Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 792–796, February 1986
Medical Sciences

Increased mRNA for low density lipoprotein receptor in livers of rabbits treated with 17α-ethinyl estradiol

(S1 nuclease assay/regulation of cell surface receptors/cholesterol metabolism)

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ABSTRACT **Pharmacologic doses of 17α-ethinyl estradiol are known to increase the number of low density lipoprotein (LDL) receptors in livers of rats, thereby producing a profound fall in plasma cholesterol levels. We now report that ethinyl estradiol exerts the same effect in livers of male and female rabbits and that the increase in receptor number is correlated with a 6- to 8-fold increase in the levels of receptor mRNA. Receptor protein was measured by ligand blotting, and mRNA levels were measured by a quantitative solution hybridization/S1 nuclease protection assay using uniformly ^{32}P-labeled single-stranded cDNA probes. These experiments demonstrate that pharmacologic induction of the mRNA for the LDL receptor in liver can lead to increased LDL receptor levels and a fall in plasma cholesterol in experimental animals.**

Regulation of the activity of the low density lipoprotein (LDL) receptor in liver constitutes a potential mechanism by which dietary and hormonal agents may alter plasma cholesterol levels (1). LDL receptors remove LDL and its precursor, intermediate density lipoprotein (IDL), from plasma (2). In rodent species (rabbits, hamsters, and rats), approximately 70% of the body's LDL receptors are expressed on liver cells (3–6). When these receptors are reduced in number, lipoprotein-bound cholesterol accumulates in plasma (7–10); conversely, an increase in hepatic receptors profoundly lowers plasma cholesterol levels (11–13).

In tissue culture cells such as human fibroblasts, the number of LDL receptors is dictated by the amount of mRNA for the receptor (14). When cultured cells have an overabundance of cholesterol, the amount of receptor mRNA declines, the synthesis of LDL receptors is reduced, and the uptake of LDL is diminished (1, 14). Conversely, when cultured cells have an increased requirement for cholesterol, they produce increased amounts of LDL receptor mRNA (14). No quantitative estimates of the mRNA for the LDL receptor in animal livers have been made. Whether changes in mRNA levels underlie regulation of the LDL receptor in the liver is also unknown.

A dramatic induction of LDL receptor activity occurs in livers of rats treated with pharmacologic doses of 17α-ethinyl estradiol (11, 12). This demonstration followed the observation of Hay *et al.* (15), who showed that pharmacologic doses of ethinyl estradiol lead to a profound drop in the total plasma cholesterol level in rats. Kovanen *et al.* (11) and Chao *et al.* (12) showed that the fall in cholesterol was attributable to a marked increase in the number of LDL receptors in the liver, with consequent rapid clearance of lipoproteins from plasma.

The present studies were designed to reveal whether high doses of ethinyl estradiol raise hepatic LDL receptor levels by producing an increase in the amount of LDL receptor mRNA. We turned from the rat to the rabbit because of the availability of a cDNA probe for the rabbit LDL receptor (T.Y., M.S.B., J.L.G., and D. W. Russell, unpublished data). Kushwaha and Hazzard have shown that estrogens decrease the plasma cholesterol level in cholesterol-fed rabbits (16) and enhance the uptake of apolipoprotein E-containing very low density lipoproteins (VLDL) into rabbit liver (17). Whether this effect is due to an enhancement of LDL receptor levels in rabbits is unknown. We here use our autologous ^{32}P-labeled cDNA to measure the amounts of mRNA for the LDL receptor in livers of rabbits that have been treated with pharmacologic doses of ethinyl estradiol. The results indicate that ethinyl estradiol does lead to a marked increase in the mRNA for the LDL receptor in rabbit liver and that this increase correlates with the increase in LDL receptor protein observed in these animals.

METHODS

Materials. 17α-Ethinyl estradiol (catalog no. E-4876, lot 1240-0269) and propylene glycol were obtained from Sigma. β-migrating VLDL (β-VLDL) was isolated from the plasma of cholesterol-fed rabbits and radiolabeled with ^{125}I (7). Other materials were obtained from previously reported sources (14, 18–20).

Rabbits. New Zealand White rabbits (1.5–1.8 kg, 5–7 weeks old) were obtained from Hickory Hill Rabbitry (Flint, TX) and fed Purina Rabbit Laboratory Chow. All rabbits were exposed to 12 hr of light (6 a.m. to 6 p.m.) and 12 hr of darkness (6 p.m. to 6 a.m.) daily for 2 weeks prior to use.

Estradiol Administration. 17α-Ethinyl estradiol was dissolved in propylene glycol (10 mg/ml) at 37°C and administered daily at 8 a.m. to rabbits subcutaneously in a final volume of 1 ml with dosages ranging from 0.1 to 5 mg/kg of body weight. Control rabbits were injected with propylene glycol only.

Ligand Blotting of LDL Receptors. LDL receptors were quantified by ligand blotting with ^{125}I-labeled β-VLDL (^{125}I-β-VLDL) as previously described (20) with minor modifications. A 100,000 × *g* supernatant fraction of Triton X-100-solubilized liver membranes (540 μg of protein per lane) was subjected to electrophoresis on a 7% polyacrylamide gel containing 0.1% $NaDodSO_4$ in the absence of reducing agents. The proteins were then transferred electrophoretically to nitrocellulose paper and incubated for 1 hr at 37°C with ^{125}I-β-VLDL (≈5 × 10^5 cpm/μg of protein) at 2.5 μg of protein per ml in buffer A [50 mM Tris·HCl/2 mM $CaCl_2$/80 mM NaCl/5% (wt/vol) Carnation nonfat dry milk at pH 8] (21). The paper was washed with buffer A, rinsed with buffer containing 50 mM Tris·HCl and 2 mM $CaCl_2$ at pH 8, dried, and subjected to autoradiography (20). Molecular weight calibration was carried out as described (20).

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Abbreviations: LDL, low density lipoprotein(s); VLDL, very low density lipoprotein(s); β-VLDL, β-migrating VLDL; HDL, high density lipoprotein(s).

Blot Hybridization of Poly(A)$^+$ RNA. Total RNA was isolated from rabbit liver by extraction with guanidinium isothiocyanate (22). Poly(A)$^+$ RNA was purified by oligo-(dT)-cellulose chromatography, denatured with glyoxal, size-fractionated by electrophoresis (20 V, 16 hr) on 1.5% agarose gels containing 40 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.0), transferred to Zeta-Probe membranes (Bio-Rad) in 20× NaCl/Cit (1× NaCl/Cit = 150 mM NaCl/15 mM sodium citrate), and prehybridized as described (23). Hybridization with a single-stranded ^{32}P-labeled rabbit LDL receptor probe was carried out at 42°C for 16 hr in a 50% (vol/vol) formamide solution containing 5× Denhardt's solution (23), 5× NaCl/Cit, 0.1% $NaDodSO_4$, denatured salmon sperm DNA at 100 μg/ml, and poly(A)$^+$ RNA at 1 μg/ml. The membranes were subsequently washed with 0.1× NaCl/Cit and 1% $NaDodSO_4$ for 75 min at 65°C, dried, and subjected to autoradiography (21). The ^{32}P-labeled rabbit cDNA probe was prepared from an M13 phage DNA template containing a 328-base-pair *Pst* I/*Pst* I fragment (see below) from the coding region of pLDLR-11, a rabbit LDL receptor cDNA. The details of the isolation of pLDLR-11 will be described elsewhere (T.Y., M.S.B., J.L.G., and D. W. Russell, unpublished data).

Measurement of LDL Receptor mRNA by S1 Nuclease Protection Assay. The amount of LDL receptor mRNA was measured by minor modification (18) of the DNA-excess solution hybridization/S1 nuclease protection method described by Newman *et al.* (24). Total RNA was isolated from rabbit liver by extraction with guanidinium isothiocyanate (22). An M13 DNA template was prepared by subcloning a 328-base-pair *Pst* I/*Pst* I fragment (sense strand) from the coding region of rabbit pLDLR-11 into the *Pst* I site of the M13mp18 vector. This M13 template was allowed to hybridize with a universal sequencing primer 17 nucleotides in length. This primer was then extended by incubation with 0.25 mM each dTTP, dATP, and dGTP; 44 μM [α-^{32}P]dCTP (specific activity ≈ 10^6 cpm/pmol); and the Klenow fragment of *Escherichia coli* DNA polymerase I. After *Eco*RI digestion, the single-stranded fragment was isolated by 7 M urea/5% polyacrylamide gel electrophoresis and hydroxylapatite chromatography (24). The single-stranded [^{32}P]cDNA fragment (4 fmol, ≈10^5 cpm/fmol) was hybridized with various amounts of total cellular RNA in 0.1 ml of 20 mM Tris·HCl, pH 7.8/20 mM EDTA/0.3 M NaCl/100 μg of denatured salmon sperm DNA per ml at 68°C for 60 hr. The sample was digested for 2 hr at 45°C with 3000 units of S1 nuclease (25), and nuclease-resistant hybrids were collected by precipitation with 7.5% trichloroacetic acid. Measured values for LDL receptor mRNA per unit of total cellular RNA were converted to mRNA copies per liver cell as described by Williams *et al.* (25), using the mean value of our own measurements of the ratio of RNA to DNA in rabbit liver (2.7:1) and using the figure of 5.5 pg per cell for the amount of DNA per diploid liver cell (24).

Other Assays. The cholesterol content of plasma lipoprotein fractions (19) and the protein content of membrane preparations (26) were measured by the indicated methods.

RESULTS

To quantify LDL receptor mRNA levels in rabbit liver by the S1 nuclease protection technique (24, 25), we prepared a uniformly ^{32}P-labeled single-stranded fragment of DNA that is complementary to 328 nucleotides from the coding region of the rabbit LDL receptor mRNA. Various amounts of total liver RNA were hybridized to completion with this probe. Excess unhybridized probe was digested with S1 nuclease, and the nuclease-resistant hybrids were precipitated with trichloroacetic acid, isolated by filtration, and subjected to scintillation counting. To determine the specific activity of the ^{32}P probe, we performed the same S1 nuclease digestion after hybridization of the probe with known amounts of the M13 template that had been used to prepare the probe. Hybridization with known amounts of this template gave a linear increase in S1 nuclease-resistant radioactive material (Fig. 1 *Inset*), and this allowed us to calculate the specific activity of the ^{32}P probe (≈10^5 cpm/fmol). This information permitted us to estimate the number of copies of LDL receptor mRNA per average liver cell (see *Methods*).

Fig. 1 shows the results of a representative S1 nuclease protection assay performed with liver RNA from a control rabbit and a rabbit treated with ethinyl estradiol for 10 days at 5 mg/kg. In both cases the amount of S1 nuclease-resistant acid-precipitable radioactive material increased linearly with increasing amounts of RNA added to the assay. The RNA from the treated animal gave an 8-fold higher amount of S1 nuclease-resistant hybrid, indicating that the amount of LDL receptor RNA was 8-fold higher in the liver of the treated animal as compared with the control animal. Polyacrylamide gel electrophoresis of the S1 nuclease-protected fragment showed that the entire cDNA insert was protected (data not shown).

Fig. 2 shows the results of receptor mRNA measurements in livers from groups of three rabbits that were treated for 10 days with various doses of ethinyl estradiol (*A*) or with ethinyl estradiol at 5 mg/kg for various numbers of days (*B*). The amount of LDL receptor mRNA in untreated animals

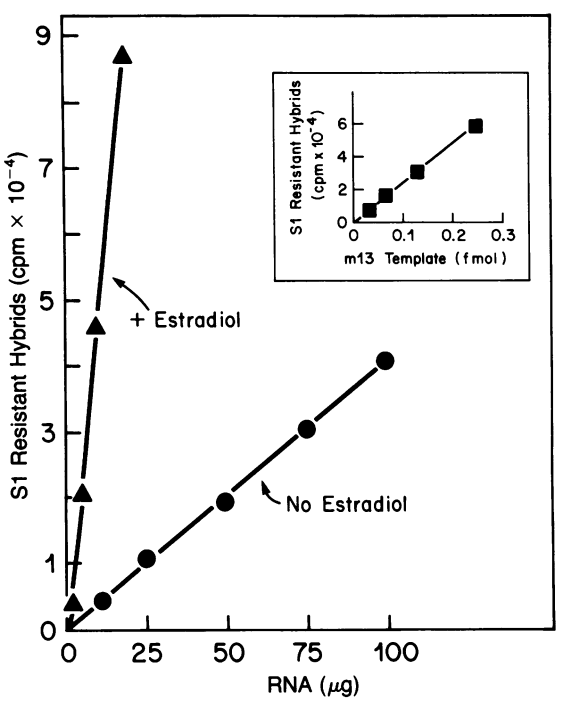

FIG. 1. Measurement of hepatic LDL receptor mRNA in untreated (●) and estradiol-treated (▲) male rabbits by the quantitative S1 nuclease technique. A daily dose of 5 mg/kg of 17α-ethinyl estradiol dissolved in propylene glycol was given subcutaneously to one rabbit for 10 days (▲). A second rabbit received daily subcutaneous injections of propylene glycol without estradiol for the same time (●). Various amounts of total liver RNA were hybridized with a single-stranded ^{32}P-labeled rabbit LDL receptor cDNA probe. The hybrids were subjected to S1 nuclease digestion, and the S1-resistant hybrids were collected by trichloroacetic acid precipitation and subjected to scintillation counting. (*Inset*) S1 nuclease-resistant hybrids formed between the ^{32}P probe and various amounts of the template DNA from which it was synthesized. A blank value of 0.4 × 10^4 cpm (representing the amount of S1-resistant radioactivity precipitated in the absence of mRNA or template) was subtracted from each value.

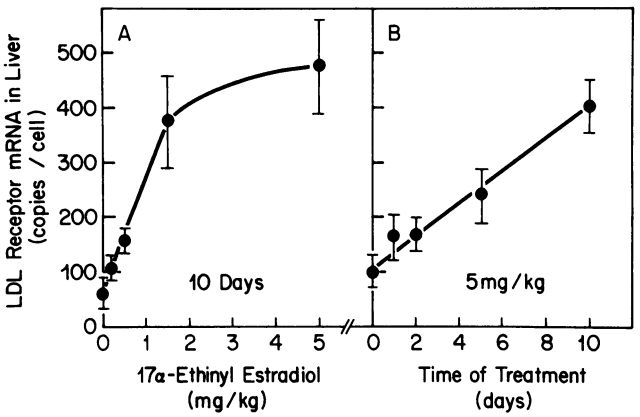

FIG. 2. Increase in hepatic LDL receptor mRNA in rabbits treated with estradiol. Male rabbits were treated subcutaneously for 10 days with the indicated daily dose of 17α-ethinyl estradiol in propylene glycol (*A*) or for the indicated time with estradiol at 5 mg/kg (*B*). After treatment, the rabbits were killed, and total cellular RNA was prepared from the liver. The concentration of LDL receptor mRNA in liver was measured by the standard S1 nuclease assay. Each value is the mean ± SEM of data from three rabbits.

averaged 80 copies per cell in the experiment of Fig. 2*A* and 109 copies per cell in Fig. 2*B*. At the 10-day time point, the lowest dose of ethinyl estradiol tested (0.1 mg/kg) produced a detectable increase in LDL receptor mRNA. The increase was almost maximal (≈6-fold) at a dose of 1.5 mg/kg. At the highest dose tested (5 mg/kg) there was a detectable increase in mRNA within 1 day, and the mRNA continued to rise in a linear fashion for 10 days (Fig. 2*B*).

We next tested the correlation between mRNA levels measured by the S1 nuclease protection technique and measured by blot hybridization. Pairs of rabbits were treated with ethinyl estradiol at 0, 5, or 10 mg/kg for 10 days. Total RNA was isolated from each liver and subjected to S1 nuclease analysis (Table 1). Another aliquot of RNA was fractionated by oligo(dT)-cellulose chromatography to yield poly(A)$^+$ mRNA, which was then subjected to agarose gel electrophoresis, transferred to blotting paper, and hybridized with the ^{32}P-labeled cDNA probe (Fig. 3). Various exposures of these autoradiograms were subjected to densitometric analysis, and the results are shown in Table 1. There it can be seen that the increase in mRNA as measured by the S1 nuclease assay (≈7-fold) was correlated with an increase in mRNA as determined by the densitometric scans of the blots (≈14-fold).

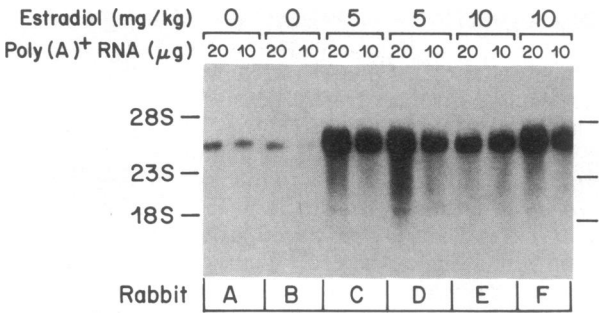

FIG. 3. Blot hybridization of ^{32}P-labeled LDL receptor cDNA to poly(A)$^+$ RNA from livers of rabbits treated with various amounts of estradiol. Male rabbits were treated subcutaneously with the indicated daily dose of 17α-ethinyl estradiol dissolved in propylene glycol for 10 days. Aliquots (20 and 10 μg) of poly(A)$^+$ RNA from liver were subjected to electrophoresis in agarose, transferred to Zeta-Probe membranes, and hybridized at 42°C for 16 hr with a ^{32}P-labeled single-stranded rabbit LDL receptor cDNA probe (10^9 cpm/μg, 2×10^6 cpm/ml). The filter was washed and exposed to x-ray film for 12 hr at −70°C. The positions to which standard 18S, 23S, and 28S ribosomal RNAs migrated are indicated. The results of densitometric scanning of various exposures of these blots are shown in Table 1. Rabbits A–F are the same animals as those in Fig. 4 and Table 1.

When the blot discussed above was reprobed with a ^{32}P-labeled plasmid containing a chicken actin cDNA insert of 1.8 kilobases (provided by Ray MacDonald of our institution), a signal corresponding to actin mRNA was detected in all lanes. No consistent differences in the intensities of the signal for actin mRNA were detected for the control (A and B) and estradiol-treated (C to F) rabbits in Fig. 3 (data not shown).

To determine whether the mRNA levels correlated with the amount of receptor protein, we subjected aliquots of the same six livers to centrifugation to isolate a total membrane fraction. The membranes were then solubilized with detergents, subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis, transferred to blotting paper, and incubated with ^{125}I-β-VLDL (Fig. 4). Under these conditions the ^{125}I-β-VLDL binds to the LDL receptor in direct proportion to the amounts of receptor present (20). Densitometric scanning of the gels in Fig. 4 showed an ≈6.5-fold increase in LDL receptors in the estradiol-treated animals (Table 1). This increase in receptor protein correlated with the quantitative estimates of mRNA for the LDL receptor in the same animals (Table 1).

Table 1. Increase in concentrations of LDL receptor mRNA and protein in livers of male rabbits treated with 17α-ethinyl estradiol

			LDL receptor mRNA		
Rabbit	Estradiol, mg/kg	Body weight, % of pretreatment	Blot hybridization assay, relative intensity	S1 nuclease assay, copies/liver cell	LDL receptor protein by ligand blot, relative intensity
A	0	119	1	63	1
B	0	106	0.25	21	1
			0.63	42	1
C	5	103	6.3	270	7
D	5	103	11	286	6
			8.7	278	6.5
E	10	97	6.9	218	7
F	10	97	11	258	6
			9.0	238	6.5

Normal male rabbits were treated subcutaneously with the indicated daily doses of estradiol in propylene glycol for 10 days as described in the legend to Fig. 3. The relative levels of LDL receptor mRNA were estimated by densitometric scanning of the blots in Fig. 3; the intensity of the band in rabbit A was assigned a value of 1. The relative levels of LDL receptor protein were estimated by densitometric scanning of the ligand blots in Fig. 4; the intensity of the band for rabbit A was assigned a value of 1. The concentration of LDL receptor mRNA (copies per liver cell) was measured by the standard S1 nuclease assay. Rabbits A–F refer to the same animals as those in Figs. 3 and 4.

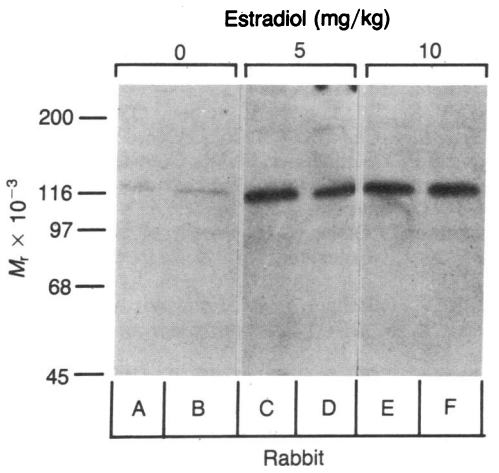

FIG. 4. Ligand blotting of LDL receptors from the livers of rabbits treated with various amounts of 17α-ethinyl estradiol. Detergent-solubilized extracts of liver membranes were prepared from the rabbits described for Fig. 3. The extracts were subjected to electrophoresis on $NaDodSO_4$/7% polyacrylamide gels under nonreducing conditions. After transfer to nitrocellulose paper, ligand blotting with rabbit ^{125}I-β-VLDL was carried out. The nitrocellulose paper was exposed to x-ray film at 20°C for 48 hr. M_r standards are indicated. The results of densitometric scanning of these blots are shown in Table 1. Rabbits A–F are the same animals as those in Fig. 3 and Table 1.

All of the above experiments were performed with male rabbits. To determine whether ethinyl estradiol also increased LDL receptor mRNA in female rabbits and to determine the effects of this agent on plasma lipoprotein levels in both sexes, we made the measurements shown in Table 2. In experiment A, lipoprotein cholesterol levels were measured in groups of male rabbits that were given various doses of ethinyl estradiol for 10 days. These rabbits are the same as the ones shown in the dose–response curve of Fig. 2*A*. In experiment B, female rabbits were treated with ethinyl estradiol at 5 mg/kg for 7 days. The females showed a 7-fold increase in LDL receptor mRNA that was similar to the increase observed in the males at the same dose of ethinyl estradiol. There was some scatter in the lipoprotein cholesterol measurements that could be attributed to the small number of animals in each group. Nevertheless, it is clear that in both sexes the ethinyl estradiol produced a marked reduction in total plasma cholesterol levels. This reduction affected LDL-cholesterol levels most significantly (approximately a 75% reduction in males and females), but it also affected HDL levels (approximately a 55% reduction in the two sexes).

During the course of these studies, we measured hepatic LDL receptor mRNA levels and simultaneous plasma cholesterol levels in a total of 16 untreated rabbits and 29 rabbits that were treated with various doses of ethinyl estradiol for various times (total number of rabbits = 45, including both males and females). Statistical analysis of these pooled data showed a significant negative correlation between LDL receptor mRNA levels and total plasma cholesterol levels. The highest correlation was obtained when the logarithms of the mRNA levels and cholesterol levels were compared. The correlation coefficient (r) between these two variables was −0.55 and the P value was less than 0.001.

DISCUSSION

The current data demonstrate that pharmacologic doses of ethinyl estradiol can produce a 6- to 8-fold elevation in the amount of mRNA for the LDL receptor in rabbit liver. This elevation appears to account for the increase in LDL receptor protein levels that follows administration of ethinyl estradiol.

The current studies in rabbits, as well as the previous ones in rats (12, 13), were not designed to determine whether ethinyl estradiol stimulates LDL receptors by virtue of its activity as an estrogen or to determine whether estrogens in general are physiologic regulators of LDL receptor mRNA levels. Rather, ethinyl estradiol was used in very high pharmacologic doses in a model system to determine whether alterations in LDL receptor mRNA levels can be detected in rabbits and to determine whether the mRNA levels correlate with increased amounts of LDL receptor protein. The data indicate that the quantitative S1 nuclease protection method is adequate to detect such changes in mRNA levels. This method has advantages over other methods based on densitometric scans of RNA blots in that large amounts of total RNA can be used, which permits detection of very low abundance mRNAs. Moreover, the data are sufficiently quantitative so that changes of less than 2-fold can be reliably measured.

The expression of mRNA levels in terms of copies per cell should be taken only as an approximation. This value represents an average value for all liver cells; in reality, the LDL receptor mRNA is likely to be distributed nonuniformly among different cell types. Moreover, the calculation is based on gross estimates of the amount of total RNA and DNA per liver cell. Nevertheless, expression of data in this fashion is valid for comparisons between experimental groups in which all calculations use the same set of assumptions.

It seems likely that increases in hepatic LDL receptor mRNA levels similar to those observed in this study may account for the fall in plasma cholesterol levels and increase in hepatic uptake of VLDL that was previously observed after estrogen administration to cholesterol-fed rabbits (16,

Table 2. Effect of 17α-ethinyl estradiol on LDL receptor mRNA in liver and on plasma cholesterol levels in male and female rabbits

Exp.	Sex	No. of animals	Estradiol treatment: Dose, mg/kg	Estradiol treatment: Duration, days	Body weight, % of pretreatment	Concentration of LDL receptor mRNA, copies/liver cell	Plasma cholesterol, mg/dl: Total	VLDL	LDL	HDL
A	Male	3	0	—	117 ± 1	80 ± 24	60 ± 9	3 ± 1	21 ± 2	27 ± 4
		3	0.1	10	111 ± 2	127 ± 24	24 ± 3	1 ± 0.2	8 ± 4	21 ± 3
		3	0.5	10	99 ± 11	168 ± 8	28 ± 8	1 ± 1	10 ± 8	16 ± 2
		3	1.5	10	106 ± 3	403 ± 81	12 ± 1	1 ± 0.9	0.2 ± 0.2	9 ± 1
		3	5	10	93 ± 2	497 ± 92	24 ± 3	1 ± 0.4	5 ± 1	12 ± 1
B	Female	5	0	—	104 ± 3	49 ± 13	71 ± 11	3 ± 2	37 ± 9	25 ± 3
		5	5	7	92 ± 3	364 ± 78	27 ± 6	2 ± 1	10 ± 4	11 ± 1

Rabbits were treated subcutaneously with the indicated dose of estradiol in propylene glycol for the indicated time. After treatment, the animals were killed, plasma was obtained, and total cellular RNA was prepared from the liver. The concentration of LDL receptor mRNA in liver was measured by the standard S1 nuclease assay. Each value is the mean ± SEM. HDL, high density lipoprotein.

17). However, VLDL from cholesterol-fed rabbits (so-called β-VLDL) can also enter liver through a separate receptor, the chylomicron remnant (6, 27). Whether or not estrogen affects the activity or number of chylomicron remnant receptors is unknown.

In the current studies in rabbits, as in the earlier studies in rats (11, 12), ethinyl estradiol lowered plasma levels of HDL as well as LDL. Rabbit HDL contains apolipoprotein E (28), a known ligand for the LDL receptor. It is possible that the fall in HDL levels is produced, at least in part, by enhanced clearance of apolipoprotein E-containing HDL via the LDL receptor. However, in view of the complexities of HDL metabolism (29), it is not possible to assign a definite mechanism for its reduction at the present time.

Richard Gibson provided excellent technical assistance with the animal experiments. We thank Gregorio Gil for his helpful advice in setting up the S1 nuclease assay, Wolfgang Schneider for help with ligand blotting, and David W. Bilheimer and Randall R. Romig for performing lipoprotein fractionations. This research was supported by grants from the National Institutes of Health (HL 20948) and the Moss Heart Fund.

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