Erα (A) and Erβ (B) genes in AKR and C57L mice with intact ovaries and in the ovarioectomized mice treated with 17β-estradiol (E2)-releasing pellets at 0, 3, or 6 μg/day, or E2 (6 μg/day) plus antiestrogenic ICI 182,780 at 125 μg/day. Each value represents the mean ± SD of data that were measured in triplicate by quantitative real-time PCR assays from the pooled small intestine tissues (n = 4 per group). It should be emphasized that under the conditions of intact gonads, the intestines of female AKR and C57L mice display ~1.5-fold higher expression levels of Erα and Erβ mRNAs compared with those in males of the same strains (data not shown). Most notably, expression levels of Erα are ~10-fold higher than those of Erβ. The relative mRNA levels for Erα are similar between mice with intact ovaries and mice receiving E2 at 3 μg/day, both being slightly higher than the E2-deficient mice. Furthermore, expression levels of Erα are significantly (P < 0.01) increased in mice treated with E2 at 6 μg/day. However, the E2 effects on Erα expression are completely blocked by ICI 182,780. Compared with mice with intact ovaries, expression levels of Erβ are slightly (P = not significant) up-regulated by E2 at 6 μg/day, but not by E2 at 0 or 3 μg/day, or E2 (6 μg/day) plus antiestrogenic ICI 182,780 at 125 μg/day.
Real-time PCR assays (4, 36) were performed in triplicate on a GeneAmp 5700 Sequence Detection System (Applied Biosystems). Relative mRNA levels were calculated using the threshold cycle of an unknown sample against a standard curve with known copy numbers. To obtain a normalized target value, the target amount was divided by the endogenous reference rodent glyceraldehyde-3-phosphate dehydrogenase (Gapdh) as the invariant control (part no. 4308313, Applied Biosystems).

Statistical methods. All data are expressed as means ± SD. Statistically significant differences among groups of mice were assessed by Student’s t-test or Mann-Whitney U-tests. If the F value was significant, comparison among groups of mice was further analyzed by a multiple-comparison test. Analyses were performed with SuperANOVA software (Abacus Concepts, Berkeley, CA). Statistical significance was defined as a two-tailed probability of <0.05.

RESULTS

Effects of estrogen and its antagonist on gene expression of intestinal ERS. Our previous study (36) has found that ovariectomy reduced plasma E2 concentrations to below detection limits. The E2 replacement at 3 μg/day achieved equivalent and physiological levels of E2 in the ovariectomized females compared with those in mice with intact ovaries, and, at 6 μg/day, it attained plasma E2 levels in a higher physiological range. Using quantitative real-time PCR techniques, we investigated expression levels of the intestinal Eρα and Eρβ genes in gonadectomized mice of both genders treated with various amounts of E2 or its antagonist. As displayed in Fig. 1, expression levels of Eρα mRNAs are ~10-fold higher compared with those of Eρβ, suggesting that Eρα is a major steroid hormone receptor producing the biological effects of estrogen in the small intestine. Furthermore, there were no significant differences in the relative mRNA expression for the Eρα gene among the duodenum, jejunum, and ileum. This is also the case in the expression of Eρβ. It should be emphasized that under intact gonads conditions, the small intestines of female mice (Fig. 1) displayed approximately threefold higher expression levels of Eρα and Eρβ mRNAs compared with those in males (data not shown). We observed that the relative mRNA levels for the Eρα and Eρβ genes were essentially similar between mice with intact gonads and mice receiving E2 at 3 μg/day, being slightly higher than those in the E2-deficient mice. Furthermore, expression levels of Eρα were significantly (P < 0.01) upregulated in mice treated with E2 at 6 μg/day. However, the E2 effects on expression of the intestinal Eρα and Eρβ mRNAs could be completely blocked by the antagonist ICI 182,780.

Effects of estrogen and its antagonist on intestinal cholesterol absorption. Using cholesterol balance techniques, we investigated cholesterol absorption in gonadectomized AKR and C57L mice of both genders treated with E2 at 0, 3, or 6 μg/day or E2 (6 μg/day) plus antiestrogenic ICI 182,780 (125 μg/day). As shown in Table 1, all mice ate basically similar

Table 1. Cholesterol balance data in gonadectomized AKR and C57L mice treated with estrogen and its antagonist during metabolic steady-state conditions

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol Intake, mg/day</th>
<th>Biliary Cholesterol, mg/day</th>
<th>Steroid Excretion, mg/day</th>
<th>Absorbed Cholesterol*, mg/day</th>
<th>Cholesterol Absorption*, %</th>
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<tbody>
<tr>
<td></td>
<td>Male</td>
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<tr>
<td>AKR mice</td>
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<tr>
<td>0 μg/day E2</td>
<td>0.81 ± 0.02</td>
<td>1.26 ± 0.09</td>
<td>1.86 ± 0.08</td>
<td>0.21 ± 0.01</td>
<td>26 ± 2</td>
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<tr>
<td>3 μg/day E2</td>
<td>0.79 ± 0.03</td>
<td>1.28 ± 0.12</td>
<td>1.86 ± 0.11</td>
<td>0.21 ± 0.02</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>6 μg/day E2</td>
<td>0.85 ± 0.02</td>
<td>1.97 ± 0.11^ad</td>
<td>2.50 ± 0.12^ad</td>
<td>0.32 ± 0.02^ad</td>
<td>38 ± 3^ad</td>
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<tr>
<td>6 μg/day E2+ICI</td>
<td>0.77 ± 0.04</td>
<td>1.21 ± 0.06^b</td>
<td>1.77 ± 0.07^b</td>
<td>0.21 ± 0.04^b</td>
<td>27 ± 4^b</td>
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<tr>
<td>C57L mice</td>
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<tr>
<td>0 μg/day E2</td>
<td>0.80 ± 0.05</td>
<td>2.29 ± 0.26^k</td>
<td>2.79 ± 0.31^l</td>
<td>0.30 ± 0.01^b</td>
<td>38 ± 4^b</td>
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<td>3 μg/day E2</td>
<td>0.79 ± 0.03</td>
<td>2.47 ± 0.15^k</td>
<td>2.95 ± 0.16^k</td>
<td>0.31 ± 0.03^k</td>
<td>39 ± 3^k</td>
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<tr>
<td>6 μg/day E2</td>
<td>0.84 ± 0.04</td>
<td>3.36 ± 0.34^ik</td>
<td>3.70 ± 0.33^ik</td>
<td>0.50 ± 0.04^m,n</td>
<td>60 ± 4^j^o,m</td>
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<tr>
<td>6 μg/day E2+ICI</td>
<td>0.77 ± 0.03</td>
<td>2.02 ± 0.18^p</td>
<td>2.47 ± 0.19^p</td>
<td>0.32 ± 0.02^p,r</td>
<td>41 ± 2^p,r</td>
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<tr>
<td></td>
<td>Female</td>
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<tr>
<td>AKR mice</td>
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<tr>
<td>0 μg/day E2</td>
<td>0.77 ± 0.07</td>
<td>1.19 ± 0.13</td>
<td>1.75 ± 0.15</td>
<td>0.20 ± 0.04</td>
<td>26 ± 3</td>
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<tr>
<td>3 μg/day E2</td>
<td>0.80 ± 0.02</td>
<td>1.24 ± 0.14</td>
<td>1.83 ± 0.13</td>
<td>0.22 ± 0.03</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>6 μg/day E2</td>
<td>0.83 ± 0.03</td>
<td>1.92 ± 0.19^s</td>
<td>2.43 ± 0.20^s</td>
<td>0.33 ± 0.03^td</td>
<td>39 ± 3^d</td>
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<tr>
<td>6 μg/day E2+ICI</td>
<td>0.79 ± 0.06</td>
<td>1.22 ± 0.14^s</td>
<td>1.77 ± 0.14^s</td>
<td>0.24 ± 0.04^s</td>
<td>30 ± 4^s</td>
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<td>C57L mice</td>
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<tr>
<td>0 μg/day E2</td>
<td>0.79 ± 0.06</td>
<td>2.15 ± 0.17^t</td>
<td>2.61 ± 0.09^t</td>
<td>0.32 ± 0.04^t</td>
<td>41 ± 9^t</td>
</tr>
<tr>
<td>3 μg/day E2</td>
<td>0.79 ± 0.03</td>
<td>2.26 ± 0.18^t</td>
<td>2.72 ± 0.21^t</td>
<td>0.33 ± 0.02^t</td>
<td>42 ± 4^t</td>
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<tr>
<td>6 μg/day E2</td>
<td>0.85 ± 0.03</td>
<td>3.23 ± 0.26^k,m</td>
<td>3.56 ± 0.25^k,o</td>
<td>0.51 ± 0.05^k,o</td>
<td>60 ± 4^j^o,m</td>
</tr>
<tr>
<td>6 μg/day E2+ICI</td>
<td>0.80 ± 0.02</td>
<td>2.21 ± 0.18^p</td>
<td>2.68 ± 0.17^p</td>
<td>0.34 ± 0.02^r</td>
<td>42 ± 3^p</td>
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</table>

Values are means ± SD. *Absorbed cholesterol was determined by subtracting the daily fecal neutral steroid output from the daily cholesterol intake and the daily biliary cholesterol output as measured by HPLC methods (31). ^The cholesterol absorption was determined by the cholesterol balance analysis according to published methods (31). ICI, ICI 182,780. ^P < 0.001 compared with AKR mice treated with 17β-estradiol E2 at 0 μg/day. ^P < 0.01 compared with AKR mice treated with E2 at 3 μg/day. ^P < 0.01 compared with AKR mice treated with E2 at 6 μg/day. ^P < 0.01 compared with AKR mice treated with E2 at 6 μg/day. ^P < 0.01 compared with AKR mice treated with E2 at 6 μg/day. ^P < 0.01 compared with AKR mice treated with E2 at 6 μg/day. ^P < 0.01 compared with AKR mice treated with E2 at 6 μg/day. ^P < 0.01 compared with AKR mice treated with E2 at 6 μg/day. ^P < 0.01 compared with AKR mice treated with E2 at 6 μg/day. ^P < 0.01 compared with AKR mice treated with E2 at 6 μg/day. ^P < 0.01 compared with AKR mice treated with E2 at 6 μg/day. ^P < 0.01 compared with AKR mice treated with E2 at 6 μg/day. ^P < 0.01 compared with AKR mice treated with E2 at 6 μg/day.
amounts of food (cholesterol content = 0.77–0.85 mg/day). However, daily biliary cholesterol outputs (2.15–2.47 mg/day) in gonadectomized C57L mice treated with E2 at 0 or 3 μg/day were significantly (P < 0.01) higher compared with AKR mice (1.19–1.28 mg/day) because C57L mice are a gallstone-susceptible strain (32). Because of higher biliary cholesterol secretion, daily fecal total neutral steroid excretion was significantly (P < 0.01) greater in C57L mice (2.61–2.95 mg/day) than in AKR mice (1.75–1.86 mg/day). Moreover, an input-output analysis revealed that absorbed mass of cholesterol daily in C57L mice (0.30–0.33 mg/day) was significantly (P < 0.05) higher compared with AKR mice (0.20–0.22 mg/day). The calculated percent cholesterol absorption in C57L mice was 38–42%, being significantly (P < 0.05) greater than that in AKR mice (26–27%). As reported in our previous study (36), the E2 treatment at 6 μg/day significantly (P < 0.01) increased biliary cholesterol outputs and augmented output and pool size of biliary bile salts, favoring C57L mice more than AKR mice. Consequently, percent cholesterol absorption was significantly increased in C57L mice (60%), still being significantly (P < 0.05) higher than in AKR mice (38–39%). In contrast, the biological actions of estrogen on intestinal cholesterol absorption could be fully abolished by the treatment of antiestrogenic ICI 182,780. Furthermore, no gender differences in percent cholesterol absorption were found in the gonadectomized mice treated with the same amount of E2 or E2 plus the antagonist.

**Regulation of expression of intestinal sterol transporters by estrogen and its antagonist.** To further examine the responses of intestinal sterol efflux transporters to high levels of estrogen, we studied expression levels of Abcg5, Abcg8, and Npc1l1 in the gonadectomized AKR and C57L mice treated with various doses of estrogen and its antagonist. Figure 2A exhibits that expression levels of Abcg5 and Abcg8 are essentially similar among mice with intact gonads, E2-deficient mice, and mice receiving E2 at 3 μg/day. However, E2 at a dose of 6 μg/day significantly (P < 0.01) increased expression levels of Abcg5 and Abcg8 in the jejunum and ileum and to a lesser extent in the duodenum. Figure 2B shows that E2 at 6 μg/day (P < 0.01) increased gene expression levels of intestinal sterol efflux transporter Npc1l1. On the contrary, the E2 effects on gene expression of these intestinal transporters could be totally blocked by ICI 182,780. Taken together, high doses of E2 (6 μg/day) influence the relative mRNA levels for Abcg5, Abcg8, and Npc1l1 in the small intestines of both AKR and C57L mice as well as fecal total neutral steroid excretion and percent cholesterol absorption. Thus our results suggest a possible “estrogen-Er-sterol transporter” pathway for regulating intestinal cholesterol absorption.

**Effects of estrogen and its antagonist on small intestinal transit time.** Because small intestinal motility is an important factor influencing cholesterol absorption efficiency (21, 24, 34), we studied small intestinal transit times in these mice. We examined the distribution of radioactivity along the small intestine at 30 min after intraduodenal installation of a lipid mixture containing [3H]sitostanol. The geometric center of the distribution profile of radioisotope in the small intestine was essentially similar between AKR (geometric center = 11.0–12.3) and C57L mice (geometric center = 11.3–12.7), whatever the mice had intact gonads or were gonadectomized and in the setting of treatment with E2 at 0 or 6 μg/day. Furthermore, the distribution of radioactivity in the small intestine displayed peaks between segments 7 and 16 in all mice. These studies suggest that various amounts of E2 do not influence small intestinal transit time in mice.

**Aging-related effects on intestinal cholesterol absorption.** Table 2 shows cholesterol absorption efficiency determined by the cholesterol balance method in male and female AKR and C57L mice of different ages and with intact gonads. Again, we found that all mice ate essentially similar amounts of food (cholesterol content = 0.72–0.81 mg/day), irrespective of the changes in age. As found in our previous study (30), with increasing age, C57L mice produced significantly (P < 0.001)
higher biliary cholesterol outputs compared with AKR mice of the same age and of the same gender. Furthermore, an input-output analysis found that cholesterol mass absorbed from the small intestine was significantly ($P < 0.05$) greater in C57L mice than in AKR mice. Overall, we observed that intestinal cholesterol absorption increased significantly with age in C57L mice of both genders (40–43% in the young adult, 46–49% in the older adult, and 54–60% in the aged; $P < 0.05$ compared between the young adult and the aged mice) and in female AKR mice (31 ± 4% in the young adult, 36 ± 4% in the older adult, and 40 ± 2% in the aged; $P < 0.01$ compared between the young adult and the aged mice). However, this is not the case in male AKR mice (27 ± 3% in the young adult, 30 ± 1% in the older adult, and 32 ± 4% in the aged; $P = 0.19$ compared between the young adult and the aged mice). Nonetheless, these results suggest that C57L mice absorb more cholesterol than AKR mice, and aging augments intestinal cholesterol absorption efficiency, consistent with our previous studies (30) as measured by the plasma dual-isotope ratio method.

**Aging-related effects on gene expression of intestinal sterol transporters.** To study whether aging influences gene expression of intestinal sterol transporters, we investigated expression levels of the Abcg5, Abcg8, and Npc1l1 genes in AKR and C57L mice of different ages. Figure 3 exhibits that the relative mRNA levels for Abcg5 (Fig. 3A) and Abcg8 (Fig. 3B) in the jejunum and ileum are slightly higher in AKR mice compared with those in C57L mice of the same age and of the same gender. Furthermore, there was a remarkably negative correlation between age and expression levels of the jejunal and ileal Abcg5 and Abcg8 in mice; however, they do not reach statistically significant differences. Of note is that the relative mRNA levels for duodenal Abcg5 and Abcg8 were essentially similar in mice of different ages. As shown in Fig. 3C, our results revealed that aging significantly ($P < 0.05$) increased expression levels of Npc1l1 in the duodenum and jejunum, but not in the ileum, especially in C57L mice, which are associated with increased intestinal cholesterol absorption (see Table 2 and Ref. 30).

**DISCUSSION**

Recent progress in understanding the molecular basis of intestinal sterol transporters has provided many new insights into the complex physiological mechanisms involved in intestinal cholesterol absorption (16). The present study in two unique mouse models highlights three important issues: 1) as shown by dose-response studies, high doses of E2 delivered via subcutaneous time-release pellets significantly augment intestinal cholesterol absorption, mostly due to an upregulated expression of intestinal sterol influx transporter Npc1l1 via the intestinal Erα pathway and an increase in biliary cholesterol secretion through the hepatic Erα pathway; 2) the biological actions of estrogen on increased intestinal cholesterol absorption and biliary cholesterol secretion can be fully abolished by the treatment of antiestrogenic ICI 182,780 as a result of inhibiting the intestinal and hepatic Erα activities; and 3) aging enhances intestinal cholesterol absorption by suppressing expression levels of the jejunal and ileal Abcg5 and Abcg8 and upregulating expression levels of the duodenal and jejunal Npc1l1. Our studies show that the responses of gene expression of Abcg5, Abcg8, and Npc1l1 to high levels of estrogen and an increase in age are different, suggesting two different regula-
The biological effects of estrogen are generally ascribed to transcriptional modulation of target genes through two ER subtypes, ERα and ERβ (8, 15), which are encoded by two distinct genes, *Era* and *Erβ*. Both receptors are members of the steroid hormone receptor superfamily of ligand-activated transcription factors and have tissue expression patterns that overlap but are not identical (13, 17). Our results show that the mouse intestinal epithelium is characterized by high levels of ERα mRNAs and low levels of ERβ mRNAs. E2 is the most potent agonist at ERs, and in the present study, its biological effects on cholesterol absorption via the intestinal Erα pathway are observed in exogenously E2-treated mice. In a previous study (4), there was a remarkably negative correlation between percent cholesterol absorption and expression levels of the jejunal and ileal *Abcg5* and *Abcg8* in chow-fed mice. However, it is interesting to find in the current study that under conditions of high levels of estrogen, increased expression levels of *Abcg5* and *Abcg8* are associated with an increase in the efficiency of intestinal cholesterol absorption. A possible reason is that Erα functions as a transcriptional regulator to stimulate gene expression of *Abcg5* and *Abcg8* in the intestine. In addition, our results do not exclude the possibility that E2 per se may produce a specific enhancing effect on the expression of the genes for these sterol efflux transporters. Furthermore, we found that high doses of E2 upregulate expression levels of *Npc1l1*, which is associated with augmented intestinal cholesterol absorption. Therefore, our results support the concept that the efficiency of cholesterol absorption could be determined by the net effect between influx and efflux of intraluminal cholesterol molecules crossing the brush border of the enterocyte (4, 16). We also observed that these biological effects of E2 are completely blocked by the antiestrogenic ICI 182,780, suggesting that this response is most likely a direct effect of Erα and the *Abcg5*, *Abcg8*, and *Npc1l1* genes may be a potential target gene of Erα. Overall, these studies suggest a possible estrogen-Erα-sterol transporter pathway regulating the efficiency of intestinal cholesterol absorption.

In addition, we observed that the Erα activated by E2 promotes hepatic outputs of biliary lipids, consistent with previous results (18, 36). Accumulated evidence from human and animal studies showed that estrogen could stimulate HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis so that more total-body cholesterol is produced in the liver and small intestine. The increase in hepatic cholesterol biosynthesis is associated with a significant increase in secretion rates of biliary total and newly synthesized cholesterol (37). Consequently, there is an increased delivery of cholesterol from bile into the intestinal lumen for absorption by the enterocyte. Also, increased de novo synthesis in the intestine provides an important source of cholesterol for incorporation into nascent chylomicron particles. In addition, we found that high doses of E2 greatly increase hepatic outputs of biliary bile salts and bile salt-dependent bile flow rates (36). An increase in bile salt concentrations augments intraluminal micellar cholesterol solubilization, and accordingly, this alteration results in increased cholesterol absorption (35). Again, these biological effects of estrogen are fully blocked by antiestrogenic ICI 182,780 (36), which induces a significant reduction in intestinal cholesterol absorption as shown in Table 1. These results indicate that biliary factors also play a major role in the regulation of cholesterol absorption in mice in response to high levels of estrogen.

We observed that aged mice display significantly higher cholesterol absorption efficiency compared with the young adult groups. Our findings are in agreement with the results of Hollander and Morgan (12), showing that the absorption rate of cholesterol by the small intestine increased linearly with aging, with a twofold increase in intestinal cholesterol absorption in aged rats (42 mo old) compared with young adult rats (1 mo old). It should be emphasized that although aged mice probably have much lower estrogen levels compared with young adult mice, hepatic cholesterol synthesis and biliary lipid secretion are significantly increased with age (5, 30), suggesting that aging is an independent factor influencing cholesterol metabolism. Increased biliary lipid secretion contributes, in part, to higher absorption of cholesterol from the intestine in aged mice.
compared with young adult mice. In the present study, we found that aging enhances cholesterol absorption by suppressing expression of the jejunal and ileal sterol efflux transporters Abcg5 and Abcg8 and upregulating expression of the putative duodenal and jejunal sterol influx transporter Npc1l1. These alterations are associated with increased intestinal cholesterol absorption in aged mice. Therefore, this work provides a basic framework for further investigating the molecular mechanisms whereby longevity (aging) genes influence these intestinal sterol transporters to enhance cholesterol absorption. Our studies also suggest that aging and high levels of estrogen are two independent factors influencing intestinal cholesterol absorption.

It has been observed that slow small intestinal transit time enhances intestinal cholesterol absorption in humans (21) and animals (24, 34). In a previous study (30), we observed that there are slightly slower small intestinal transit rates in aged C57L mice compared with their young adults and such alterations may partly contribute to increased intestinal cholesterol absorption in aged mice. In addition, we found that small intestinal transit times are essentially similar in the gonadectomized mice of both genders treated with various doses of E2. These results demonstrate that estrogen does not influence small intestinal motility in mice.

Under conditions of high levels of estrogen and aging, biliary cholesterol is significantly increased in mice (see Tables 1 and 2), which provides a large amount of endogenous cholesterol from bile into the intestinal lumen for absorption by the enterocyte. Thus total cholesterol mass absorbed by the intestine is significantly increased and daily excretion of fecal neutral steroids is markedly increased as well. Furthermore, increased excretion of fecal neutral steroids may reduce cholesterol accumulation in the body. It should be emphasized that in the present study, we investigated the effects of estrogen and aging on gene expression levels, but not protein concentrations, of the intestinal Abcg5, Abcg8, and Npc1l1 genes because of possible similarity between transcriptional and translational actions in these intestinal sterol transporters. Furthermore, we observed that the molecular mechanisms, whereby intestinal cholesterol absorption is regulated by estrogen and aging, are basically similar between C57L and AKR mice.

We conclude that Erα stimulated by estrogen and aging augment the efficiency of intestinal cholesterol absorption by increasing biliary lipid output and mediating intestinal sterol transporters favoring influx of intraluminal cholesterol molecules across the apical membrane of the enterocyte. This work therefore may lead to novel approaches to the treatment of hypercholesterolemia, especially in aged populations and in patients being exposed to high levels of estrogen.

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