Development, food intake, and ethinylestradiol influence hepatic triglyceride lipase and LDL-receptor mRNA levels in rats

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Abstract The influence of development and ethinylestradiol on low density lipoprotein (LDL)-receptor mRNA and hepatic triglyceride lipase (HTGL) activity and mRNA levels was studied in rat liver and intestine. Intestinal LDL-receptor mRNA levels are maximal in the perinatal period, whereas liver LDL-receptor and HTGL mRNA levels are highest after weaning in adult life. All mRNA levels reach a maximum between day 15 and 20 when rats still consume a lipid-rich diet, and increase twofold during weaning. Liver and intestinal LDL-receptor mRNA levels are not influenced by ovariectomy, but increase after ethinylestradiol treatment. Liver LDL-receptor mRNA shows a dose-dependent increase after ethinylestradiol and a sevenfold rise in liver LDL-receptor mRNA is attained with a dose of 2000 μg/day. Intestinal LDL-receptor mRNA increases slightly more than twofold after ethinylestradiol and this increase is not dose-dependent. Changes in LDL-receptor mRNA are independent of changes in food intake induced by ethinylestradiol treatment, since they are still observed after pair-feeding. The ethinylestradiol-induced increases in LDL-receptor mRNA levels are reflected by decreased serum apoB levels. HTGL mRNA levels increase after ovariectomy and show a dose-dependent decrease after ethinylestradiol. Pair-feeding abolishes the increase seen after ovariectomy, while the estrogen-mediated decrease is attenuated. These alterations in HTGL mRNA are reflected by similar changes in liver HTGL activity. In conclusion: *1*) estrogens increase LDL-receptor mRNA levels and decrease HTGL mRNA levels in rats, resulting in a decrease in serum apoB concentration and liver HTGL activity; *2*) changes in food intake are important in the in vivo regulation of HTGL mRNA levels, since the increased food intake after ovariectomy is in part responsible for the increased mRNA levels. — **Staels, B., H. Jansen, A. van Tol, G. Stahnke, H. Will, G. Verhoeven, and J. Auwerx.** Development, food intake, and ethinylestradiol influence hepatic triglyceride lipase and LDL-receptor mRNA levels in rats. *J. Lipid Res.* 1990. **31:** 1211–1218.

Supplementary key words LDL-receptor • liver • gene regulation • sex steroids • apoB • intestine • development

The liver plays a central role in lipoprotein metabolism (1, 2). Besides the production of several apolipoproteins, the liver also produces receptors and enzymes involved in lipoprotein metabolism such as the LDL-receptor and HTGL. HTGL is located on the sinusoidal surface of liver endothelial cells (3), where it exerts a major regulatory influence on the concentration of atherogenic lipoproteins in plasma. HTGL influences the physical characteristics of several lipoproteins by hydrolyzing triglycerides and phospholipids in intermediate density lipoprotein (IDL) and high density lipoprotein 2 (HDL_2) resulting, respectively, in the production of LDL (4–7) and HDL_3 (8, 9). HTGL activity is reported to be regulated by sex steroids (10). Estrogens suppress postheparin hepatic lipase activity (11) and, concomitantly, increase plasma HDL_2 cholesterol, whereas androgens increase HTGL activity (12), thereby decreasing plasma HDL_2 cholesterol. This might explain the higher serum HDL_2 cholesterol concentrations found in women relative to men (13).

LDL cholesterol is primarily removed from plasma by receptor-mediated endocytosis of the LDL particle via the LDL-receptor (1, 2). Regulation of liver LDL-receptor activity constitutes a major mechanism by which dietary and hormonal agents may alter plasma cholesterol (2, 14). A dramatic induction of LDL-receptor activity occurs in livers of rats treated with pharmacological doses of ethinylestradiol (15, 16). In the rabbit, this increase in LDL-receptor protein is caused by the induction of the mRNA for the LDL-receptor by ethinylestradiol (17).

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; IDL, intermediate density lipoprotein; HDL, high density lipoprotein; HTGL, hepatic triglyceride lipase; OVX, ovariectomy; EE, ethinylestradiol; apo, apolipoprotein.

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Both HTGL and LDL-receptor cDNAs have been cloned and sequenced recently (18–23). The availability of these probes has made it possible to study mRNA levels and consequently the regulation of the synthesis of these proteins. The present study was designed to investigate whether developmental changes in liver and intestinal mRNA levels occur and whether sex hormones regulate LDL-receptor and HTGL mRNA levels in the rat. The increase in liver LDL-receptor mRNA observed in the present study is in accord with the estrogen-induced rise of LDL-receptor activity observed previously in the rat (15, 16). Concomitantly, serum apoB levels are decreased by estrogen treatment. Furthermore, it is demonstrated that treatment with ethinylestradiol in vivo provokes a dose-dependent decrease of HTGL mRNA levels, resulting in decreased HTGL activity, an effect that may contribute to the well-known sex differences in lipoprotein profile (13).

MATERIALS AND METHODS

Animals and treatment

Animals and treatments were exactly the same as described previously (24). Adult male Wistar rats of the same age as the intact control female rats were used for determination of sex differences in HTGL and LDL-receptor mRNA levels. At the end of each experiment the animals were fasted overnight, weighed, and killed by exsanguination under ether anesthesia. Blood was allowed to clot. The serum was stored −20°C for determination of apoB levels. The liver was removed immediately, rinsed with 0.9% NaCl, and frozen in liquid nitrogen. The intestine was removed, rinsed with ice-cold 0.9% NaCl, and the epithelium was scraped off and frozen in liquid nitrogen.

Serum apoB determination

Serum apoB was measured by radial immunodiffusion according to Cheung and Albers (25), using a specific antiserum raised in rabbits against purified rat LDL-apoB (26). Serum apolipoprotein concentrations, expressed in arbitrary units (A. U.), were calculated as percentages of a rat serum standard pool (obtained from 50 rats) run simultaneously with the serum samples on the plates. Six different dilutions of the serum standard pool were run on each plate. All serum samples were run in triplicate. The standard pool was kept at −80°C in batches of 0.25 ml. The data were expressed in arbitrary units, because of the insolubility of purified apoB and the lack of sufficient quantities of pure rat apoB for use as an absolute standard. The within-day and between-day coefficients of variation of the apoB assay are 2% and 6%, respectively. Serum levels as low as 5 A. U. can be measured.

RNA analysis

Total liver and intestinal cellular RNA was prepared exactly as described previously (24). The HTGL and LDL-receptor mRNA levels were quantified by dot-blot hybridization as described (24). The 0.3 kb Bam HI restriction fragment of the human LDL-receptor clone pLDLR-3 (a kind gift from Drs. M. S. Brown and J. L. Goldstein), corresponding to nucleotide position 1079–1450 and containing part of the EGF-precursor homology domain, was used for hybridization (22). This domain shares a sequence homology of 84% with the rat LDL-receptor sequence (23). It detects a single mRNA species of approximately 4.9 kb (Fig. 2B), which is slightly smaller than the human LDL-receptor mRNA (22). An Ava I-Hind III full-length HTGL cDNA constructed from partial cDNA fragments (18, and G. Stahnke, unpublished results) was used for HTGL mRNA determination. With this probe a mRNA species with size similar to those previously reported in the literature for human and rat HTGL (18, 19, 21, 27) was detected (Fig. 3B). Both probes were labeled to a specific activity of 2.8×10^9 cpm/μg using random prime labeling (Boehringer Mannheim). Filters were hybridized for 72 h to 1.5×10^6 cpm/ml of the probe as described (28). They were washed in 500 ml of 0.5 × SSC and 0.1% SDS for 10 min at room temperature and twice for 30 min at 65°C and subsequently exposed to X-ray film (Hyperfilm-βmax, Amersham). Autoradiograms of filters were analyzed by quantitative scanning densitometry (LKB 2202 Ultrascan Laser Densitometer) as described (24). Northern blot hybridization of glyoxal-treated total cellular RNA was performed as described previously (28). To ascertain that equivalent amounts of RNA were loaded, the same filters that were used previously (24) were rehybridized after stripping. On these filters apoE mRNA levels have been shown not to change after ovariectomy and ethinylestradiol treatment (24).

Assay of liver HTGL activity

HTGL activity was determined as described previously (29). Briefly, liver (about 100 mg) was homogenized in 4 ml ice-cold 0.154 M NaCl containing 5 IU heparin (Thromboliquine, Organon, Oss, The Netherlands) using a Polytron PT 10 homogenizer (10 sec). The homogenates were centrifuged in plastic reaction vessels in an Eppendorf 3200 centrifuge at 4°C for 2 min at 8000 *g*. Lipase activity of the tissue extracts was measured following the release of fatty acids from a ^{3}H-labeled trioleoylglycerol emulsion in the presence of 1 M NaCl (29).

Statistical methods

Multiway analysis of variance was used to evaluate the results of the ethinylestradiol treatment and the route of administration. Values observed using different dosages

and routes of administration were compared by contrast statements. A two-tailed unpaired Student's *t*-test was used to evaluate differences between means in the experiments on pair-fed animals.

RESULTS

Developmental changes in LDL-receptor and HTGL mRNA levels in rat liver and intestine

Liver LDL-receptor mRNA levels were elevated during the first 10 days after birth and decreased during the late suckling period. After weaning (around day 20) a sharp increase occurred and thereafter the mRNA levels remained elevated (**Fig. 1**). Intestinal LDL-receptor mRNA levels, on the other hand, were very high during the postnatal period (days 5–10) and decreased during the late suckling period. This decrease was more pronounced in the intestine relative to the liver. As in the liver, an increase in LDL-receptor mRNA could be observed starting from day 30; thereafter the levels remained constant (Fig. 1).

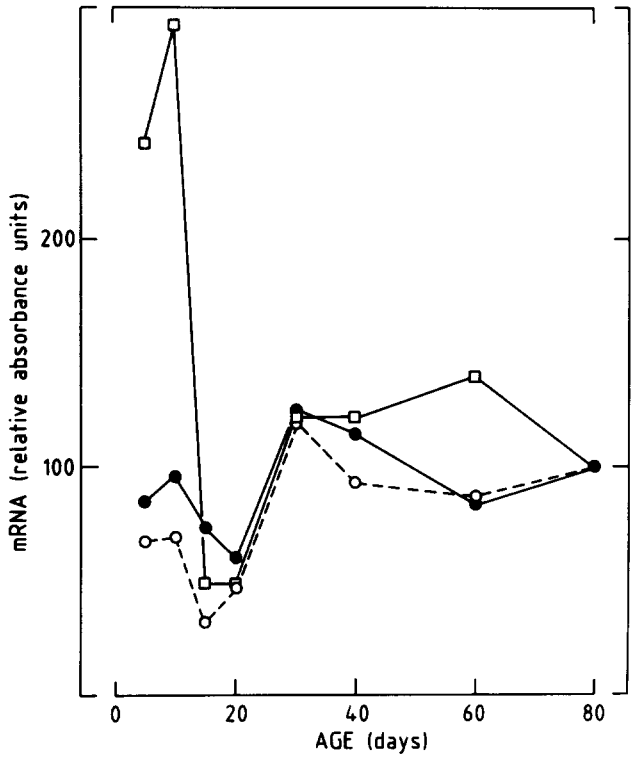

Fig. 1. Developmental changes in the mRNA levels for HTGL (●——●) and LDL-receptor in liver (○---○) and intestine (□——□) of rats. RNA was prepared from pooled livers and intestines of male rats of different ages (5 days old: n = 40; 10 days old: n = 20; 15 days old: n = 10; 20 and 30 days old: n = 6; 40, 60, and 80 days old: n = 4). mRNA levels of HTGL and LDL-receptor were measured using dot-blot analysis as described in Materials and Methods. Values are expressed in relative absorbance units taking the amount of mRNA measured at day 80 as 100%.

HTGL mRNA levels showed a pattern similar to liver LDL-receptor mRNA, but generally the changes were less pronounced than for the LDL-receptor mRNA (Fig. 1). The developmental changes in HTGL mRNA levels observed in this study are in accord with the results reported by Semenkovich et al. (27) during the preparation of this manuscript.

Influence of ethinylestradiol on liver LDL-receptor and HTGL mRNA levels

Female rats, ovariectomized for 34 days, were treated for 7 days with a wide range of estrogen doses (0.02–2000 μg ethinylestradiol). The results were compared to those for intact male and female animals of identical age as well as to those for ovariectomized animals receiving the vehicle only. Each treatment group consisted of six rats; half of the animals received the hormone intragastrically, whereas the other half was treated subcutaneously (24). Two-way analysis of variance demonstrated no significant influence of the route of ethinylestradiol administration on liver LDL-receptor and HTGL mRNA levels and accordingly the results were combined. Alterations in serum lipid, apoA-I, and apoE levels were reported previously (24). Briefly, serum cholesterol and apoA-I increased after ovariectomy, whereas serum cholesterol, triglycerides, apoA-I, and apoE levels decreased after ethinylestradiol treatment.

Male rats tended to have lower LDL-receptor mRNA levels than females, although the difference was not significant (**Fig. 2A**). Ovariectomy or low doses of ethinylestradiol did not influence LDL-receptor mRNA levels in females, while higher doses of ethinylestradiol increased LDL-receptor mRNA levels. At 20 μg/day the LDL-receptor mRNA levels almost doubled, and at 2000 μg/day a 10-fold increase was observed (Fig. 2A and B).

HTGL mRNA levels in male and female rats were similar. However, the levels in female rats showed a larger variation than those in males, which could be caused by the fact that the female animals were in different periods of the estrous cycle. When female animals were ovariectomized for 41 days, a 50% increase was observed. Ethinylestradiol substitution of these animals decreased HTGL mRNA in a dose-dependent way (**Fig. 3A**). At a dose of 2 μg/day, HTGL mRNA reached the same level as those in intact animals and at 2000 μg/day HTGL mRNA decreased to less than one-third of the level in untreated ovariectomized animals (Fig. 3A and B).

While after ovariectomy HTGL mRNA is regulated by the induced changes in food intake, ethinylestradiol influences LDL-receptor and HTGL mRNA directly

Since ovariectomy and estrogens influence food intake significantly (30), the effects of ovariectomy and estrogen therapy were investigated in pair-fed animals. Food intake

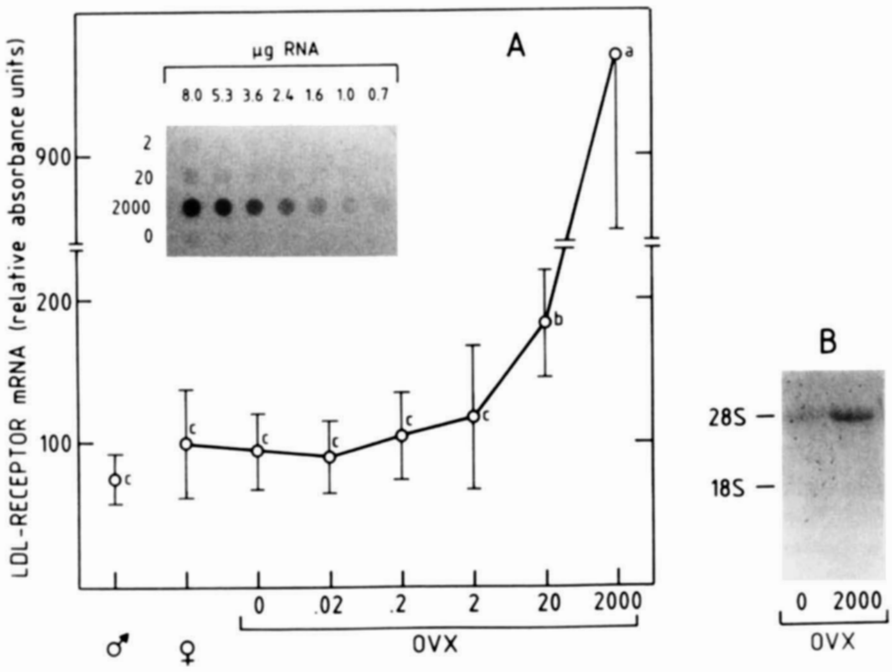

Fig. 2. Influence of sex, ovariectomy, and ethinylestradiol on rat liver LDL-receptor mRNA levels. Panel A: Rats were 131 days old at the end of the experiment. Female animals were ovariectomized (OVX) on day 90; treatment with the indicated doses of ethinylestradiol (µg/day) was started 34 days later and continued for 7 days. The intact control male and female rats and the ovariectomized control rats received vehicle (0) only. Total RNA was extracted from livers of individual animals and LDL-receptor mRNA levels were measured by dot-blot hybridization technique as described in Materials and Methods. Values are expressed in relative absorbance units taking the mean value of the intact female rats as 100%. Each value represents the mean ± SD of six animals. Statistically (ANOVA) significant differences ($P<0.05$) are observed between values followed by different letters. Panel A, inset: a representative dot-blot of RNA from ovariectomized and ethinylestradiol-treated animals is shown. Panel B: Northern blot analysis of the influence of ovariectomy and ethinylestradiol (2000 µg/day) on liver LDL-receptor mRNA levels. Northern blot hybridization of glyoxal-treated total cellular RNA was performed as described (28) on the same filters used previously (24). The hybridization conditions of the LDL-receptor cDNA probe are as described in Materials and Methods. The localization of the 18 and 28 S rRNAs are indicated.

of the ovariectomized rats was restricted to the amount consumed by intact rats during the entire period (41 days). Similarly, the sham-injected ovariectomized controls received, during the 7 days of treatment, the amount of food consumed by the ethinylestradiol-treated animals. At the end of the treatment there was no significant difference between the body weights of the treated and control animals (24). Serum lipid and apolipoprotein levels changed in a similar fashion after ovariectomy and ethinylestradiol as in ad libitum-fed animals (24). In order to determine whether the increase in serum cholesterol after ovariectomy and the decrease after ethinylestradiol treatment were reflected not only by changes in HDL-apolipoproteins (24) but also by changes in VLDL- and LDL-apolipoproteins, serum apoB levels were measured. A significant increase in serum apoB was observed after ovariectomy, whereas apoB levels dropped to less than 20% of the control after ethinylestradiol treatment (**Table 1**).

Pair-feeding did not influence liver or intestinal LDL-receptor mRNA levels after ovariectomy (Table 1). An intermediate dose of ethinylestradiol (20 µg/day) resulted in slightly higher levels of liver LDL-receptor mRNA in pair-fed animals compared to ad libitum-fed animals (Fig. 2A and Table 1), while there was a dramatic increase at the highest dose (Table 1). Intestinal LDL-receptor mRNA levels increased more than twofold after ethinylestradiol. In contrast to the liver, the increase in intestinal LDL-receptor mRNA was independent of the dosage of ethinylestradiol, at least in the range tested (Table 1).

HTGL mRNA levels no longer increased after ovariectomy in pair-fed animals and liver HTGL activity was not significantly influenced (Table 1). Ethinylestradiol treatment, however, decreased HTGL mRNA levels regardless of changes in food intake, but the decrease was less pronounced than in ad libitum-fed animals. Indeed at 2000 µg/day the decrease in HTGL mRNA levels was only slightly more than 40% compared to the control ovariectomized animals (Table 1), whereas the mRNA levels fell to less than one-third of the ovariectomized controls when the animals were not pair-fed (Fig. 3). These changes in HTGL mRNA levels were reflected by altered HTGL activity (Table 1). HTGL activity decreased by more than

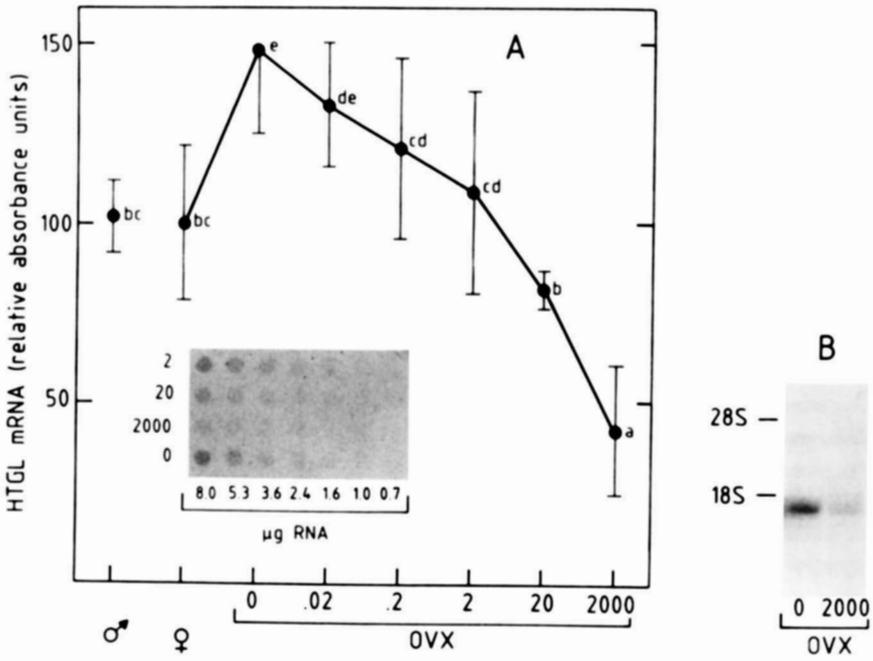

Fig. 3. Influence of sex, ovariectomy, and ethinylestradiol on rat HTGL mRNA levels. Animals and treatments are identical to those described in Fig. 2. Panel A represents a dot-blot of RNA obtained from animals from the different treatment groups, while panel B represents a Northern blot. Northern blot hybridization of glyoxal-treated total cellular RNA was performed as described (28) on the same filters used previously (24). The blots were hybridized with the HTGL probe as described in Materials and Methods. Values are expressed in the same way as in Fig. 2. Statistically (ANOVA) significant differences ($P<0.05$) are observed between values followed by different letters.

30% after 20 µg/day and fell to less than 50% of the control values at 2000 µg ethinylestradiol per day.

DISCUSSION

HTGL and LDL-receptor mRNA levels change during development and estrogen treatment. Estrogens decrease HTGL gene expression and activity. Furthermore, it is shown that the estrogen-provoked increase in liver LDL receptor number is mediated by an increase in LDL-receptor mRNA levels in rats.

During the first 10 days after birth, liver LDL-receptor and HTGL mRNA levels are elevated. Both mRNAs decrease between days 10 and 20, a period when plasma lipid concentrations are highest (31), while after day 20 a

TABLE 1. Influence of ovariectomy and ethinylestradiol on liver HTGL activity and mRNA levels, serum apoB, and liver and intestinal LDL-receptor mRNA levels in pair-fed rats

	HTGL			LDL-Receptor mRNA	
Treatment	Activity	mRNA	ApoB	Liver	Intestine
	mU/mg protein	*R.A.U.*	*A.U.*	*R.A.U.*	
Intact control	1.93 ± 0.29	100 ± 5	58 ± 6	100 ± 20	100 ± 54
OVX pair-fed	2.44 ± 0.31	91 ± 20	83 ± 10[a]	110 ± 30	85 ± 29
OVX + 20 µg EE	0.86 ± 0.18[a]	75 ± 6	8 ± 2[a]	391 ± 90[a]	265 ± 127[a]
OVX pair-fed	1.37 ± 0.30	100 ± 29	65 ± 16	100 ± 14	100 ± 30
OVX + 2000 µg EE	0.60 ± 0.10[a]	57 ± 11[a]	9 ± 3[a]	724 ± 86[a]	231 ± 52[a]
OVX pair-fed	1.36 ± 0.11	100 ± 13	51 ± 10	100 ± 36	100 ± 7

Female rats (90 days old) were ovariectomized (OVX) and treatment with the indicated dosage of ethinylestradiol (EE) or vehicle was started 34 days later and was continued for 7 days. Each group consisted of four rats and four pair-fed controls. Serum apoB and liver HTGL activity were determined as described in Materials and Methods. HTGL and LDL-receptor mRNA levels were measured in livers of individual animals by a dot-blot hybridization technique as described in Materials and Methods. mRNA values are expressed in relative absorbance units (R.A.U.) taking the mean value of the control group as 100. All values represent the mean ± SD.

[a]Statistically significant differences between groups (two-tailed unpaired Student's *t*-test, $P < 0.05$).

marked increase is observed. It is conceivable that these changes are the result of the high fat and cholesterol intake during the suckling period (31, 32). The increase in both mRNAs after weaning, when the rat changes from a relative lipid-rich to a carbohydrate-rich diet (32), is in accord with this hypothesis. However, these alterations may be mediated by differences in hormone secretion and/or responsiveness during pubertal development. The high levels of LDL-receptor mRNA in the intestine of postnatal rats, on the other hand, may be a consequence of the sudden transition from the inactive to the active state of this organ immediately after birth.

Since sex hormone secretion increases drastically after puberty and influences plasma lipid levels during adulthood, the hormonal regulation of the synthesis of two key proteins in lipoprotein metabolism, the LDL-receptor and HTGL, was investigated. It is demonstrated that ethinylestradiol administration down-regulates HTGL activity by decreasing its mRNA levels in rats (Table 1). In humans, HTGL also seems to be regulated by estrogens (10, 11). After the estrogen peak during the luteal phase of the menstrual cycle, postheparin-HTGL activity decreases (33). Furthermore, administration of estradiol valerate to postmenopausal women increases the HDL_2 cholesterol concentration and decreases postheparin plasma HTGL activity (34). In addition, women have lower HTGL activity compared to men (35), which may explain their higher HDL_2 cholesterol levels (13, 36–38), and their lower risk for coronary heart disease (13, 39).

Ethinylestradiol increases LDL-receptor mRNA levels. These results are in accord with the results of Kovanen, Brown, and Goldstein (15) and Windler et al. (16), who demonstrated increased binding of apoE- and apoB-containing lipoproteins to liver membranes and a marked increase in the number of LDL-receptors in the livers from ethinylestradiol-treated rats. In rabbits a similar increase in LDL-receptor number and mRNA levels was described after administration of pharmacological doses of ethinylestradiol (17). In addition, the dramatic decrease in serum apoB (Table 1) and the complete disappearance of serum apoE (24) point to an increased clearance of these apolipoproteins, since both apoB and apoE mRNA levels are unchanged in the livers from ethinylestradiol-treated rats (24, and B. Staels, L. Chan, G. Verhoeven, and J. Auwerx, unpublished results). In humans, treatment of males with pharmacological doses of estrogens provokes an increased fractional catabolic rate of LDL and a decrease in plasma LDL cholesterol levels (40). Furthermore, estrogens have been shown to induce LDL-receptor activity in the human hepatoma cell-line, HepG2 (41). If one is allowed to extrapolate these studies to humans, the increase in receptor mRNA by estrogens could provide one mechanism that could explain in part why women have lower levels of LDL cholesterol, a known risk factor for atherosclerosis, than men (13).

The increased intestinal LDL-receptor mRNA levels, on the other hand, are probably more related to the extreme decrease in serum cholesterol levels than to a direct effect of ethinylestradiol on the intestine (24). First, the increase in LDL-receptor mRNA is not as pronounced as in the liver (Table 1). Second, in contrast to the hepatic LDL-receptor mRNA levels, no dose-dependent effect of ethinylestradiol was observed on intestinal LDL receptor mRNA. Third, there are indications that the gastrointestinal tract does not contain estrogen receptors (42).

At increasing doses of ethinylestradiol, serum cholesterol and apoB levels are decreased (24, Table 1). In the rat, 90% of serum cholesterol is carried by HDL. The absence of serum lipid transfer protein activity in the rat is thought to account for the lower LDL cholesterol levels in these animals compared to humans and for the increased cholesterol content of an apoE-rich HDL-fraction of lower density (43, 44). Since the liver expresses about 70% of the body's LDL receptors (2, 45, 46), the regulation of LDL receptor expression in this organ influences serum cholesterol levels significantly (2, 15, 16). It is conceivable that in the rat the estrogen-induced decrease in HTGL levels increases the flux from HDL_3 over HDL_2 to apoE-rich HDL. The rise in LDL-receptors might then facilitate the clearance of this particle by apoE-mediated binding to the LDL-receptor, which in turn might account for the fall in serum cholesterol and apoE (24). In addition, the decrease in serum apoB levels points to an increased clearance of apoB-containing lipoproteins, thus contributing to the decreased serum cholesterol levels.

Although LDL-receptor mRNA levels in adult rats do not seem to be influenced by food intake, a clear effect of food intake was visible on HTGL activity and mRNA levels. On the one hand, the increase in HTGL mRNA levels seen after ovariectomy disappeared when the animals were pair-fed. On the other hand, the decrease in HTGL mRNA after estrogen treatment was less pronounced when these animals were pair-fed. In addition, HTGL activity was lower in the ovariectomized rats that were pair-fed with the ethinylestradiol-treated rats than in those pair-fed with the intact controls (Table 1). The latter animals consumed significantly higher amounts of food than the ethinylestradiol-treated rats (24). Reduced food intake (estrogen treatment) and fasting seem to decrease whereas increased food intake (ovariectomy) seems to increase HTGL mRNA levels and activity, pointing to the fact that both HTGL activity and mRNA levels are controlled, at least in part, by food intake. These findings are in agreement with the reported decrease in HTGL activity following a period of fasting for 24 h (47).

In conclusion, HTGL mRNA levels are regulated by development, food intake, and estrogens, whereas LDL-receptor mRNA levels are influenced by development and

estrogens. Although fairly high doses of ethinylestradiol are required to induce the observed changes in mRNA, it is possible that more subtle sex hormone-induced changes in HTGL and LDL-receptor activity might predispose women to a less atherogenic lipoprotein profile than men, if these results can be extrapolated to humans.

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