In vivo regulation of apolipoprotein A-I gene expression by estradiol and testosterone occurs by different mechanisms in inbred strains of mice[1]

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Abstract We tested the hypothesis that testosterone and estrogen modulate apoA-I gene expression and metabolism by different mechanisms that may be influenced by genetic factors. Male and female C3H/HeJ (atherosclerosis-resistant) and C57BL/6J (atherosclerosis-susceptible) mice (n = 5/group) were castrated (Placebo). Castrates were given 17β-estradiol (E2) at 0.16 μg/g (E2L) or 5 μg/g (E2H) body weight per day, or testosterone (Testo) 1 μg/g per day, 14 days after surgery, for 14 days. Plasma total cholesterol concentrations (TC) were higher in male Placebo mice than in females. Testosterone altered TC and high density lipoprotein (HDL) cholesterol by gender and strain; however (HDL-C)/TC ratios and apoA-I concentrations were unaltered. Testosterone did reduce HDL particle diameters in both genders of C3H mice only. Low density lipoprotein-cholesterol (LDL-C)/TC ratios remained constant and apoB increased in males only. E2L and E2H decreased TC, HDL-C/TC ratios, and apoA-I. Decrements varied by strain. HDL diameters decreased in both genders in C3H mice only; however, HDL size distributions were altered in both strains. LDL-C/TC ratios increased in all groups. E2L mice showed variable responses of apoB, but apoB rose uniformly in all E2H groups. Testosterone increased and E2H decreased hepatic apoA-I synthesis. ApoA-I mRNA concentrations remained stable in both Testo and E2 groups. ApoA-I gene transcription varied by strain and gender, but all changes were < twofold. Testosterone did not affect hepatic apoB or LDL receptor mRNA, however, E2H increased both mRNAs in males but not in females. On Western blotting of liver membranes, E2H had little effect on mouse LDL receptor protein mass; by contrast, E2H increased LDL receptor ~threefold in rats. In summary, responsiveness of mouse lipids to testosterone and E2 vary by strain and gender. Testosterone and E2 differ in their regulation of apoA-I production mainly at the level of translation. Hormones operate at several levels of gene regulation, suggesting that complex mechanisms are involved. Mice differ from rats and rabbits in their LDL receptor responsiveness to estradiol treatment.—**Tang, J., R. A. K. Srivastava, E. S. Krul, D. Baumann, B. A. Pfleger, R. T. Kitchens, and G. Schonfeld.** In vivo regulation of apolipoprotein A-I gene expression by estradiol and testosterone occurs by different mechanisms in inbred strains of mice. *J. Lipid Res.* 1991. **32:** 1571–1585.

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Plasma HDL concentrations are potent predictors of coronary disease in humans (1, 2). Apolipoprotein (apo) A-I is the major apolipoprotein of HDL and its concentrations also are useful predictors of coronary disease risk. Men have a greater risk of coronary heart disease than pre-menopausal women perhaps because of the lower concentrations of HDL and apoA-I in their plasma, whereas post-menopausal or castrated women have coronary risk and plasma lipoprotein profiles that more closely resemble those of men. Estrogen replacement therapy returns the lipoprotein profiles and perhaps the coronary risk towards pre-menopausal levels. Thus, some of the differences between men and women with respect to lipoprotein and coronary risk may be due to sex hormones rather than gender.

ApoA-I is synthesized in liver as well as in intestine (3), and its hepatic synthesis is influenced by estrogens and androgens (4–8). These hormones also alter plasma total cholesterol and HDL-cholesterol levels in many species (9–13). In some studies, these hormones influence the rate of synthesis of apoA-I (14–16), while in others, they alter its clearance from plasma (17). Differences in hormone responsiveness between individuals and species exist and

Abbreviations: apo, apolipoprotein; HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; HTGL, hepatic triglyceride lipase; LPL, lipoprotein lipase; FPLC, fast protein liquid chromatography; DEPC, diethyl pyrocarbonate; SDS, sodium dodecyl sulfate; EDTA, ethylenediamine tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; BSA, bovine serum albumin; TCA, trichloroacetic acid; PMSF, phenylmethylsulfonyl fluoride; HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; DTT, dithiothreitol; PIPES, piperazine-N,N'-bis[2-ethanesulfonic acid]; EGTA, ethylene glycol-bis(β-amino ethyl)ether; SSC, 0.15 M sodium citrate, pH 7.0; HSS, high speed supernatant; PC, personal computer; LDL-R, LDL receptor.

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probably reflect the genetic diversity of the subjects under study.

Relatively little is known about the hormonal regulation of apoA-I production at the molecular level. Even less is known about any interactions of hormonal and genetic factors, which are difficult to study in outbred humans or experimental animals. However, using inbred strains of mice, comparisons can be made between identically treated, but genetically distinct strains to determine what role genetic factors may play in the animals' responsiveness to various experimental perturbations. In inbred mice, strain-related differences exist in susceptibility to diet-induced atherosclerosis and in lipoprotein profiles (18–22). For example, the mouse strains C57BL/6J and C57L/J are susceptible to diet-induced atherosclerosis, while the C3H/HeJ and NZB/BINJ strains are resistant (19). Plasma lipoprotein profiles also are affected in characteristic ways when these mice are administered atherogenic diets.

We hypothesized that C57BL/6J and C3H mice, two of the strains known to have differing lipoprotein profiles and differing susceptibilities to diet-induced atherosclerosis, would also differ with respect to the regulation of their apoA-I metabolism by sex steroids. We further hypothesized that testosterone and estrogen may differ in their effects on the molecular regulation of apoA-I. Accordingly, we measured plasma concentrations and compositions of HDL, plasma concentrations of apoA-I, rates of hepatic apoA-I synthesis and the in vivo regulation of apoA-I gene expression in castrated male and female C3H/HeJ and C57BL/6J mice administered either testosterone propionate or 17β-estradiol. We found that indeed there were differences in responsiveness by mouse strain, gender, and hormone, and that the regulation of apoA-I production occurred at both transcriptional and translational steps.

METHODS

Animals and groups of treatment

Five- to six-week-old, male and female C3H/HeJ and C57BL/6J mice were obtained from Jackson Laboratory, Bar Harbor, ME. All mice were housed at 24°C in a room illuminated in 12-h light-dark cycles and maintained on Purina chow 5015 diet and tap water ad libitum. Mice of each strain were divided into four groups (n = 5/group); placebo, castrated mice treated with vehicle injections; Testo, castrated mice administered testosterone propionate (Tp) at 1 μg/g body weight per day; E2L, castrated mice administered 17β-estradiol (E2) 0.16 μg/g body weight per day; and E2H, castrated mice administered 17β-estradiol 5 μg/g body weight per day. Castrations were performed under metofane anesthesia at least 7 days after the arrival and housing of mice in our local facility and animals were allowed to recover for 14 days after surgery. The indicated doses of testosterone propionate and 17β-estradiol dissolved in propylene glycol were injected subcutaneously along the back at the same time each day for 14 days, while placebo mice received propylene glycol only. Animals were weighed before and after 14 days of hormone administration. At the end of the experiment, animals were fasted overnight and killed by exsanguination under ether anesthesia. Plasma (anticoagulated by EDTA) from individual mice was pooled in each group for FPLC and HDL particle size determination. Serum was collected individually for determination of total cholesterol, sex hormones, and apolipoprotein concentrations. Livers were removed immediately and pooled in each group for determining hepatic apoA-I synthesis and for isolating nuclei and total RNA.

Testosterone and 17β-estradiol levels in the serum

Testosterone and estradiol radioimmunoassay kits were purchased from Amersham (DSL 4100 and IMB 100). Standard curves were constructed by plotting the log hormone levels (pg/ml) versus cpm of ^{125}I-labeled testosterone or estrogen. The standard curves ranged from 100 to 25,000 pg/ml for testosterone and 20 to 3,000 pg/ml for estradiol. One hundred μl of individual mouse sera were used for each assay.

Nondenaturing gradient gel electrophoresis

Nondenaturing gradient gel electrophoresis was performed as previously described (22). Briefly, plasma pools from each group (40 μl) were adjusted to d 1.21 g/ml by adding solid KBr and overlayered with d 1.21 g/ml KBr solution containing 1 mmol/l EDTA. Total lipoproteins (d < 1.21 g/ml) were collected (in 20 μl) after ultracentrifugation in a Beckman 42.2 Ti rotor at 42,000 rpm for 24 h at 10°C. The d < 1.21 g/ml lipoprotein fractions (10 μl per lane) were electrophoresed in PAA 4/30 polyacrylamide gradient gels (Pharmacia, Uppsala, Sweden). Electrophoreses were carried out at 125 V at 10°C for 24 h, and the gels were stained in 0.04% Coomassie brilliant blue G-250. Thyroglobulin and bovine serum albumin (BSA) were added to each sample as internal size standards. Five reference proteins were used for calibrating particle sizes: thyroglobulin (Stoke's diameter 17.0 nm), apoferritin (12.2 nm), catalase (9.6 nm), lactate dehydrogenase (8.2 nm), and BSA (7.1 nm). Standard curves were plotted as the relative migration of the reference proteins compared to BSA versus the diameter of the reference proteins. Standard curves were used to determine the sizes of the mouse lipoprotein particles.

Gel permeation chromatography and analysis of fractions

Separation of mouse plasma lipoproteins on FPLC Superose-6 columns was performed as previously described

(18). Briefly, 400 μl of pooled plasmas from each group was centrifuged at 100,000 *g* min and the clear supernatants were loaded onto two Superose-6 columns (FPLC system, Pharmacia) connected in series. Fractions were eluted at 0.5 ml/min with 1 mmol/l EDTA, 154 mmol/l NaCl, and 0.02% NaN_3 (pH 8.2). The first 12 ml of effluent was discarded, and 50 fractions of 0.5 ml each were subsequently collected. Cholesterol analyses on the column fractions and individual mouse serum samples were performed using enzymatic kits from Wako Chemicals USA (Richmond, VA).

Determination of serum apoA-I and apoB concentrations

Serum apoA-I and apoB concentrations were determined by electroimmunoassay according to the method of Laurell (23) with some modifications. For apoA-I, electroimmunoassay plates were prepared as follows: 1.5% agarose (Seakam) with 5% Dextran T10 (Pharmacia) in Tris-Tricine buffer (0.8 M Tris, 0.024 M Tricine, 0.034 mM calcium lactate, 0.05% sodium azide, pH 8.6). For apoB, the plates contained 1% agarose, 3% PEG (mol wt 8000, Sigma), and 0.16% Triton. Agarose solutions were boiled and allowed to cool to 55°C, after which rabbit anti-mouse apoA-I or apoB monospecific polyclonal antibodies were added at 1:150 and 1:200 dilutions, respectively. The agarose/antibody solutions were poured onto GelBond film (FMC) (cast gel dimensions = 21.6 × 10 × 0.15 cm). Twenty-three wells of 4 mm diameter were punched out with center-to-center distances of 9 mm. Pooled plasmas from each mouse group or individual mouse serum samples were diluted 1:25 with Tris-Tricine buffer containing 1% BSA (Sigma A-7030) and 8 M urea for apoA-I. No urea was added for apoB. Five μl of standard or diluted samples was applied to the gel wells. Electrophoresis was carried out in Tris-Tricine buffer at 2 V/cm for 16–18 h in an LKB Multiphor chamber connected to a water circulator maintained at 15°C. Washing, staining, and drying of the electroimmunoassay plates were performed as described in the LKB 2117 Multiphor II Electrophoresis System Laboratory Manual (24). Results were calculated by area in mm^2 (rocket height × ½ width at ½ height) per μl sample applied. Mouse apoA-I and LDL isolated from plasma were used as standards. The standard curves were linear over the range of 50–400 ng for apoA-I and 300–1200 ng for apoB.

Hepatic apoA-I synthesis

ApoA-I synthesis rates in mouse livers were determined according to the method of Williams and Dawson (25). Freshly isolated liver was chopped into 5-mg slices with a razor blade, and rinsed twice with bicarbonate-buffered Krebs-Ringer solution (KRB, 120 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, pH 7.4), containing 50 U/ml penicillin and 50 μg/ml streptomycin. The washes removed blood and minimized the contamination with plasma lipoproteins. Forty mg of pooled total liver slices from each group (equal aliquots from each mouse) was incubated for 1 h at 37°C in 200 μl KRB containing antibiotics, 0.1% glucose, and 200 μCi[^{35}S]methionine (1 mCi/ml) which was lyophilized and dissolved in the buffer (sp act: 1106 Ci/mmol, TRAN ^{35}S LABEL, ICN, Irvine, CA) under an atmosphere of 95% O_2/5% CO_2. The tissue incubation was brief (1 h) to ensure that the spectrum of newly synthesized proteins was representative of the proteins being synthesized at the time the tissue was removed from the animal and so that more than 95% of synthesized protein would remain in the tissue. After 1 h of incubation, the liver slices were washed twice with 2 ml ice-cold KRB and homogenized at 0–2°C with 600 μl homogenization buffer (0.02 M sodium phosphate, pH 7.4, 0.15 M NaCl, 0.005 M EDTA, 200 μg/ml phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100). The homogenate was ultracentrifuged at 2°C at 112,000 *g* for 1 h to prepare a high-speed supernatant (HSS). The HSS of each group were stored at −70°C until analysis.

ApoA-I hepatic synthesis in each group was measured by immunoprecipitation. Briefly, 10 μl of rabbit anti-mouse apoA-I antiserum (prepared in this laboratory) in 1% Triton X-100 and 200 μg/ml PMSF were incubated with 20 μl HSS overnight at 4°C (performed in triplicate). Immune complexes were precipitated with 30 μl of a solution of 10% Immuno-Precipitin (Formalin-fixed Staph A cells) (Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, MD) in homogenization buffer for 30 min at room temperature. The antibody-antigen complexes were pelleted by spinning at 12,000 rpm for 3 min, and the pellets were washed twice with 1 ml homogenization buffer. Fifty μl of SDS gel-loading buffer (26 mM Tris, pH 6.8, 10.6% glycerol, 1.1% SDS, 2% s-mercaptoethanol, 0.024% bromophenol blue) was added to the pellets after the last wash and the solutions were boiled for 5 min. The immunoprecipitates were then run on 10% SDS polyacrylamide gels as previously described (25). After staining with Coomassie blue, the gel was treated with EN^{3}HANCE (NEN Research Products, Boston, MA) for 1 h, followed by a cold water precipitation for 30 min and 1% glycerol for 1 h. The gel was then dried and exposed to Kodak XAR-5 film at −70°C for autoradiography. The peaks corresponding to apoA-I were quantified using the video analysis system (JAVA Jandel Scientific, Cortex Madera, CA) on a PC. ApoA-I synthesis was expressed as the area under the apoA-I peaks (cm^2)/total protein synthesis (cpm)/h. Total protein synthesis was assessed by trichloroacetic acid (TCA) precipitation. One ml dH_2O was added to 20 μl HSS. One ml 20% TCA was then added to this mixture, and after incubating at 4°C for 15 min, the precipitates were isolated by centrifugation and washed twice with 10% cold

TCA. The precipitates were dissolved in 200 μl Soluene 350 (Packard, Downers Grove, IL) and counted for ^{35}S radioactivity in a Beckman LS 2800 scintillation counter. ApoA-I synthesis in placebo group was considered as 100%.

Determination of apoA-I mRNA concentrations in liver and intestine by solution hybridization

Hepatic and intestinal total RNAs were isolated by the method of Chirgwin et al. (26). The rat apoA-I cDNA probe, which was constructed in pGEM-2, was kindly provided by Dr. J. Gordon (Department of Biological Chemistry, Washington University). The probe showed good cross-reaction with mouse apoA-I mRNA on Northern blots. A 552 bp insert including 469 bp of rat apoA-I cDNA, a 17-mer poly C sequence, and the 66 bp polylinker of pGEM-2 was cut out by EcoRI and purified by 1% low melting agarose gel (Bethesda Research Laboratories) and then subcloned into the EcoRI site of the pGEM-3Zf(+) vector. The orientation of the ligated rat apoA-I cDNA in pGEM-3Zf(+) was tested both by sequencing and by hybridizing the ^{32}P-labeled apoA-I cRNA probe (obtained by transcribing the vector with T7 RNA polymerase, see below) with ssDNA standard made from the recombinant vector.

The pGEM-3Zf(+) containing the apoA-I cDNA insert was linearized by Kpn I and followed by conversion of the 3' overhang produced by Kpn I to a blunt end by adding Klenow DNA polymerase before transcription. A ^{32}P-labeled rat apoA-I cRNA probe was then transcribed by T7 RNA polymerase from the linearized vector according to Promega Guide protocol (27). The reaction mixture contained (Promega transcription kit): 4 μl of 5× transcription buffer, 2 μl of 100 mM DTT, 20 U RNAsin (ribonuclease inhibitor), 1 μl linearized template (1 μg/μl), and Klenow DNA polymerase at 5 U/μg DNA. The mixture was incubated at 22°C for 15 min and then the following was added: 1 μl each of 10 mM ATP, GTP, and CTP, 2.4 μl 100 μM UTP, 5 μl[α-^{32}P]UTP (50 μCi at 650 Ci/mmol, ICN), 1 μl T7 RNA polymerase (15–20 U/μl). The mixture was incubated at 37°C for 60 min. In order to remove the DNA template, 1 μl RQ1 RNase free DNase I (1 U/μG DNA) (Boehringer Mannheim Biochemicals) and 0.5 μl RNAsin (20 U) (Promega) was added after the transcription reaction and incubated for a further 15 min at 37°C. To purify the transcribed probes, the reaction mixture was extracted with phenol-chloroform followed by an extraction with chloroform. The cRNA probe was precipitated by ethanol and dissolved in 20 μl DEPC-treated H_2O. The unincorporated nucleotide triphosphates were removed from the probe solution by chromatography on an RNA Sephadex G-50 (BMB) quick-spin column. The eluted probe was then ready for use.

An 858 bp cRNA standard was transcribed from the same vector by SP6 RNA polymerase after linearization with NaeI (which is a restriction enzyme site on the pGEM-3Zf(+) vector outside the polylinker region). The cRNA standard was then purified as described above and dissolved in DEPC-treated water. Aliquots were stored at −70°C until use.

The solution hybridization assay for apoA-I mRNA was carried out at 65°C (optimal temperature determined in earlier experiments) (28). Initial experiments were also carried out to determine the range of total RNA concentrations that yielded a linear response. Ten μg of total cellular RNA was chosen in subsequent experiments to quantify the apoA-I message. The range of the standard curve was from 10 pg to 200 pg of RNA standard. The RNA samples (10 μg) in triplicate were dried in a SpeedVac (Savant, Farmingdale, NY) and dissolved in 25 μl hybridization buffer (40% formamide, 400 mM NaCl, 1 mM EDTA, 40 mM PIPES, pH 6.5–6.7). Five μl ^{32}P-labeled apoA-I probe (40,000 cpm) was added to each tube and the content of the tube was covered by 5 μl paraffin oil. Tubes were incubated at 65°C overnight. Three hundred μl of ice-cold RNase solution (40 μg/ml RNase A, 2 μg/ml RNase T1, 0.3 M NaCl, 5 mM EDTA, 10 mM Tris.HCl, pH 7.5) was added and incubated at 30°C for 1 h. One ml cold TCA solution (10% TCA, 1.5% sodium pyrophosphate) was then added and the tubes were placed on ice for 15 min. The samples were then filtered through a G F/L glass fiber filter (Schleicher & Schuell, Keene, NH). The filters were washed with 10% cold TCA, dried at 80°C for 1 h, and counted for ^{32}P radioactivity. The calculation factor based on this assay was 1100 bp (length of apoA-I cDNA) divided by 858 bp (length of cRNA standard used in the assay) which equals 1.28. The hepatic apoA-I mRNA concentrations were expressed as pg apoA-I mRNA/μg total RNA.

Determination of apoA-I mRNA transcription rates in isolated liver nuclei

Mouse liver nuclei were isolated according to the method of Groudine, Peretz, and Weintrauts (29) with some modification. A section of mouse liver was excised immediately after killing and rinsed with ice-cold buffer A (60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM 2-mercaptoethanol, 0.5 mM EGTA, 2 mM EDTA, 15 mM HEPES, pH 7.5). After rinsing, the liver (total of 1 g pooled from each group) was homogenized in 10 volumes of ice-cold buffer A containing 0.3 M sucrose and centrifuged for 10 min at 2,500 rpm in a swinging-bucket rotor. The pellet containing crude nuclei was resuspended in 2 ml of 2 M sucrose in buffer B (same as buffer A but with 0.1 mM each of EGTA and EDTA). This was then layered over a cushion of 2 M sucrose in buffer B and centrifuged for 20 min at 25,000 rpm at 4°C in a Beckman TL 100 ultracentrifuge. The clean nuclear pellet was resuspended in storage buffer (20 mM Tris-HCl, pH 7.9, 7.5 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM PMSF, 50% glycerol). The concentra-

tion of the nuclei was about $5 \times 10^5/\mu l$. Nuclei in storage buffer could be stored for several weeks at −70°C without loss of activity.

In vitro transcription rates for apoA-I in isolated nuclei were determined according to the method of Schibler et al. (30). One to 2×10^7 nuclei equivalent to 200–250 μg DNA, in triplicate, were used in the in vitro elongation reactions. The reaction buffer contained: 100 mM Tris-HCl, pH 7.9, 50 mM NaCl, 0.4 mM EDTA, 0.1 mM PMSF, 1.2 mM PMSF, 1.2 mM DTT, 1 mg/ml heparin sulfate, 2 mM $MnCl_2$, 4 mM $MgCl_2$, 1 mM each of GTP, ATP, CTP, 10 mM creatine phosphate, 130 U/ml ribonuclease inhibitor, 30% glycerol, and 100 μCi[α-^{32}P]UTP (650 Ci/mM, ICN). The transcription reaction was allowed to occur at 30°C for 40 min and was terminated by the addition of DNase 1 (RNase free) and further incubated at 30°C for 5 min. Twenty μg Proteinase K, 5 mM EDTA, and 1% SDS were then added and the reaction mixtures were incubated at 37°C for a further 30 min. The reaction mixtures were extracted by hot (65°C) phenol–chloroform–isoamylalcohol 25:24:1 (v/v/v), and the RNA was subsequently precipitated by ethanol. The transcription products were checked by running aliquots on urea SDS-polyacrylamide gels.

The hybridization assay was carried out according to the method of Chazenbalk, Wadsworth, and Rapoport (31). Recombinant pGEM-3Zf(+) vector containing the 550 bp rat apoA-I cDNA was linearized with Kpn I, denatured in 0.2 M NaOH, and neutralized with 1 M HEPES, pH 6.5. Two μg of treated plasmid DNA was applied to nitrocellulose paper and dried at 80°C. The nitrocellulose papers containing the apoA-I probe were prehybridized for 2 h at 42°C in hybridization buffer (20 mM PIPES, pH 6.7, 50% formamide, 2 mM EDTA, 0.8 M NaCl, 0.2% SDS, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 100 μg/ml poly(A), and 0.02% BSA). The hybridization reaction was carried out by adding 100 μl hybridization buffer with $1–10 \times 10^6$ cpm of extracted nuclear [^{32}P]RNA to the prehybridized nitrocellulose paper and incubating for 60 h at 42°C. Fifty μl of paraffin oil was added to each tube to prevent evaporation. After hybridization the nitrocellulose papers were washed twice with 2× SSC and 0.1% SDS for 30 min at room temperature and treated with RNase A solution (10 μg/ml in 2× SSC) at 37°C for 30 min followed by washing with 2× SSC twice at room temperature. Further washings of the filters were done twice at 65°C in 0.1× SSC containing 0.1% SDS for 30 min. The filters were dried at room temperature and exposed to Kodak XAR-5 film using intensifying screens at −70°C. To elute the hybridized RNA for quantitation, the filters were incubated with 200 μl of 0.3 M NaOH for 15 min at 65°C followed by the addition of 50 μl glacial acetic acid and 4 ml scintillation fluid and counted. β-Actin mRNA transcription rates were used as internal standards. The hepatic apoA-I mRNA transcription rates in the groups of mice were expressed as a percentage of the transcription rate in the placebo group.

Comparison of LDL-R up-regulation by estrogen between rats and mice

Male C3H/HeJ mice (n = 3/group) and 250–275 g male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN) (n = 1/group) were divided into three groups: placebo administered at 1.6 μl/g body weight propylene glycol per day; 17 α-ethinyl estradiol (17α-EE) administered at 5 μg/g body weight per day; and 17β-estradiol (17β-E2) administered at 5 μg/g body weight per day. The 17α-ethinyl estradiol and the 17β-estradiol were dissolved in propylene glycol and delivered subcutaneously at the same time each day for 6 days. After an overnight fast, the animals were exsanguinated under ether anesthesia on the seventh day. Plasma, anticoagulated with EDTA, was collected for total cholesterol concentration determination. Livers were immediately removed, and liver membrane preparations were performed as described by Kovanen, Brown, and Goldstein (32). Briefly, the livers were homogenized in 10 ml Tris buffer (150 mM NaCl, 1 mM $CaCl_2$, and 10 mM Tris-HCl, pH 7.5) with two 10-sec pulses in a Tekmar homogenizer (Cincinnati, OH) at 4°C. The whole homogenates were then centrifuged at 500 *g* for 5 min. The 500 *g* supernatant was centrifuged at 8,000 *g* for 15 min, and the 8,000 *g* supernatant was recentrifuged at 100,000 *g* for 60 min. The 100,000 *g* pellets were resuspended in 6 ml Tris buffer by repeatedly passing the pellet through a 21-gauge needle. This suspension was centrifuged at 100,000 *g* for 60 min, and the pellet was frozen quickly in liquid nitrogen and stored at −80°C.

LDL-R protein determination

Within a week, the frozen pellets were resuspended in 10 ml buffer (50 mM NaCl, 1 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.5) and solubilized using a 21-gauge needle. The solubilized pellets were sonicated for 20 sec in a Branson Sonifier cell disruptor (Model W-185E, Heat Systems-Ultrasonics, Inc., Plainview, NY) using a microprobe at a setting of 6, and protein concentrations were determined by a modification of the Lowry procedure (33).

The solubilized liver membranes were mixed with a solution of 2.5% SDS, 50% glycerol, and 0.5% bromophenyl blue in a ratio of 4:1. Two hundred μg of total liver membrane protein was loaded onto each lane of a 6% polyacrylamide gel containing 0.1% SDS and electrophoresed without preheating and in the absence of dithiothreitol for 3 h at 4°C in a 25 mM Tris-glycine (pH 8.6) and 0.1% SDS running buffer.

Transfer to nitrocellulose paper was accomplished using an alteration of the method of Towbin, Staehelin, and Gordon (34) except that methanol was omitted from

the transfer buffer. Nitrocellulose membranes (Micron Separations, Inc., Westboro, MA) presoaked in transfer buffer (25 mM Tris-glycine) for 30 min, were placed on the anode side of the gel between two sheets of porous filter paper. Electrotransfer was performed overnight at 4°C at 4 V/cm.

After the transfer, the nitrocellulose paper was dried and then immersed in Bovine Lacto Transfer Technique Optimizer (BLOTTO) blocking buffer consisting of 5% (w/v) nonfat powdered dry milk in phosphate-buffered saline (PBS) with 0.01% antifoam A emulsion (Sigma Chemical Co.) and 0.001% merthiolate (Sigma Chemical Co.) for 2 h. The nitrocellulose paper was then incubated overnight with rabbit anti-bovine adrenal LDL-R antiserum (#4526, generously provided by Dr. Janet Boyles, San Francisco, CA) diluted 1:1000 in BLOTTO at room temperature. The nitrocellulose paper was washed with 250 ml of 0.05% NP-40 in PBS, and a ^{125}I-labeled goat anti-rabbit IgG antibody diluted in BLOTTO to a concentration of approximately 7.5×10^5 cpm/ml was incubated with the nitrocellulose paper for 4 h at room temperature. The nitrocellulose paper was again washed with 0.05% NP-40 in PBS, dried, and placed in film cassettes for exposure at −80°C for autoradiography. Densities of the bands on autoradiographs corresponding to LDL-R were quantified on a video analysis system (JAVA Jandel Scientific).

Determination of LDL-R and apoB mRNA in liver by solution hybridization

Rat LDL-R cDNA clone was obtained from Dr. A. D. Cooper (Palo Alto Medical Foundation, Palo Alto, CA). A 550 bp fragment was cut out by Pst I and subcloned into Pst I site of pGEM-3Zf(+). The ^{32}P-labeled LDL-R cRNA probe was transcribed by T_7 RNA polymerase after linearization by HindIII from pGEM-3Zf(+) containing LDL-R cDNA. The cRNA standard was transcribed from the same vector by sp6 RNA polymerase, after linearization by NaeI (27). Rat apoB cDNA clone was obtained from Dr. A. J. Lusis (University of California, Los Angeles, CA). A 240 bp apoB cDNA was subcloned into EcoRI site of pGEM-3Zf(+) and linearized by BamHI and NaeI for standard and probe, respectively, apoB cRNA standard and probe were transcribed by T_7 and sp6 RNA polymerase, respectively (27). Fifty μg of total RNA was used for both LDL-R and apoB. The solution hybridization assays were carried out in triplicate as above and reference 28. The hybridization temperature was 60°C for LDL-R and 55°C for apoB. Both LDL-R

TABLE 1. Effects of testosterone and estradiol on serum hormone concentrations and body weights of castrated male and female mice

Strains and Groups	Serum		Body Weight	
	Testosterone	Estradiol	Before Treatment	After Treatment
	pg/ml		*g*	
Male				
C3H/HeJ				
Placebo	52 ± 6	24 ± 9	30 ± 1	27 ± 1*
Testo	289 ± 239*		29 ± 1	27 ± 2
E2L		58 ± 27*	29 ± 2	27 ± 1
E2H		3642 ± 406**	29 ± 2	23 ± 2*
C57BL/6J				
Placebo	123 ± 51	18 ± 3	25 ± 2	24 ± 3
Testo	266 ± 248*		25 ± 2	22 ± 0.4*
E2L		33 ± 8*	25 ± 1	27 ± 1*
E2H		3324 ± 1275**	26 ± 2	27 ± 2
Female				
C3H/HeJ				
Placebo	76 ± 10	31 ± 6	20 ± 2	19 ± 2
Testo	123 ± 28*		20 ± 3	18 ± 3
E2L		42 ± 9*	22 ± 2	20 ± 1
E2H		1503 ± 881**	21 ± 1	19 ± 1
C57BL/6J				
Placebo	97 ± 24	26 ± 7	18 ± 1	17 ± 1
Testo	231 ± 48*		19 ± 1	17 ± 1
E2L		38 ± 9*	19 ± 1	19 ± 1
E2H		2766 ± 997**	19 ± 1	18 ± 0.2

Serum hormone concentrations were determined after 14 days of administration as described in Methods. Body weights were determined immediately prior to the first hormone injection and after 14 days of treatment. Data represent the means ± standard deviations from five mice in each group; *, $P < 0.05$; **, $P < 0.001$ by either the unpaired Student's *t*-test or Mann-Whitney two-sample test.

and apoB cRNA probes showed good cross-reaction with mouse mRNA on Northern blots.

RESULTS

Body weights and hormone levels

Body weights of both castrated male and ovariectomized female mice were measured before and after estradiol or testosterone propionate administration (**Table 1**). Body weights did not change in female mice, but there were statistically significant differences in four of the eight groups in male mice that could not be attributed simply to hormonal administration since the placebo group received propylene glycol injections only. Serum estradiol and testosterone concentrations were comparable in both genders and mouse strains after castration. Administration of testosterone propionate increased testosterone levels significantly to mimic physiological ranges in the testosterone groups. Estradiol levels were increased more than 1.4-fold with low dose estradiol treatment (to physiologic ranges for females), and 50-fold or more with high dose treatment (Table 1).

Effects of hormones on plasma lipid, apoA-I, and apoB concentrations

Total cholesterol. Baseline total plasma cholesterol concentrations were 19% higher in male C3H/HeJ (205 mg/dl, placebo) than in female C3H/HeJ mice (172 mg/dl, placebo) and 14% higher in male C57BL/6J (142 mg/dl, placebo) than female C57BL/6J mice (125 mg/dl, placebo) (Table 1). Total cholesterol concentrations were variably affected by testosterone administration. In male C3H/HeJ and female C57BL/6J mice, there were no changes, while significant increases were seen in male C57BL/6J and significant decreases in female C3H/HeJ mice. Both low and high doses of estradiol decreased plasma total cholesterol by more than 33% in C3H/HeJ mice of either gender, while the effects were very small in either male or female C57BL/6J mice.

Testosterone. Lipoprotein profiles obtained by gel permeation chromatography on plasmas of mice from each strain and gender are shown in **Fig. 1.** Lipoprotein cholesterol was calculated from these profiles as indicated in the figure. Testosterone propionate altered plasma HDL-cholesterol concentrations to the same extent as total cholesterol depending upon the strain and gender. Therefore, HDL-cholesterol to total cholesterol ratios remained constant (**Table 2**). On the other hand, plasma apoA-I concentrations were not altered significantly by testosterone in either strain or gender, so that HDL-cholesterol to apoA-I mass ratios changed in the same direction as HDL-cholesterol and total cholesterol. Mean HDL diameters remained the same in both genders of C57BL/6J mice, while diameters decreased in both genders of C3H mice (from 11.3 to 11.0 for males and 11.3 to 11.1 for females). The size distribution of the HDL particles was also altered in C3H mice (Table 2 and **Fig. 2**).

Testosterone treatment significantly increased plasma apoB concentrations (by 1.2- and 1.5-fold, respectively) in males of both strains, but apoB did not change in females. Moreover, LDL cholesterol to total cholesterol ratios remained stable for both strains and both genders.

Estradiol. Estradiol treatment reduced total cholesterol by more than 1.4-fold in C3H mice while smaller changes were observed in C57BL mice. However, HDL-cholesterol concentrations and HDL-cholesterol/total cholesterol ratios decreased in both strains and both genders. The effects were considerably greater in C3H mice (Table 2 and Fig. 1). Plasma apoA-I concentrations decreased significantly in males of both strains, but not in parallel with HDL cholesterol. Thus, HDL-cholesterol to apoA-I mass ratios decreased in male C3H mice, but increased in male C57BL mice. ApoA-I and HDL-cholesterol ratios changed in the same direction in females, as in the respective males, but the changes were much smaller in magnitude. It should be noted that plasma apoA-I concentrations in both strains of male mice were significantly correlated with the total plasma cholesterol concentrations ($r = 0.86$, $P < 0.001$ and $r = 0.77$, $P < 0.001$), and HDL-cholesterol concentrations ($r = 0.92$, $P < 0.01$ and $r = 0.99$, $P < 0.001$ for C3H/HeJ and C57BL/6J mice, respectively).

Diameters of HDL particles in C3H mice diminished after estradiol treatment from 11.3 nm to 10.7 nm and 11.3 nm to 10.8 nm for males and females, respectively, while the respective diameters of HDL in C57BL mice were unchanged at 10.5 nm and 10.6 nm. There were estradiol-induced changes in the size distributions of HDL particles in both genders and strains (Fig. 2 and Table 2).

Low dose estradiol produced increases in LDL-cholesterol and apoB concentrations in C57BL males, while it raised LDL-cholesterol but reduced apoB in C3H males (see Table 2 and Fig. 1). LDL-cholesterol was increased by low-dose estradiol in females of both strains, but no significant changes in apoB were observed. High-dose estradiol increased apoB and LDL-cholesterol in both genders of both strains (Fig. 2, lanes 9 and 10, note the heavy bands near the tops of the gels that correspond to LDL), and LDL-cholesterol to total cholesterol ratios increased in all groups (Table 2). There were no significant correlations between apoB and total plasma cholesterol concentrations, or between apoB and LDL-cholesterol concentrations (data not shown). The two-dose estradiol experiment was done twice, 6 months apart, in castrated C57BL males with compatible results (data not shown).

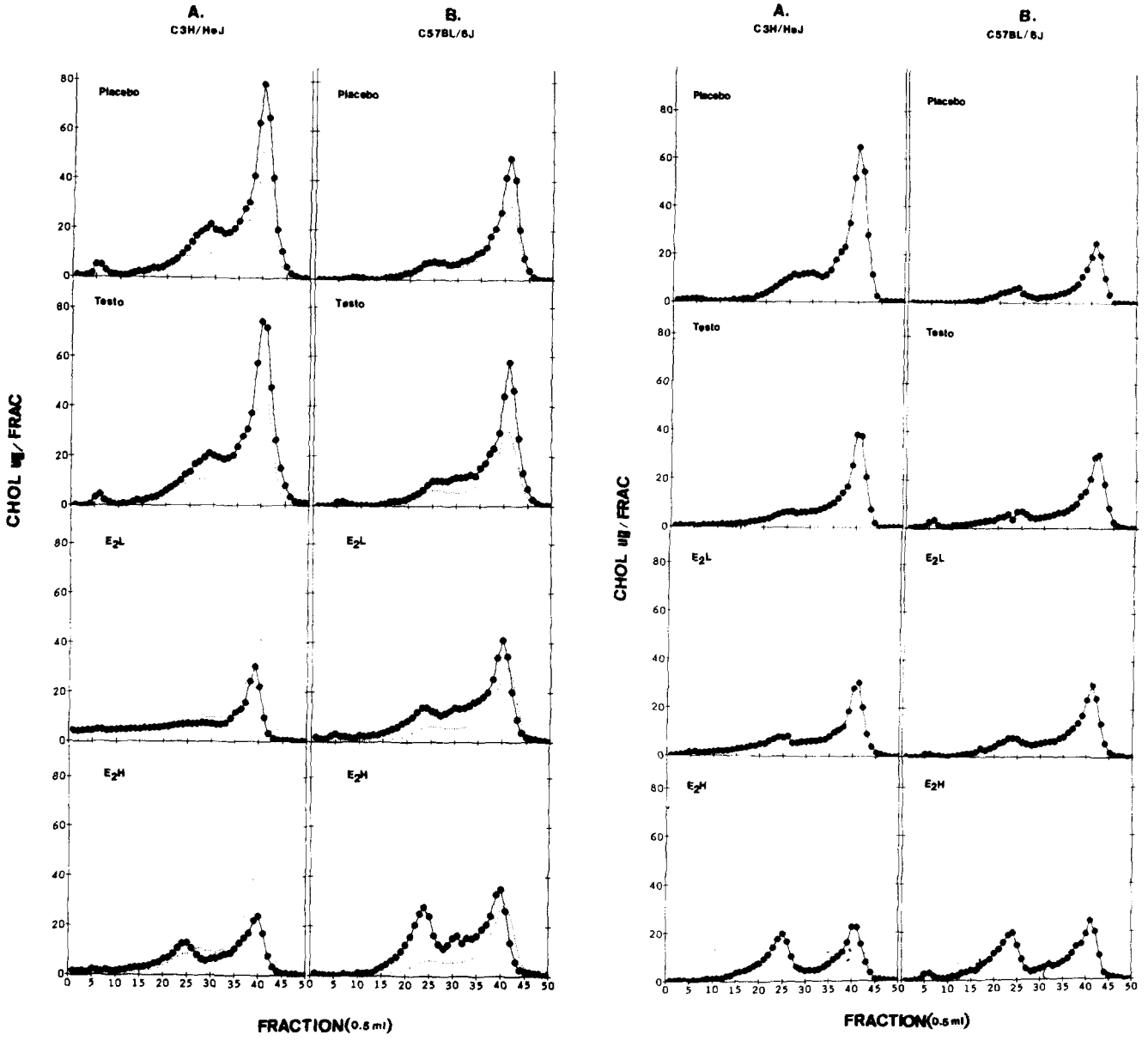

Fig. 1. Elution profiles of lipoproteins by gel permeation chromatography on Superose columns (FPLC system, Pharmacia); males, left and females, right. Pooled plasmas from each experimental group were applied to two Superose 6 columns attached in series as described in Methods. Lipoproteins were eluted at 0.5 ml/min with 1 mmol/l EDTA, 154 mmol/l NaCl, and 0.02% NaN_3 (pH 8.2). Fractions (0.5 ml) were used analyzed for cholesterol and values represent the µg of cholesterol per fraction. Isolated human lipoproteins were used to calibrate the columns. Peaks of human LDL and HDL eluted between fractions 25–30 and 42–46, respectively. Profiles of plasma cholesterol from untreated non-castrated female mice fed Purina Chow 5105 diet are shown by the dashed line in (A) for comparison (see reference 18). To calculate LDL- and HDL-cholesterol concentrations in mouse plasma, the cholesterol contents in fractions 16 through 32 and 33 through 50 were summed, respectively (see Table 2).

Hepatic apoA-I synthesis and its regulation

Testosterone. Hepatic apoA-I synthesis was increased 1.4-fold or more by testosterone treatment. This was true in both strains and both genders (**Table 3** and **Fig. 3**). (It should be noted that although synthesis rates for apoA-I were expressed as a percent of the total TCA-precipitable amino acid radioactivity, similar amounts (~40 mg) of liver tissue were used for each determination. Therefore, although it is possible that values for the absolute amount of apoA-I synthesized per mg tissue may be different than the values expressed as a percent of total protein synthesis, changes in apoA-I synthesis after hormone treatment relative to the placebo controls would still hold.) While hepatic apoA-I mRNA concentrations remained unchanged or decreased slightly, apoA-I transcription rates were significantly increased by 21–75% (except in female C57BL/6J mice). Thus, testosterone influences both tran-

TABLE 2. Effects of testosterone and estradiol administration on plasma HDL- and LDL-cholesterol and apolipoprotein A-I and apolipoprotein B concentrations of castrated male and female mice

Strains and Groups	TC	HDL-C	HDL-C[a] / TC	ApoA-I	HDL-C[a] / ApoA-I	HDL Dia.	LDL-C[a] / TC	ApoB
	mg/dl	*mg/dl*		*mg/dl*		*nm*		*mg/dl*
Male								
C3H/HeJ								
Placebo	205 ± 22	135	0.66	161 ± 16	0.84	11.3	0.30	33 ± 2
Testo	208 ± 10	139	0.67	175 ± 15	0.79	11.0	0.30	39 ± 4*
E2L	113 ± 12**	51	0.45	112 ± 13**	0.46	10.7	0.34	22 ± 4*
E2H	109 ± 13**	53	0.49	82 ± 7	0.65	10.7	0.40	65 ± 7**
C57BL/6J								
Placebo	142 ± 14	105	0.74	164 ± 13	0.64	10.5	0.24	30 ± 2
Testo	184 ± 6*	132	0.72	174 ± 18	0.76	10.5	0.26	44 ± 5**
E2L	157 ± 11	84	0.54	107 ± 9**	0.79	10.5	0.38	42 ± 4**
E2H	149 ± 37	70	0.47	95 ± 21**	0.74	10.5	0.50	82 ± 14**
Female								
C3H/HeJ								
Placebo	172 ± 28	121	0.70	141 ± 14	0.86	11.3	0.28	22 ± 3
Testo	125 ± 12**	84	0.68	120 ± 11*	0.70	11.1	0.28	25 ± 7
E2L	129 ± 12**	75	0.58	119 ± 13*	0.63	10.8	0.34	27 ± 6
E2H	125 ± 17**	57	0.46	79 ± 6**	0.72	10.8	0.50	72 ± 8**
C57BL/6J								
Placebo	125 ± 8	84	0.69	136 ± 34	0.62	10.6	0.30	36 ± 13
Testo	116 ± 13	74	0.64	124 ± 25	0.60	10.6	0.28	31 ± 5
E2L	111 ± 4*	71	0.64	134 ± 34	0.53	10.6	0.33	30 ± 2
E2H	137 ± 10	61	0.44	95 ± 13*	0.64	10.6	0.47	65 ± 5**

Plasma total cholesterol (TC), apoA-I, and apoB concentrations were determined as described in Methods. HDL diameters were determined by non-denaturing gradient gel electrophoresis as described in Methods. HDL-cholesterol (HDL-C) and LDL-C concentrations were calculated by summing the cholesterol contents of the appropriate column fractions after chromatography of mouse plasma on the FPLC system (see Methods and the legend to Fig. 1). Pools of plasma consisting of equal volumes derived from each of five animals per group were subjected to FPLC. Therefore, HDL-C and LDL-C concentrations consist of single determinations per experimental group. Data represent means ± standard deviations from five mice in each group; *, $P < 0.05$; **, $P < 0.001$ by either unpaired Student's *t*-test or Mann-Whitney two-sample test.

[a]*Results are given as mass ratios.*

scriptional and translational mechanisms in apoA-I gene expression. The increased apoA-I synthesis probably resulted from increased translation. However, simultaneously there must have been an increase in apoA-I catabolism from plasma to account for the unchanged plasma apoA-I concentrations noted in the testosterone-treated animals (Table 2).

Estradiol. In livers of estradiol-treated males apoA-I synthesis was decreased, e.g., to 36% and 11% of placebo in the E2L and E2H C3H/HeJ groups and to 29% and 7% in the E2L and E2H C57BL/6J groups, respectively (Table 3). However, hepatic apoA-I mRNA levels showed relatively little change. Transcription rates increased in C3H males and decreased in C57BL males (Table 3). In female mice of both strains, on the other hand, low dose estrogen increased apoA-I synthesis rates by more than 1.9-fold and apoA-I transcription rates increased as well, while mRNA concentrations decreased slightly (Table 3). Large doses of estrogen in female mice decreased apoA-I synthesis (as was also seen in the males), while transcription rates were increased and the mRNA levels remained unchanged (Table 3). Thus, regulation of hepatic apoA-I gene expression by estradiol is complex and occurs via both transcriptional and post-transcriptional mechanisms. Despite an increase in apoA-I synthesis by hepatic tissue in some cases, gradually decreasing plasma levels of apoA-I in mice of both genders indicates increased catabolism of apoA-I in these animals after estradiol administration. Since an appreciable proportion of plasma apoA-I arises from the intestine, intestinal mRNA concentrations were also measured to see whether changes in intestinal apoA-I mRNA could account for the reductions in plasma apoA-I concentrations. No significant estradiol-related changes were noted (data not shown).

Hepatic apoB mRNA and LDL-R mRNA levels

Since plasma concentrations of apoB were affected by administration of the hormones, hepatic apoB and LDL-R mRNA levels were measured. Hepatic LDL-R mRNA and apoB mRNA levels were not changed by testosterone treatment in any of the mice with the exception of a 36% decrease in apoB mRNA in C57BL male mice (**Table 4**). In males, both low and high doses of estrogen increased apoB mRNA concentrations ~2-fold, while LDL-R mRNA levels remained unchanged or increased by <50%

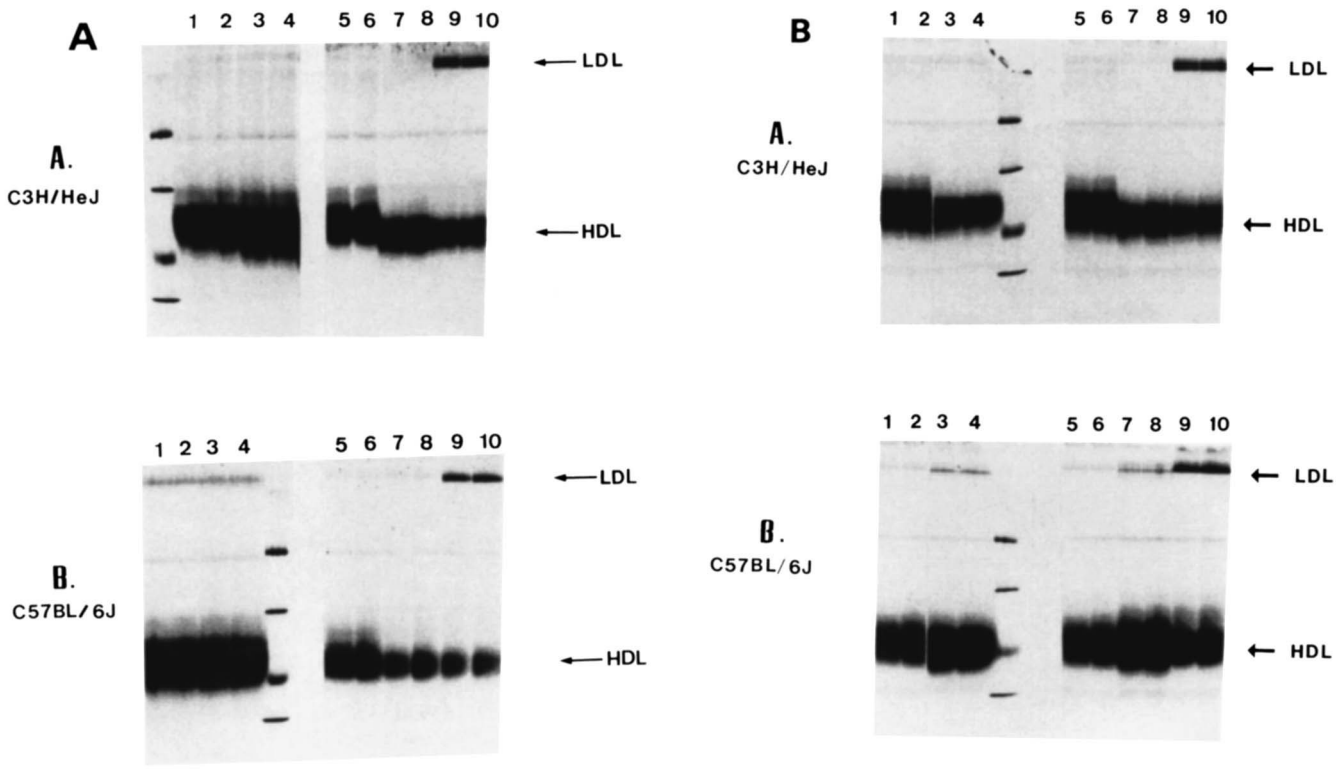

Fig. 2. Nondenaturing gradient gel electrophoresis of the d<1.21 g/ml fraction of mouse plasma. Ten μl of the d<1.21 g/ml fractions of pooled plasmas from each mouse group was applied to 4–30% polyacrylamide gradient gels. Reference proteins were run as size calibrators (unmarked lanes). Thyroglobulin and BSA were added to each sample as internal standards. Single plasma pool samples from each group were run in duplicate. A, castrated male mice; B, ovariectomized female mice. Lanes 1, 2, 5, and 6, placebo; lanes 3 and 4, TESTO; lanes 7 and 8, E2L; lanes 9 and 10, E2H.

(Table 4). In female mice, by contrast, both LDL-R and apoB mRNA concentrations remained unchanged in the E2L an E2H groups.

Liver cholesterol concentrations also affect apoB production and LDL-R activity (35). Therefore, liver total cholesterol concentrations were determined for individual livers from all animals, using gas chromatography with 5α-cholestane as internal standard according to the method of Ishikawa et al. (36). There were no significant differences noted between the treatment groups, strains or genders (data not shown).

Comparison of LDL-R activities between estrogen-treated rat and mouse

Large doses of 17α-ethinyl estradiol administered to rats are known to produce large decreases in the concentrations of all plasma lipoproteins and apolipoproteins. This is associated with increases in LDL-R activity. While in response to estradiol, HDL-cholesterol and apoA-I fell in some mice, apoB and LDL did not. In fact, plasma apoB concentrations rose in the E2H groups. In order to assess whether the mouse LDL-R responds differently to estrogen treatment than the rat LDL-R, a comparison experiment was carried out wherein 17α-ethinyl estradiol or 17β-estradiol were injected in large doses (5 μg/g/day) into both species. Total cholesterol levels fell by ~70% in the rats and remained unchanged in mice. (Note these were not castrates.) In rat, LDL-R protein was increased with 17α-ethinyl estradiol and 17β-estradiol treatments by 2.7- and 3.1-fold, respectively, while mouse LDL-R activity was not up-regulated (**Fig. 4** and **Table 5**). LDL-R mRNA levels were not strikingly increased in either rat or mouse.

DISCUSSION

The C3H/HeJ and C57BL/6J mice used in the present study were chosen because they have different genetic backgrounds and susceptibilities to diet-induced atherosclerosis. Thus, we could test whether genetic factors that affect susceptibility to atherogenesis had any influence on responsiveness to sex steroids. We used castrated animals to simplify the interpretation of the data, because we found in preliminary experiments that castration per se affected lipid levels and apoA-I regulation (data not shown). Since castration affects sex hormone metabolism and the pituitary-gonadal axis in a complex way, we felt the interpretation of the results of castration would be complicated, whereas the results of replacement or "cross

TABLE 3. Effects of testosterone and estradiol administration on hepatic apoA-I synthesis, mRNA concentrations, and transcription rates of castrated male and female mice

Strains and Groups	Hepatic Synthesis (% of Placebo)	mRNA Concentrations (pg A-I mRNA/μg Total RNA)	Transcription Rates (% of Placebo)
Male			
C3H/HeJ			
Placebo	100 ± 7	20.6 ± 4.5	100 ± 10
Testo	137 ± 7**	20.1 ± 1.5	144 ± 6
E2L	36 ± 4**	28.3 ± 3.3	110 ± 7
E2H	11 ± 1**	24.6 ± 2.0	188 ± 5**
C57BL/6J			
Placebo	100 ± 8	24.0 ± 1.1	100 ± 10
Testo	249 ± 11**	21.0 ± 2.3	175 ± 12**
E2L	29 ± 2**	21.7 ± 1.2	70 ± 9*
E2H	7 ± 1**	27.4 ± 1.6*	89 ± 6
Female			
C3H/HeJ			
Placebo	100 ± 16	16.9 ± 1.9	100 ± 10
Testo	181 ± 19**	13.2 ± 1.3*	121 ± 4*
E2L	222 ± 9**	12.8 ± 0.6*	150 ± 12**
E2H	53 ± 6**	14.2 ± 2.0	177 ± 14**
C57BL/6J			
Placebo	100 ± 14	15.6 ± 1.5	100 ± 14
Testo	269 ± 26**	17.6 ± 4.1	71 ± 10*
E2L	186 ± 40*	10.8 ± 1.6*	146 ± 4**
E2H	74 ± 7*	12.9 ± 3.0	169 ± 10**

Hepatic apoA-I synthesis rates, mRNA concentrations, and apoA-I mRNA transcription rates were determined as described in Methods. Hepatic synthesis rates were determined on pooled liver slices from five mice in each group. Values represent the means ± standard deviations of triplicates, expressed as a percent of the values obtained in placebo (100%). ApoA-I mRNA concentrations were determined by RNA-excess solution hybridization and transcription rates were determined in vitro as described in Methods. To measure apoA-I mRNA concentrations and transcription rates, livers were pooled in each mouse group and analyses were performed in triplicate. The transcription rates are expressed as a percent of the values obtained in placebo (100%); *, $P < 0.05$; **, $P < 0.001$ by either unpaired Student's *t*-test or Mann-Whitney two-sample test.

hormonal" therapy of castrates could be attributed to direct or secondary effects of the administered hormones themselves.

The strains differed even after castration in their total plasma cholesterol, HDL-cholesterol, apoA-I concentrations, and in HDL size distributions (Table 2 and Fig. 1). There were also differences in these parameters by gender. These gender- and strain-related differences were similar in direction to those seen in intact animals (18–22). Studies were conducted on atherosclerotic lesions and HDL-lipid levels in male, female and testosterone-treated female mice from strains C57BL, Balb/c, and C3H by

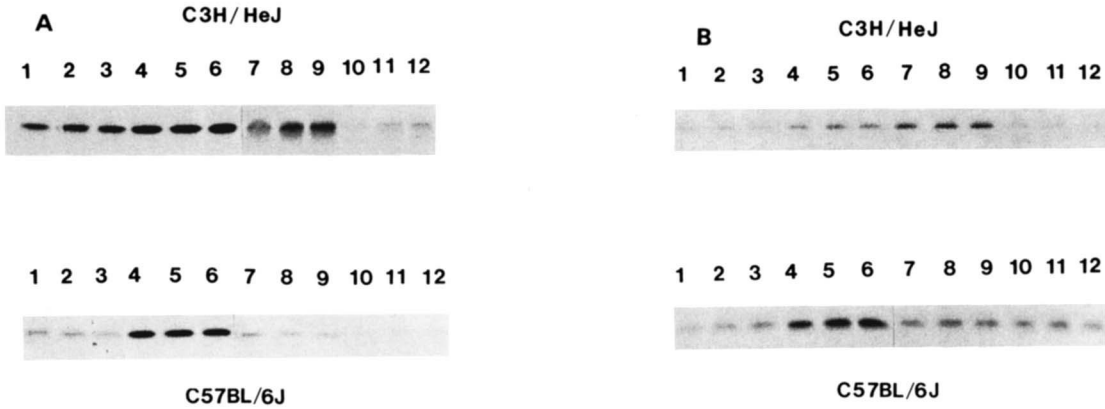

Fig. 3. Hepatic synthesis of apoA-I. A, results for males; B, results for females. Forty mg of pooled liver slices from each mouse group was incubated for 1 h at 37°C in 200 μl KBR containing 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1% glucose, and 200 μCi [^{35}S]methionine under an atmosphere of 95% O_2/5% CO_2 as described in Methods. The liver slices were homogenized and the homogenate was spun at 112,000 *g* to obtain the high-speed supernatant (HSS). Immunoprecipitation of apoA-I was performed as described in Methods. The immunoprecipitates were run on 10% SDS polyacrylamide gels. After autoradiography of the dried gels, the film was scanned. ApoA-I synthesis was expressed as the area under the apoA-I peaks (cm^2)/total protein synthesis (cpm). Total protein synthesis was assessed by TCA precipitation. Pooled samples from each incubation were run in triplicate on the gels. The mean values for apoA-I synthesis (cm^2/cpm) ± standard deviations for each mouse group are expressed as a percent of the value obtained for the placebo mouse group (100%). Lanes 1, 2, 3, placebo; lanes 4, 5, 6, testo; lanes 7, 8, 0, E2L; lanes 10, 11, 12, E2H.

TABLE 4. Effects of testosterone and estradiol administration on hepatic apoB and LDL-R mRNA concentrations of castrated male and female mice

Strains and Groups	ApoB mRNA Concentration (pg B mRNA/μg total RNA)	LDL-R mRNA Concentration (pg LDL-R mRNA/μg total RNA)
Male		
C3H/HeJ		
Placebo	133.0 ± 16.1	5.86 ± 0.57
Testo	117.7 ± 5.3	5.06[a]
E2L	251.3 ± 32.5**	7.41 ± 0.04*
E2H	256.4 ± 11.5**	7.51 ± 1.01
C57BL/6J		
Placebo	182.6 ± 2.5	6.21 ± 0.09
Testo	135.2 ± 30.5*	5.14[a]
E2L	216.2 ± 11.2**	5.77 ± 1.35
E2H	270.1 ± 8.0**	7.43 ± 0.28**
Female		
C3H/HeJ		
Placebo	59.4 ± 12.4	6.7 ± 1.7
Testo	62.4 ± 9.4	6.6 ± 1.5
E2L	54.1 ± 4.4	5.5 ± 2.8
E2H	41.2 ± 1.6*	6.2 ± 0.3
C57BL/6J		
Placebo	73.7 ± 10.1	6.5 ± 0.9
Testo	60.4 ± 4.7	9.7 ± 1.9
E2L	49.8 ± 4.2*	5.1 ± 0.7
E2H	59.5 ± 4.2*	5.9 ± 1.3

Hepatic apoB, LDL-R mRNA concentrations were determined as described in Methods by RNA-excess solution hybridization assays. Livers were pooled in each mouse group and analyses were performed in triplicate. Values represent the means ± standard deviations of the triplicate; *, $P < 0.05$; **, $P < 0.001$ by either unpaired Student's *t*-test or Mann-Whitney two-sample test.

[a]Indicates the single datum point due to lack of the RNA sample.

Paigen et al. (37); they show that higher levels of testosterone are associated with higher HDL-lipid levels and reduced risk of heart disease. They also reported that male mice have more HDL-lipid than the females.

In the present study, testosterone in castrated males raised HDL-cholesterol slightly and to a differing extent than apoA-I levels, whereas in castrated females HDL-cholesterol and apoA-I levels tended to fall (Table 2, Fig. 1). Because the changes in HDL-cholesterol and apoA-I were disproportionate, HDL-C/apoA-I ratios changed, falling in C3H and rising or remaining the same in C57BL. HDL mean diameters fell and size heterogeneity was changed in C3H but not in C57BL (Fig. 2). Thus, there were differences in the responses of lipoproteins to testosterone, both by strain and by gender.

Testosterone increased the hepatic synthesis of apoA-I in all groups, however, to different extents (Fig. 3, Table 3). Since plasma concentrations of apoA-I did not reflect changes in synthetic rates, either secretion from hepatocytes did not keep pace with synthesis, i.e., there was increased intracellular degradation, or there was enhanced clearance of apoA-I from plasma. The dissociation between rates of synthesis and plasma concentrations has been noted before in other animals (4).

Regulation of synthesis by testosterone is complex. Increases in apoA-I mRNA transcription rates matched changes in synthesis in direction, but they were smaller in magnitude, and apoA-I mRNA steady-state levels were not significantly changed, suggesting that the hormone affects the apoA-I production sequence at more than one site.

We chose both physiological (E2L) and pharmacological (E2H) doses of 17β-estradiol for the hormone treatment. In contrast with testosterone, estradiol uniformly decreased HDL-cholesterol and apoA-I concentrations (Tables 2). Lipoprotein profiles of males came to resemble

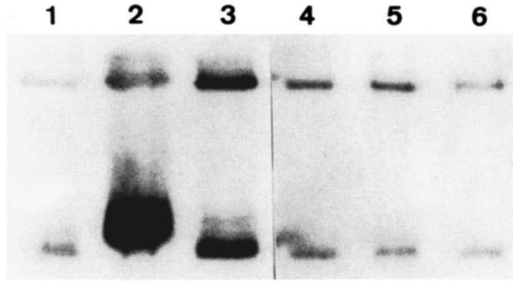

Fig. 4. LDL-receptor protein determination by Western blotting using a rabbit polyclonal anti-bovine adrenal LDL-R antibody. Two hundred μg of total liver membrane protein was loaded onto each lane, as described in Methods. The upper band represents the mature LDL-R, while the lower band is a precursor of the LDL-R, which also cross-reacts with the antibody. Lane 1, rat placebo; lane 2, rat 17α-EE; lane 3, rat 17β-E2; lane 4, mouse placebo; lane 5, mouse 17α-EE; lane 6, mouse 17β-E2.

TABLE 5. Effect of 17α-ethinyl estradiol and 17β-estradiol administration on hepatic LDL-R protein and mRNA levels in intact male rats and mice

Groups and Treatments	LDL-R Protein (% of control)	LDL-R mRNA Concentrations (pg LDL-R mRNA/μg total RNA)
Rats (Sprague-Dawley)		
Control	100	7.1 ± 0.6
17α-EE	272	6.5 ± 0.7
17β-E2	301	6.9 ± 0.5
Mice (C3H/HeJ)		
Control	100	7.9 ± 0.7
17α-EE	93	11.3 ± 0.4*
17β-E2	83	7.9 ± 0.2

Rat and mouse hepatic membrane LDL-R proteins were quantitated on an autoradiograph as described in Methods after 6 days of estrogen administration. Hepatic LDL-R mRNA concentrations of rat and mouse were determined by RNA-excess solution hybridization assays as described in Methods. Livers were pooled in each group. Concentrations of LDL-R mRNA represent the means ± standard deviations of triplicates; *, $P < 0.05$ by unpaired Student's t-test.

those of females (Fig. 1, left). The C57BL strain seemed to be more resistant to this effect of estradiol than C3H, and females were more resistant than males, with C57BL females being the most resistant. As in the case of testosterone, the effects on HDL-cholesterol and apoA-I were not proportionate for the various groups, resulting in characteristic HDL-C/apoA-I ratios.

ApoA-I synthesis fell significantly in both groups of males on both doses of estradiol (Table 3). In females, the lower doses produced increases in synthetic rates (Table 1), and only the high dose caused reductions. Thus, as in the case of plasma concentrations, females responded differently than males after estradiol treatment. Regulation of apoA-I production by estrogen also is complex. Transcription rates, mRNA steady state levels, and synthesis rates did not match each other either in direction or magnitude, and there were differences by strain and gender. This suggests that estrogen also affects the apoA-I production sequence at more than one site.

Rats and rabbits treated with large doses of 17α-ethinyl estradiol experience decreases in all their plasma lipoproteins probably due to up-regulation of their hepatic LDL-receptors (32, 38). Thus, the 17β-estradiol-induced decreases in mice plasma apoA-I and HDL-cholesterol may have been explainable by a hormone-induced up-regulation of LDL-receptors, leading to an enhanced clearance of HDL (particularly that fraction containing apoE) from plasma. However, mice LDL-cholesterol and apoB concentrations either did not change or rose with low dose estradiol, and apoB rose dramatically in all groups on high-dose estradiol. To explain these apparently anomalous findings, we examined the regulation of hepatic apoB and LDL-receptors in these animals.

In response to 17β-estradiol, apoB mRNA levels were increased in males and unchanged or slightly decreased in females (Table 4). Thus, changes in apoB mRNA could not explain the rise in plasma apoB concentrations. LDL-receptor mRNA levels rose slightly in males and did not change in females, suggesting that LDL-receptors may not be as susceptible to estradiol-induced up-regulation in mice as they are in rats. To test this possibility more directly, a comparison experiment was carried out between intact (non-castrated) rats and mice (Table 5). The up-regulation of LDL-receptor protein in rat by large doses of either 17α-ethinyl estradiol or 17β-estradiol was confirmed as protein increased by ~3-fold. In mice, neither estrogen compound produced an up-regulation, as LDL-receptor protein did not increase. Whether the rat/mouse differences are due to differences in the LDL-receptor gene, in cellular estrogen receptors, or in the metabolism of estradiol is not known, but these findings do provide a rationale for the absence of 17β-estradiol-induced decreases in apoB-containing lipoproteins in mice, but they leave unanswered the mechanism by which decreases in plasma apoA-I and HDL-cholesterol were produced by estrogen. Perhaps other receptors such as the LDL-receptor-related protein or the HDL-receptors were up-regulated by estrogen, or as mentioned, apoA-I secretion or catabolism may not reflect rates of hepatic synthesis.

Due to the small amounts of samples available in mice, data in Tables 3 and 4 were derived from testing the pooled samples in triplicate and therefore the standard deviations do not describe the population variance but rather the measurement variance. Therefore, we repeated the E2L and E2H experiments using male and female C57BL/6J mice. Changes compatible with those presented were obtained (data not shown).

The above results obtained with mice differ from those seen in other species. For example, in post-menopausal women, unopposed estrogen treatment produces elevations in plasma VLDL and HDL concentrations and decreased LDL concentrations (39), which may be accounted for by enhanced hepatic production of VLDL

and HDL. In addition, estradiol produces marked reductions in the activity of HTGL (hepatic triglyceride lipase) with little or no change in LPL (lipoprotein lipase) activity (39). Since HTGL possesses phospholipase activity that catalyzes the hydrolysis of HDL-phospholipids which in turn destabilizes HDL and promotes its catabolism, estrogen-induced inhibition of HTGL activity in humans may stabilize HDL particles and also contribute to the rise in plasma HDL concentrations. Reduced LDL concentrations are attributed to estrogen-induced increases in hepatic LDL-receptors (40), and this has been well documented in rabbits and rats treated with large doses of ethinyl estradiol as mentioned above. Androgens, in general, produce effects opposite to those produced by estrogens, but there is less available information on androgen effects. The effects of sex hormones in mice on the lipase enzymes remains to be determined.

Rats respond differently to estradiol treatment than do humans. For example, in contrast with humans, replacement doses of estrogens given to castrated female rats result in reductions in total triglyceride, cholesterol, and apoA-I, while HDL concentrations are unaffected (41). In castrated male rats, estrogens in doses meant to mimic female plasma concentrations of estradiol produce decreases in VLDL and increases in HDL concentration (41). Supraphysiologic doses of 17α-ethinyl estradiol drastically lower plasma concentrations of all lipoprotein classes probably due to up-regulation of the LDL-receptor. Clearly, the lipoprotein responses of mice to sex steroid administration appear to differ from both humans and rats as is seen in this report. Whether these species differences are due to differences in the metabolism of sex hormones or the regulation of the genes in question remains to be elucidated.

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