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Estrogen Up-regulates Apolipoprotein E (ApoE) Gene Expression by Increasing ApoE mRNA in the Translating Pool via the Estrogen Receptor α-Mediated Pathway*

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The antiatherogenic property of estrogens is mediated via at least two mechanisms: first by affecting plasma lipoprotein profiles, and second by affecting the components of the vessel wall. Raising plasma apolipoprotein E (apoE) in mice protects them against diet-induced atherosclerosis (Shimano, H., Yamada, N., Katsuki, M., Gotoda, T., Harada, K., Murase, T., Fukuzawa, C., Takaku, F., and Yazaka, Y. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 1750–1754). It is possible that estrogen may be antiatherogenic at least in part by increasing plasma apoE levels. Therefore, we studied the regulation of apoE by estrogen. A survey of 15 inbred strains of mice showed that some mouse strains responded to injections or subcutaneously implanted pellets of estradiol by raising their apoB and apoE levels and some did not. We performed detailed studies in two "responder" strains, C57L and C57BL, and two "non-responder" strains, C3H and BALBc. Responders increased their plasma apoE levels 2.5-fold. Non-responders' levels were altered ±10%. In the responders the distribution of apoE among the plasma lipoproteins shifted from high density lipoprotein toward the apoB-containing lipoprotein fractions. In nonresponders the shift was toward high density lipoprotein. Hepatic apoE mRNA levels and relative rates of apoE mRNA transcription were unchanged in all strains, suggesting that apoE regulation occurred at posttranscriptional loci. Therefore, we measured apoE synthesis in fresh liver slices and on isolated hepatic polysomes. Two-fold increases were noted but only in responders accompanied by selective 1.5-fold increases in polysomal apoE mRNA levels. Similar increases in apoE synthesis were also observed in castrated C57BL mice given either physiological or pharmacological replacement doses of estradiol, but not testosterone, suggesting that the effect of estradiol was specific on the distribution of apoE mRNA in the translationally active polysomal pool. Next, we examined whether the effects of estrogen on apoE translation were mediated by estrogen receptors (ER). ER-α knock-out mice and their wild-type littermates were administered estradiol. As expected, apoE levels and hepatic apoE synthesis increased more than 2-fold in the wild-type littermates, but only 20% increases in the plasma apoE and hepatic synthesis were observed in the ER knock-out mice. Hepatic apoE mRNA levels did not change in either the wild-type or the ER knock-out mice. Thus, estradiol up-regulates apoE gene expression by increasing levels of apoE mRNA in the polysomal translating pool. Furthermore, the increased polysomal recruitment of apoE mRNA is largely mediated by estrogen receptors.

Estrogens may exert antiatherogenic effects either by modulating lipoprotein profiles (1) or by affecting atherosclerosis-promoting factors in the vessel wall (2). Estradiol produces marked alterations in the lipoprotein profiles of mice, raising the concentrations of apoB[1]-containing particles in plasmas of strains susceptible to atherosclerosis (3, 4). In part the rise is due to an increase in the abundance of apoB100 mRNA relative to B48 in liver (5) as estrogen down-regulates apobec1 mRNA (6). Hepatic LDL receptor protein expression is not affected (3). We hypothesized that since apoE is an important component of apoB-containing lipoproteins (7), the levels of apoE could also be raised by estrogen treatment in susceptible strains of mice.

Mouse apoE is composed of 285 amino acids residues as deduced from the cDNA sequence (8), compared with 289 amino acid residues for human apoE (9). It is synthesized by a variety of tissues, thus differing from other apoproteins that are synthesized exclusively in liver and intestine (9, 10). ApoE plays central roles in mammalian cholesterol transport by serving as a ligand for the cell surface LDL and LRP receptors that mediate the endocytosis of apoB- and apoE-containing lipoproteins (11, 12). LDL receptors recognize both apoB-100 and apoE, but not apoB48, whereas LRP recognize apoE but neither apoB-100 nor apoB48 (13, 14). Since apoB48 in the chylomicrons lack the LDL receptor binding domain, the uptake of chylomicron remnants by hepatocytes, enterocytes, and peripheral tissues occurs via apoE binding to apoE receptors (15). Thus, apoE plays a key role in cholesterol metabolism.

ApoE is also important in the development and progression of atherosclerosis. Studies of apoE knock-out (16) and apoE-

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[1] The abbreviations used are: apo, apolipoprotein; ER, estrogen receptor; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; FPLC, fast protein liquid chromatography; WT, wild type; LRP, LDL receptor-related protein.

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TABLE I
Plasma apolipoprotein E, B, and AI levels in mice treated with estradiol

Mouse strains	ApoE		ApoB		ApoAI	
	P	E	P	E	P	E
				mg/dl		
C57L	7 ± 2	16 ± 3[a]	18 ± 2	31 ± 3[a]	134 ± 18	80 ± 8[a]
C57BL	6 ± 1	14 ± 4[a]	23 ± 3	35 ± 4[a]	136 ± 12	121 ± 9[a]
C3H	10 ± 2	12 ± 2	21 ± 2	26 ± 3	141 ± 21	109 ± 19[a]
BALB	8 ± 2	7 ± 1	13 ± 2	15 ± 1	139 ± 10	116 ± 16[a]

[a] Significantly different compared to placebo group ($p < 0.02$). Each group contained five mice, and the results presented are from two independent experiments.

transgenic mice (17) showed that lack of apoE induced atherosclerosis, whereas overexpression of apoE was protective. A recent study showed an inverse correlation in apoE staining in the vessel wall and extent of lesion formation (18), suggesting that apoE may be involved in the removal of lipid-rich particles from the vessel wall by enhancing cholesterol efflux (19). Thus, factors that stimulate apoE production may retard atherosclerotic lesion formation.

Several factors modulate apoE synthesis including hormones and dietary cholesterol and fat (20–22). Estradiol stimulates VLDL secretion from rat hepatocytes, and the VLDLs contain apoE (23). Regulation of apoE gene expression by estradiol occurs both at the transcriptional and post-transcriptional loci in rats (24, 25).

As noted we expected apoE levels to be affected by estrogens. Accordingly, we administered estradiol to 15 strains of inbred mice and found the apoE levels of some strains to respond and others to be resistant (26). Here we report on the regulation of the apoE gene in more detail in two responder and two nonresponder strains. Our results demonstrate that the regulation of apoE gene expression by estrogen is primarily translational. Using estrogen receptor knock-out mice, we also show that estrogen-induced increased synthesis of apoE in the responder strains requires estrogen receptor activity.

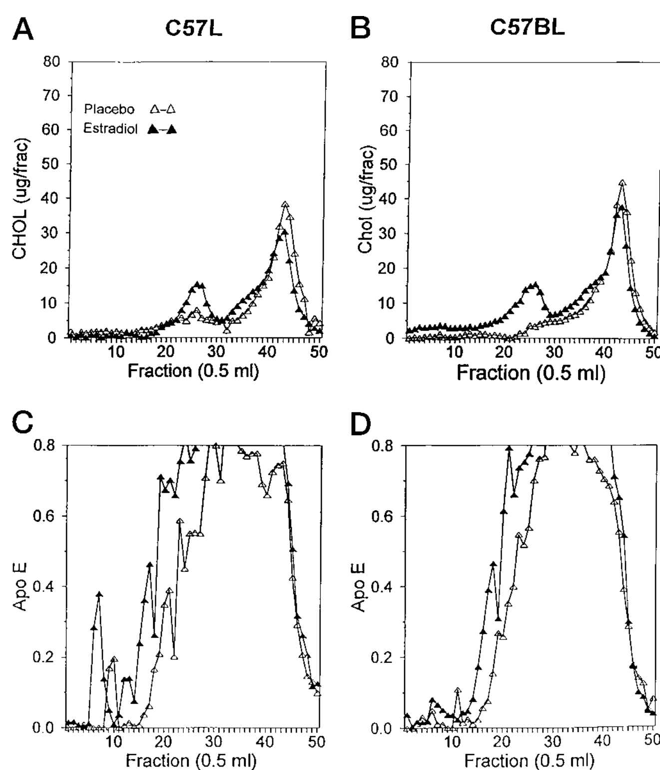

FIG. 1. **Effects of estradiol on FPLC profiles of plasma lipoproteins of C57L and C57BL strains.** Equal volumes of plasma from five individual mice were pooled and 400 microliter plasma were chromatographed on 2 Superose columns connected in series. Five hundred microliter fractions were collected, and in each fraction cholesterol concentrations were quantified by using commercial kit (*A* and *B*). *C* and *D*, distribution of plasma apoE in fractions eluted from FPLC column. In each fraction apoE was quantified by enzyme-linked immunosorbent assay (24) using serial dilutions, and results are in optical density units. Results >0.8 OD were off the standard curve of the assay and are not shown. Fractions 1–16, VLDL; 17–32, LDL; 33–50, HDL.

MATERIALS AND METHODS

Animals and Treatments—Inbred strains of male mice were purchased from Jackson Laboratories, Bar Harbor, ME. For the initial survey, three male mice of each strain were used for each treatment group. The placebo group was administered vehicle only and the treatment groups 17β-estradiol at 3 μg/g body weight/day for 5 consecutive days by subcutaneous implantation (Innovative Research of America, Toledo, OH). Animals were allowed to eat a standard rodent chow *ad libitum* (ICN Biochemicals, Cleveland, OH). Mice were fasted 4–6 h and sacrificed on the 6th day under ether anesthesia. Apoproteins and lipids were measured in plasma. RNA were prepared from liver and small intestine (27). Plasma apoE levels were quantified by enzyme-linked immunosorbent assay using rabbit anti-rat apoE antibody (28). Plasma apoB and apoAI levels in individual mouse sera were quantified by electroimmunoassay (3, 29). Total cholesterol and triglycerides were quantified using a commercial kit (Wako Pure Chemicals, Richmond, VA). Two strains (C57L and C57BL) that increased their plasma apoB levels >2-fold and two strains that showed a maximum change of <20% (BALB and C3H) were administered 17β-estradiol as described above, and regulation of apoE was compared in more detail. Each group contained five mice. In a third experiment 16 C57BL mice were castrated, allowed to recover for 14 days, and divided into four groups of four animals per group: group 1 received estradiol at 0.16 μg/g body weight/day, group 2 at 3.0 μg/g body weight/day, group 3 was administered dihydroxytestosterone at 1 μg/g body weight/day, and group 4 received the vehicle.

In the ER knock-out mice exon 2 of the estrogen receptor gene was disrupted by insertion of the *Neo* gene which also served as a selection marker (30). The ES cell line E14TG2a was derived originally from 129/J mouse strain. The targeted ES cells were injected into blastocysts from C57BL/6J mice and returned to pseudopregnant C57BL/6J females to complete their development. Thus, the ER knock-out mice had mixed genetic background of 129/J and C57BL/6J. We used wild-type littermates to control for any potential background effects. Estrogen administration and other manipulations were as described above.

Determination of Elution Profiles of Plasma Cholesterol and ApoE by Gel Permeation Chromatography—Equal volumes of plasma from individual mice were pooled and analyzed on the FPLC system as described (3, 29). In each elution fraction, total cholesterol, triglycerides, and apolipoprotein E were quantified as described before (28).

Preparation of RNA—Total RNA was prepared from fresh livers (27). In addition RNA was extracted by a one-step method (23) from nuclei, polysomes, and cytoplasmic fractions containing both monosomes and polysomes (3).

Analysis of RNA—ApoE mRNA was quantified by an RNase protection assay (31). The integrity of RNA samples was determined by Northern blotting analysis using a mouse apoE riboprobe (32). The recombinant pGEM3Zf(+) plasmid containing 298-base pair apoE cDNA fragment was linearized by *Hin*dIII for RNA polymerase T7 directed synthesis of antisense apoE RNA and by *Bam*HI for SP6 directed sense apoE RNA synthesis. The details of the *in vitro* transcription and purification of the transcript have been described in our earlier reports (3, 22, 31). The purity of the riboprobe was examined in a sequencing gel. For Northern blotting analysis, 10 μg of total RNA were denatured, separated in an 1.5% agarose gel containing formaldehyde, and transferred onto a nylon membrane (Nytran, Schleicher and Schuell) by capillary blotting. The membrane was baked at 80 °C for 1 h and hybridized either with apoE riboprobe or cDNA probe as described (32). As internal control, the mRNA for mouse β-actin was also determined. For preparing β-actin riboprobe, the recombinant plasmid was linearized with *Xba*I, and *in vitro* transcription was performed using T7 RNA polymerase. For preparing sense RNA strand the plasmid was linearized with *Eco*RI followed by *in vitro* transcription using SP6 RNA polymerase.

Preparation of Ribonucleoprotein Complexes—Ribosome preparations that contained pre-initiation complex (40 S), initiation complex (80 S), and polyribosomes were isolated by the magnesium precipitation

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procedure of Palmiter (33). All the procedures were carried out at 0–2 °C. One gram of liver was homogenized in 10 volumes of ice-cold buffer A (25 mM Tris, 25 mM NaCl, 5 mM $MgCl_2$) containing 1 mg/ml heparin and 2% Triton X-100. The homogenates were centrifuged for 5 min at 27,000 × *g*, and the supernatants were decanted into another container. Equal volumes of buffer B (4 volumes of buffer A diluted with 1 volume of 1 M $MgCl_2$) were added to the supernatant followed by incubation for 1 h in an ice bath. The contents were centrifuged for 10 min at 27,500 × *g* in a Sorval swinging bucket rotor. The supernatants were removed by aspiration, and the pellet containing the preinitiation, initiation, and polyribosome complexes were resuspended in 20 mM Hepes. Polysomes devoid of monosomes were prepared from mouse livers as described below (3).

In Vitro Translation of ApoE on Isolated Polysomes—Polysomes were isolated from pool of 2 individual mouse livers as described (3). In brief, 1 g of liver was homogenized in 6 ml of cold buffer (50 mM Tris/HCl, 25 mM NaCl, 5 mM $MgCl_2$, pH 7.7) containing 0.25 M sucrose and 500 μg/ml sodium heparin. The homogenate was centrifuged at 15,000 × *g* for 10 min at 4 °C, and the supernatant (S15) treated with 1 volume of solution containing 10% each of Triton X-100 and sodium cholate. This treatment solubilizes the membrane. The supernatant after treatment was subjected to discontinuous sucrose gradient as described (3). After the centrifugation, the opalescent band containing polysomes at the interface of 1.0/2.5 M sucrose was withdrawn with the help of a sterile syringe. Polysomes were used for polysome run-off assay using the wheat germ translation system (3). Parts of the polysomal preparations were used to prepare RNA to determine polysome-associated apoE mRNA. *In vitro* translation on the isolated polysomes were performed for 1 h at 30 °C using 2.5 A_{260} units of polysomes and 5 μl of [^{35}S]methionine (Amersham Corp., 15 mCi/ml) in a wheat germ translation system (Ambion Inc., Austin, TX). At the end of the reaction newly synthesized apoE were immunoprecipitated by rat apoE antibody (22) and processed exactly as described (3). Immunoprecipitates were dissolved in SDS loading buffer and run in a 5/10% denaturing polyacrylamide gel. The apoE protein bands were identified by autoradiography. The intensity of the bands were scanned by image analysis system (JAVA, Jandel Scientific, Corte Madera, CA).

Determination of Polysomal Profiles by Density Gradient Centrifugation—Polysomal profiles were determined essentially as described previously (34). S15 extracts were prepared as described above. One milliliter of S15 extracts were overlayered onto a linear 15–50% sucrose gradients and centrifuged as described (34). Aliquots were collected in 16 equal parts (300 μl each) by puncturing the bottom of the tube. Absorbance at 260 nm was monitored in each fraction after dilution. Total RNA was prepared from each fraction, and apoE mRNA as well as β-actin mRNA were determined as described above.

Measurements of Relative Rates of ApoE mRNA Transcription—ApoE and β-actin mRNA transcription rates were measured in isolated mouse liver nuclei exactly as described (3). Ten million nuclei were incubated with 150 μCi of [α-^{32}P]UTP and the other three nucleotides under optimized assay conditions (3) for 30 min at 26 °C. The reaction was stopped by the addition of RNase-free DNase I followed by the addition of proteinase K. The total RNA was isolated (27), and one million cpm of isolated RNA were hybridized with membrane-bound apoE cDNA probe. For background counts, labeled RNA were also hybridized with membrane-bound linearized non-recombinant vector under similar conditions. Hybridization and washings were performed as described (3). As an internal control the relative rates of β-actin mRNA transcription were also determined.

Synthesis of ApoE on Freshly Isolated Liver Slice—Wild-type and the ER knock-out mice treated with placebo or estradiol for 5 days were fasted for 4 h on the 6th day, anesthetized, and their livers immediately removed. About 50 mg of liver were sliced into 10–12 pieces using a sterile razor blade, and assays of the synthesis of protein were performed in the presence of [^{35}S]methionine (35). Tissue pieces were homogenized and high speed supernatants prepared, and aliquots of the supernatants were immunoprecipitated with anti-rat apoE antibody (28). Immunoprecipitates were processed as described (35) and subjected to electrophoresis in SDS-containing polyacrylamide gels. The gels were dried and exposed to x-ray film for visualization of the apoE protein band. The intensity of the band was quantified by our image analysis system.

RESULTS

Effects of Estrogen on Plasma Lipids, Apoproteins, and Lipoproteins—Body weights of the four strains were not affected significantly. Liver weights of the C57L and C57BL estradiol groups increased to 136% of the respective placebo groups. Increases in BALB and C3H strains were 114 and 122%, respectively. ApoB and E levels increased in C57L and C57BL, but not in C3H and BALB (Table I). ApoAI levels fell in all four strains. On FPLC, the cholesterol contents of VLDL-, IDL- and

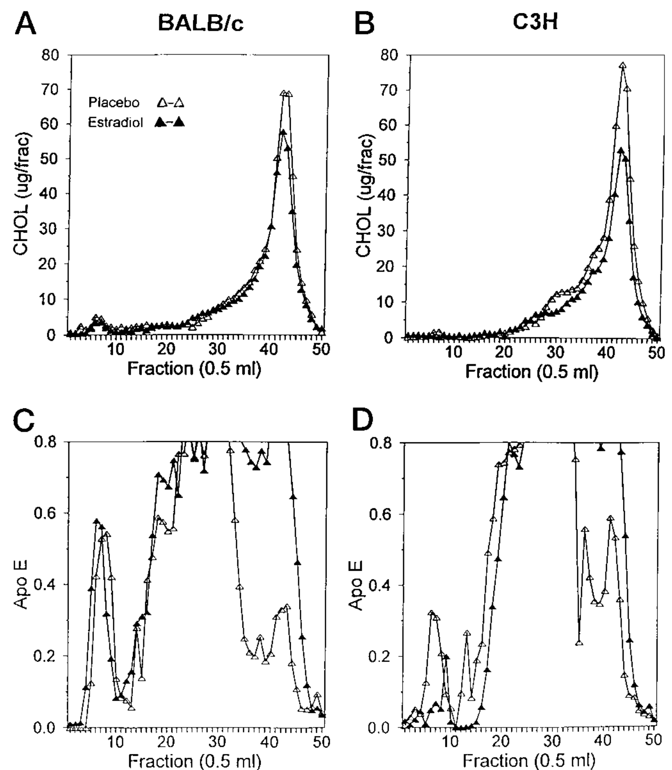

FIG. 2. **Effects of estradiol on FPLC profiles of plasma lipoproteins of C3H and BALBc strains.** Pooled plasma from five mice were fractionated as described in the legend to Fig. 1, and in each fraction, cholesterol (*A* and *B*) and apoE (*C* and *D*) were quantified. Fractions 1–16, VLDL; 17–32, LDL; 33–50, HDL.

TABLE II
Plasma apoE levels in castrated or intact WT and ER-α knock-out mice after estradiol, or testosterone treatment

Groups of C57BL male mice (n = 4 per group) were castrated (cas) or sham-operated (sham), allowed to recover, and either low dose (0.16 μg/g body weight/day, EL) or high dose (3 μg/g body weight/day, EH) of estradiol or dihydroxytestosterone (1 μg/g body weight/day, Tp) pellets were implanted subcutaneously. Estrogen receptor-α knock-out (ER-α-KO) and their wild-type (WT) littermates were administered estradiol. On the 6th day, mice were sacrificed, and apoE concentrations were measured by radioimmunoassay.

Mouse strain	No hormones		Hormones		
			EL	EH	Tp
			mg/dl		
C57BL/6J	7.5 ± 1 (sham)	4.5 ± 0.8 (cas)	13 ± 2.5 (cas)[a]	17 ± 3 (cas)[a]	4.8 ± 0.9 (cas)
WT (intact)	5.1 ± 1.1			9.5 ± 1.2[b]	
ER-α-KO (intact)	3.8 ± 1			4.5 ± 0.8	

[a] Significantly different compared to sham and cas ($p < 0.005$).
[b] Significantly different compared to placebo group.

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TABLE III
Effects of estradiol on hepatic apoE mRNA levels in subcellular fractions of four mouse strains
ApoE mRNA were quantified by RNase protection assay using 10 μg of RNA. Values of apoE mRNA are presented as pg/μg total RNA.

Mouse strains	Nuclear		Polysomal		Beta actin[a]	
	P	E	P	E	P	E
C57L	180 ± 10	192 ± 11	57 ± 5	78 ± 6[b]	24 ± 4	23 ± 3
C57BL	201 ± 11	185 ± 12	56 ± 5	80 ± 6[b]	21 ± 4	19 ± 3
BALB	257 ± 14	276 ± 13	87 ± 5	75 ± 6	32 ± 5	27 ± 4
C3H	215 ± 9	226 ± 11	71 ± 7	65 ± 5	25 ± 3	22 ± 4

[a] Beta actin mRNA were quantified on total hepatic RNA.
[b] Significantly different compared to placebo group ($p < 0.02$).

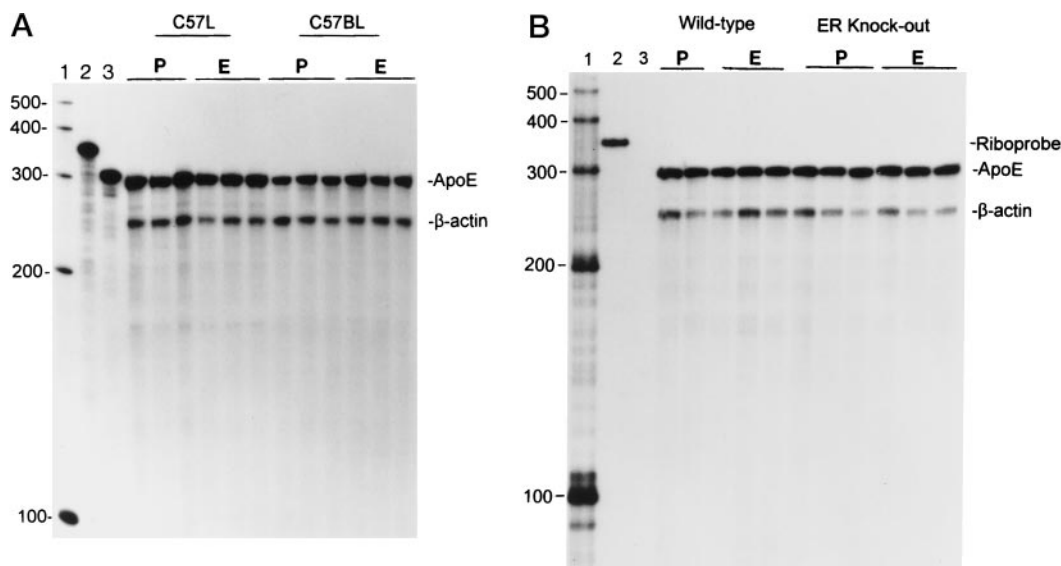

FIG. 3. **Effects of estradiol on hepatic apoE mRNA levels.** Five micrograms of total hepatic RNA were used for each RNase protection assay for apoE mRNA quantitation. *Each lane* represents an individual mouse. *P* indicates placebo, and *E* indicates estradiol treatment. Hepatic RNA and respective riboprobes were dried in a SpeedVac; hybridization was performed, and hybrids were digested with RNase. The protected RNA fragments were precipitated with isopropyl alcohol, dissolved in RNA loading buffer, and separated in a 6% sequencing gel. β-Actin mRNA was used as an internal control. *A, lane 1*, shows RNA size markers synthesized *in vitro* the same way as the riboprobes; *lanes 2* and *3* show apoE riboprobe and β-actin riboprobe, respectively. *B*, quantitation of apoE mRNA in the WT and the ER-α knock-out littermate mice. Five micrograms of liver RNA from each individual mouse was used. β-Actin was used as an internal control. *P*, placebo; *E*, estradiol-treated. *Lane 1*, RNA size markers synthesized *in vitro*; *lane 2*, apoE riboprobe; *lane 3*, riboprobe hybridized with yeast tRNA.

LDL-sized particles increased much more in the C57L and C57BL strains (Fig. 1, *A* and *B*) than in the BALB and C3H (Fig. 2, *A* and *B*). The cholesterol contents of the HDL-sized fractions decreased in all four strains, less so in the C57L and C57BL than in BALB and the C3H. These changes are compatible with alterations in apoB/apoAI ratios (Table I).

In the basal state, the majority of apoE in the plasmas of C57L and C57BL was distributed among the LDL- and large HDL-sized particles (fractions 20–40, Fig. 1, *C* and *D*), whereas in BALB/c and C3H, a larger proportion of apoE was in the LDL-sized particles (fractions 20–30, Fig. 2, *C* and *D*). Following estradiol administration in C57L and C57BL, plasma apoE shifted into the IDL- and LDL-size range (fractions >15, Fig. 1, *C* and *D*) reflecting the shifts in the major lipoprotein classes. By contrast, in C3H and BALB, apoE levels shifted toward HDL-sized particles (fractions 40–48, Fig. 2, *C* and *D*), also reflecting the estradiol induced redistributions of lipoproteins. Castration resulted in the reduction of plasma apoE levels when compared with sham-operated controls (Table II). Estradiol at either the physiologic or the higher dose increased apoE levels in the male castrated mice. Dihydroxytestosterone had smaller effects.

Estradiol was administered to ER-α knock-out and the WT littermates exactly the same way as described for the four mouse strains. More than 2-fold increases in plasma apoE levels were noticed in WT littermates, but plasma apoE levels increased only 20% in the ER knock-out mice (Table II). The 20% increase was not significant.

Effect of Estrogen on ApoE mRNA Levels and Rates of Transcription—Total hepatic and nuclear apoE mRNA levels did not change in the responder or in the nonresponder strains (Table III and Fig. 3*A*) or in the ER-α knock out mice.

In a separate experiment total hepatic apoE mRNA levels were quantified in castrated C57BL mice given low and high doses of estradiol and testosterone. Negative results, similar to those seen in the intact mice, were obtained (data not shown). Relative rates of apoE mRNA transcription measured in isolated hepatocyte nuclei also remained unaltered (Table IV). By contrast, apoE mRNA enrichments increased in polysomes of both intact and castrated mice but only in the responder strains (Table III and Fig. 4*A*). Polysomal apoE mRNA enrichments did not increase in the estrogen receptor knockout mice after estradiol treatment, although increases in polysomal apoE mRNA in the WT littermates were evident (Fig. 4*B*). As an internal control we also determined β-actin mRNA levels which did not change. These data rule out the possibility of transcriptional regulation of apoE by estrogen and point to regulation at the translational level.

We confirmed the selective estrogen-mediated enrichment of apoE mRNA in precipitated polysomal fractions using polysomal profiles prepared by density gradient ultracentrifugation. (Fig. 5). Estrogen caused >2-fold enrichment of apoE mRNA levels in the denser polysomal fractions of the responder strain C57BL.

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TABLE IV
Measurements of relative rates of apoE mRNA transcription in four inbred strains

The data represent counts/min in duplicate assays. For the measurements of relative rates of apoE mRNA transcription 10 million hepatic nuclei were used, and one million counts of newly synthesized labeled RNA were hybridized with the apoE cDNA probe. As an internal control β-actin mRNA transcription was also measured. The counts in duplicate assays were 95 and 106 in placebo and 99 and 96 in estradiol-administered C57L strain. The respective values in BALB were 91 and 76 in placebo and 79 and 93 in estradiol-administered group.

Mouse strain	Placebo	Estradiol
C57L	263,252	237,229
C57BL	274,240	290,270
BALB	215,195	218,257
C3H	264,238	279,248

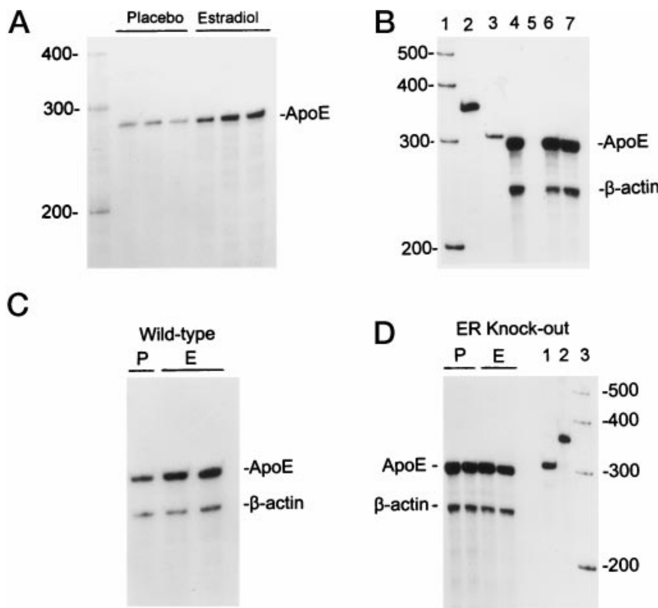

FIG. 4. **Effects of estradiol on polysomal apoE mRNA levels in the C57L strain.** *A*, polysomes were prepared from three placebo and three estradiol-treated C57L mice by precipitation, and RNA was isolated. Five micrograms of total RNA were used for the quantitation of apoE mRNA by RNase protection assay as described in the legend to Fig. 3. *B* shows distribution of apoE mRNA in monosomes and polysomes. *Lane 1*, RNA size markers; *lane 2*, apoE riboprobe; *lane 3*, β-actin riboprobe; *lane 4*, total RNA; *lane 5*, yeast tRNA; *lane 6*, polysomal RNA; *lane 7*, monosomal RNA. *C* shows estrogen's effects on polysomal RNA of the WT littermate mice; and *D* shows polysomal apoE mRNA in the estrogen receptor knock-out mice. *P*, placebo; and *E*, estradiol-treated. In *C*, total hepatic RNA from three mice were pooled from the placebo group, and RNA from two of the four estradiol-treated mice were pooled creating two pools. Both pools were used. *D*, *lane 1*, β-actin riboprobe; *lane 2*, apoE riboprobe; and *lane 3*, RNA size markers synthesized *in vitro* simultaneously with riboprobe synthesis.

Effect of Estrogen on Polysomal ApoE Translation and Hepatic ApoE Synthesis—The increased apoE mRNA levels in the polysomal fractions of responder strains warranted measurements of rates of translation on polysomes. Consonant with the changes in polysomal apoE mRNA levels, rates of translation of apoE on polysomes increased 1.6-fold in C57L and 1.8-fold in C57BL but did not change in the BALB and C3H (Fig. 6). Similarly, incorporation of [^{35}S]methionine into apoE protein by hepatic slices was increased in the estradiol-treated wild-type mice (Fig. 7*A*). Synthesis of apoE also increased 2.5-fold in the estradiol-treated castrates compared with the placebo-treated castrates (Fig. 7*B*). ApoE synthesis increased only by 20% after estrogen treatment in the ER-α knock-out mice (Fig. 7*C*) as compared with 2-fold increases in the wild-type littermates (Fig. 7*A*).

DISCUSSION

Estrogens are thought to retard atherosclerotic lesion formation by a variety of mechanisms (36). In the present studies we tested the hypothesis that one mechanism by which estrogen may inhibit lesion formation is by increasing plasma apoE levels, since overexpression of apoE protected mice from developing atherosclerosis (17), and the absence of apoE caused spontaneous atherosclerosis even on a low fat diet (16). ApoE may perhaps facilitate the removal of atherogenic particles from plasma (15), and perhaps it also facilitates removal of cholesterol from lipid-laden macrophages in lesioned arteries (19). We chose the mouse to test our hypothesis since we have shown that, unlike in rats, LDL receptors are not up-regulated by estrogen in mouse (3). Furthermore, among 15 mouse strains tested some mouse strains responded to estrogens by increasing their plasma apoE levels, and some did not (26). One of the aims of the present study was to determine the mechanisms of the strain-specific estradiol-induced increases of plasma apoE. The second aim was to determine the role of ER-α in estrogen-mediated regulation of apoE.

Plasma apoB and E concentrations and apoB- and apoE-containing particles were increased by estrogen treatment, in C57L and C57BL, termed as responder strains, but not in C3H and BALB, termed as non-responders (Figs. 1 and 2 and Table I). Estrogen also induced shifts of apoE distribution that differed by responder status. Some of the metabolic factors causing estrogen-induced elevations of apoE could be increased apoE production, altered intravascular metabolism of lipoproteins, or altered clearance of apoE-containing lipoproteins via the LDL receptor and/or LRP. Since hepatic LDL receptors in mice are not up-regulated by estrogen (3), we evaluated apoE production.

Estradiol did affect plasma apoE levels by altering hepatic apoE mRNA transcriptional mechanisms since neither total hepatic apoE mRNA levels (Table III) nor apoE mRNA transcription rates (Table IV) changed, suggesting that the locus of regulation may be posttranscriptional. Indeed, the synthesis of apoE on polysomes and in liver slices increased appropriately in response to estradiol in the "responder" C57L and C57BL strains (Fig. 7) but not in the "non-responder" BALB/c and C3H/J strains, implying that regulation by estrogen in "responders" may occur at translational loci. This was confirmed by the shift of apoE mRNA to polysomes (Figs. 4 and 5). The specificity of the observed effects was confirmed by the absence of any effects due to dihydroxytestosterone treatment in the castrated mouse (Table II). Consistent with our findings, translational (and post-translational) regulation of the apoE gene has been reported in HepG2 cells (37) and in macrophages (38). In addition, differences in the regulation of apoAII also occur at the translational level in mouse strains C57BL and BALB (39).

Ornithine-δ-aminotransferase mRNA levels in retinoblastoma cells remained constant after exposure to estrogen, but ornithine-δ-aminotransferase protein synthesis increased 3-fold, with a concomitant shift of ornithine-δ-aminotransferase mRNA toward the denser fraction of polysomes (40). Thus, estrogen can increase the recruitment of both ornithine-δ-aminotransferase mRNA and apoE mRNA on the translative pool of the polysomes. One mechanism that produces such recruitments may involve eucaryotic elongation factor eIF4E, since induction of eIF4E induces translation of proteins (41).

Estrogens are transported into nuclei by estrogen receptors residing on nuclear membranes. Estrogen-bearing receptors may then affect a number of nuclear processes. Recently, a second ER has been described. The "classical" receptor has been named ER-α and the new one ER-β (42–45). To test whether ER-α mediated the estrogen-induced increases in

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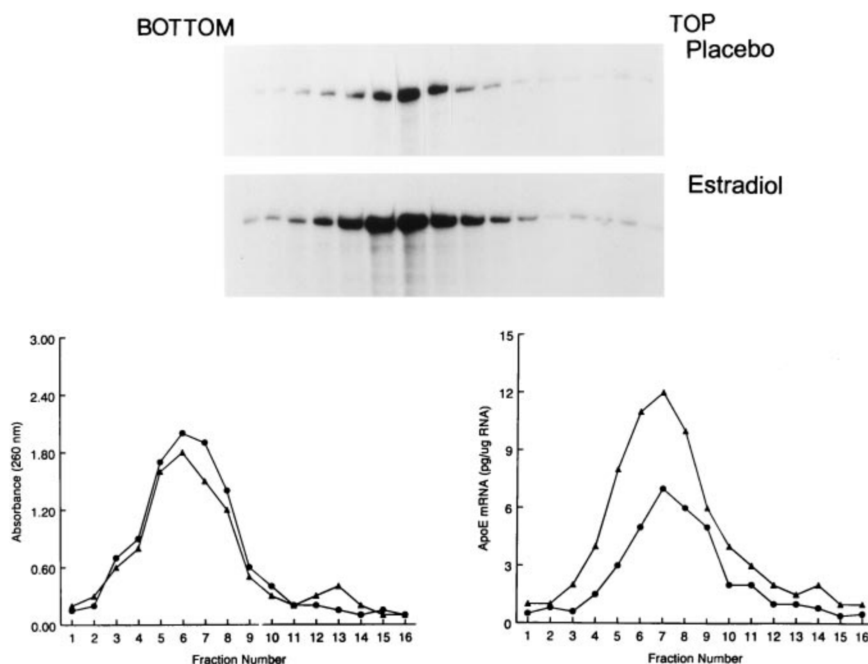

FIG. 5. **Polysome profiles of placebo and estradiol-treated C57BL mice.** Pooled livers (1 g) from three mice were used for the preparation of S15 fractions and subsequently of sucrose density gradient centrifugal profiles. After ultracentrifugation, the contents of the tube were collected in 16 equal parts from the bottom of the centrifuge tube, and in each fraction absorption at A_{260} was determined. Total RNA prepared from each fraction was used for the quantification of apoE mRNA by RNase protection assay. The intensity of the bands in the autoradiogram obtained after RNase protection assay (shown above) was determined by Image Analysis System and converted into pg/μg RNA and plotted against fraction number (*right panel*). Fraction number 1 starts from the bottom of the tube. *Top* and *bottom* in the figure represent top and bottom portion of the centrifuge tube used for polysome profile determination. *Circles* represent placebo, and *triangles* represent the estradiol-treated group.

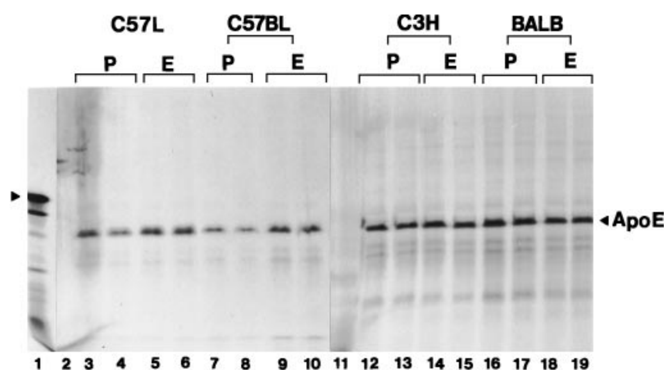

FIG. 6. **Effects of estradiol on *in vitro* translation of apoE on isolated liver polysomes.** *Lane 1*, positive control using 0.5 μg RNA of *in vitro* synthesized *Xenopus* elongation factor 1-α; *lane 2*, negative control containing all *in vitro* translation ingredients minus RNA. After the *in vitro* translation on the polysomes, apoE were immunoprecipitated, washed, and dissolved in the gel loading buffer and separated in a 5/10% SDS-polyacrylamide gel electrophoresis. Figure shows autoradiogram of the dried gel. *P*, placebo; *E*, estradiol-treated. The position of apoE has been marked on the *right*. Areas and intensities of bands were obtained by scanning, and mean ratios of estradiol- to placebo-treated animals were calculated. For C57L and C57BL strains, band ratios were 1.60 and 1.86; for C3H and BABLc strains, the ratios were 1.03 and 1.04, respectively.

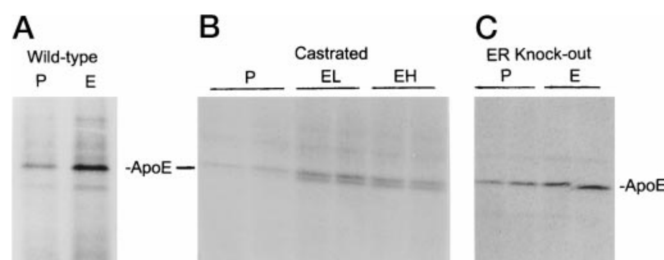

FIG. 7. **Effects of estradiol on hepatic apoE synthesis by liver slices.** Livers were excised from placebo and estradiol-treated mice immediately, and apoE synthesis was performed on liver slices (*A*). Synthesis was traced with [^{35}S]methionine (35), in two groups of mice per treatment, each group containing liver slices from two mice. Newly synthesized apoE was immunoprecipitated and processed as described (22). The intensity of the autoradiographed dried gel was scanned by Image Analysis System. *B* shows apoE synthesis in castrated mice given low (*EL*) or high (*EH*) doses of estradiol. Two mice were taken for apoE synthesis in each group. *C* shows apoE synthesis using freshly isolated liver slices from WT littermates and estrogen receptor knock-out mice given estradiol.

plasma apoE levels, we studied ER-α knock-out mice, which show only 5% estrogen binding activity of WT mice (30). Although plasma apoE increased ~2-fold in the wild-type littermates, there was only 20% increase of apoE in the ER-α knock-out mice perhaps due to the 5% residual estrogen binding activity in these mice (30), suggesting the presence of other estrogen receptors, possibly ER-β (42–45), that partially takes over estrogen receptor activity in the absence of ER-α as recently suggested (46). Lower endothelial nitric oxide production was recently reported in the ER-α knock-out mice, which was associated with the absence of ER-α (47). Although some of the functions of ER-α may be compensated by ER-β (46), many other functions of ER-α appear to be specific (47, this study). Our data suggest that ER-α is largely involved in the estrogen-induced increases of apoE synthesis. Perhaps additional anti-atherogenic activities of estrogen may also occur via ER-mediated pathways, although some antiatherogenic activities like antioxidant activity (48) may not require functional estrogen receptors.

Premenopausal women have lower levels of LDL as compared with postmenopausal women (49), and estrogen therapy in healthy (50) and moderately hypercholesterolemic (51) postmenopausal women lowers LDL and raises HDL levels. The opposite effects are seen in mice. In women, the rises in HDL could be due to lowering of hepatic lipase activities and the falls in LDL due to up-regulation of LDL receptor activities. Neither of these effects is produced by estrogens in any strain of male mice (3, 26).

In summary, we have shown that estrogen regulates apoE gene expression in a strain-specific manner in mouse. In responder strains regulation occurs primarily at the translational levels by recruitment of apoE mRNA to the translating pool of polysomes. Estrogen receptors-α are required for apoE translation to be affected by estrogens.

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Estrogen Up-regulates Apolipoprotein E (ApoE) Gene Expression by Increasing ApoE mRNA in the Translating Pool via the Estrogen Receptor α-Mediated Pathway

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