Increased Removal of β-Very Low Density Lipoproteins After Ethinyl Estradiol Is Associated With Increased mRNA Levels for Hepatic Lipase, Lipoprotein Lipase, and the Low Density Lipoprotein Receptor in Watanabe Heritable Hyperlipidemic Rabbits

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The mechanism by which ethinyl estradiol (EE) decreases the concentration of lipids in the $d<1.019$ g/ml fraction (β-very low density lipoprotein [β-VLDL]) of homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits was studied. Treatment with EE increased the activity of hepatic lipase (HL) twofold to threefold in postheparin plasma and in liver biopsies. Postheparin plasma and adipose tissue lipoprotein lipase (LPL) activities were also increased twofold to fourfold after EE. The effects of EE on HL and LPL activities were associated with a threefold to sixfold elevation in liver HL mRNA and a fourfold elevation in adipose tissue LPL mRNA steady-state levels, pointing to an effect of EE on HL and LPL gene transcription. EE also increased liver low density lipoprotein (LDL) receptor mRNA levels threefold to fivefold. These results suggest a concerted action of LPL, HL, and the LDL receptor in the removal of β-VLDL in homozygous WHHL rabbits with a defective LDL receptor. In addition, the content of apolipoprotein E in the $d<1.019$ g/ml fraction changed toward normal after EE. Because the remaining particles contained apolipoprotein B-100 almost exclusively, it is likely that apolipoprotein E-containing β-VLDLs are preferentially removed. This may be the result of the increased activity of LPL and HL influencing the conformation of apolipoprotein E on the β-VLDL particle in such a way that it is directly removed from the circulation, possibly by the induced LDL receptor. (Arteriosclerosis and Thrombosis 1991;11:1652–1659)

Ethinyl estradiol (EE) has multiple effects on lipoprotein metabolism: it influences the production of very low density lipoproteins (VLDLs) in the liver,1 it modulates the activities of the lipolytic enzymes in plasma,2-5 and it stimulates low density lipoprotein (LDL) receptor activity.6 In a previous study we found that treatment with EE resulted in a considerable decrease of serum lipids in Watanabe heritable hyperlipidemic (WHHL) rabbits.7 These animals lack normally functioning LDL receptors,8 which are involved in the uptake of intermediate density lipoproteins (IDLs) and LDL.9 While IDLs in normal rabbits are rapidly removed from the circulation directly and are only minimally converted to LDL, in homozygous WHHL rabbits IDLs are largely converted to LDL because of their low direct uptake by the LDL receptor.9 Thus, according to the present hypothesis, the activity of the LDL receptor is of major importance in determining the concentrations of IDL as well as those of LDL.

In a previous study we found that EE decreased the concentrations of lipids in the $d<1.019$ g/ml fraction of WHHL rabbits, while those in the LDL fraction were unchanged.7 Kinetic studies showed
that VLDL particles were cleared from plasma twice as fast in EE-treated animals compared with controls. In addition, it was found that the production rate of VLDL was not affected by EE. The activity of plasma postheparin hepatic lipase (HL) was shown to be increased in EE-treated animals. These findings therefore suggested a causal relation, namely that HL cooperates with the receptor in the removal and metabolism of β-VLDL in these animals. This is in line with several reports suggesting a major role for HL in the metabolism of apolipoprotein (apo) B-containing lipoproteins.10-12

The present study was designed to evaluate the mechanism by which EE treatment influences the removal of β-VLDL in WHHL rabbits. For this purpose we determined liver HL and LDL receptor mRNA levels as well as lipoprotein lipase (LPL) mRNA levels in adipose, heart, and muscle tissues. In addition, the activities of HL and LPL in postheparin plasma, as well as the apo E content in the d<1.019 g/ml fraction, were determined.

**Methods**

**Experimental Protocol**

The studies were performed with eight homozygous and eight heterozygous WHHL rabbits, 12-15 months of age with equal numbers of males and female rabbits. Animals consumed the LK-04 diet (Hope-Farms, Woerden, The Netherlands) containing 2% fat. After being matched for body weight and serum lipids, half of both groups received an implant containing 16 mg EE, and half received control implants. This 28-day treatment resulted in plasma EE concentrations that were nine to 35 times normal.7 The animals appeared healthy and showed a normal increase in body weight compared with controls during the entire study. Because sex-related differences in serum lipids and lipoproteins were absent, it was decided to place the implants in the animals without performing a gonadectomy. The physical condition and food consumption of the animals were inspected regularly and were found not to deviate from normal. In addition, when the results were analyzed, sex-specific differences in the response to EE appeared to be absent for all parameters studied. The results obtained within each group were therefore combined. The effect of the treatment was again followed for 28 days. At the end of the experiments the animals were killed after an overnight fast with a lethal dose of barbital.

**Determination of Lipolytic Activity in Various Tissues**

Immediately after the animals were killed, the abdomen was opened and was followed by perfusion of the liver with ice-cold saline. Liver and paravertebral adipose tissue as well as heart, skeletal muscle, and buttock tissues were excised, and part of them were immediately frozen in liquid N2 for RNA isolation. Another part of liver tissue was homogenized in ice-cold saline containing 10 IU heparin/ml15 and frozen until assay with the 1.0 M NaCl substrate, as described for plasma HL. LPL in paravertebral adipose tissue was determined by incubating 30 μg tissue in 250 μl 0.1 M substrate containing 20 IU heparin for 2 hours. Released fatty acids were extracted as described.14 All lipase assays were performed in duplicate; the results obtained showed good agreement. To minimize the influence of analytical variation, all postheparin plasma samples were analyzed within one series, and the same was done for all biopsies.

**Determination of mRNA Levels in Liver and Adipose Tissue**

Total cellular RNA was prepared from liver and adipose tissues of individual animals. Northern and dot blots of total cellular RNA, labeling of probes, hybridizations, and washing of filters were performed exactly as described.16-17 The following probes were used: a human HL cDNA probe, a human LPL cDNA clone, human LDL receptor clone pLDLR-3, and a chicken β-actin cDNA clone.17-21 Autoradiograms of filters were analyzed by quantitative scanning densitometry in the linear range of film sensitivity exactly as described.16 Values are expressed in absorbance units relative to the levels in the control animals.

**Analytical Methods**

Blood samples were drawn into EDTA-containing tubes (1 mg/ml). The d<1.019 g/ml fraction and the LDL fraction were isolated by sequential ultracentrifugation for 18-24 hours at 168,000g.22 Lipoproteins were isolated by aspiration.

Cholesterol was measured by the enzymatic CHOD-PAP method (No. 237574, Boehringer Mannheim GmbH, Mannheim, FRG). Triglycerides were determined by the sera-PAK kit of Miles (No. 6684, Miles Laboratories, Milano, Italy). Phospholipid and free
cholesterol were measured with enzymatic kits (Nos. 691844 and 310328, respectively, from Boehringer). Protein was determined according to the method described by Lowry et al.23

Electrophoresis of lipoproteins was performed with the Paragon-Lipo electrophoresis kit (Beckman Instruments, Palo Alto, Calif.). The bands were stained with Sudan black. Sodium dodecyl sulfate gel electrophoresis of apolipoproteins was performed in 3%/4% discontinuous polyacrylamide gels.15

Statistical Methods

Student's unpaired t test was used to test differences for significance. Unless indicated otherwise, results are expressed as mean±SEM.

Results

Effect of Ethinyl Estradiol on Body Weight, Serum Lipids, and Lipoproteins in Watanabe Heritable Hyperlipidemic Rabbits

Treatment with EE did not result in significant differences in body weight. EE caused a considerable decrease in total plasma triglycerides, from 3.80±0.91 to 0.82±0.42 mmol/l (mean±SD, p<0.001) in homozygotes and from 1.48±0.57 to 0.69±0.34 mmol/l (p<0.05) in heterozygotes. Concentrations of triglycerides in the d<1.019 g/ml fraction decreased considerably; those in the LDL fraction decreased similarly. The decrease of triglycerides in both fractions is probably the effect of a rapid exchange of triglycerides between these fractions in the WHHL rabbits (Figure 1). In both fractions the maximal decrease was already obtained 10 days after implantation of EE.

In the EE-treated homozygotes, total plasma cholesterol decreased from 20.45±2.07 to 11.15±2.51 mmol/l (mean±SD, p<0.001) and in the heterozygotes from 2.78±0.91 to 1.22±0.34 mmol/l (p<0.05). In the homozygous rabbits the decrease was exclusively in the d<1.019 g/ml fraction; LDL cholesterol did not change during the first 20 days of treatment (Figure 1). In the heterozygous rabbits cholesterol mainly decreased in the LDL fraction; the largest decrease was already observed within 10 days after implantation (Figure 1).

The chemical composition data showed that the d<1.019 g/ml fraction of the homozygotes was extremely rich in cholesteryl esters and poor in triglycerides compared with that of heterozygotes (Figure 2). This abnormality was even more pronounced in the EE-treated homozygous rabbits. Similar differences were observed in the composition of the LDL fraction in homozygotes before and during EE treatment compared with heterozygous rabbits. Both the chemical composition data of the d<1.019 g/ml fraction and the results of the agarose gel electrophoresis (Figure 3) show that the d<1.019 g/ml fraction almost exclusively contained β-VLDL, which had largely disappeared in the EE-treated homozygotes. Chemical composition data reveal that the changes induced by EE are more quantitative than qualitative (Figure 2).

Analysis of Apolipoproteins in the d<1.019 g/ml Fraction

Sodium dodecyl sulfate gel electrophoresis revealed that the d<1.019 g/ml fraction of homozygous
controls contained relatively more apo E than did the same fraction of EE-treated homozygotes (Figure 4). The d<1.019 g/ml fraction from control and EE-treated heterozygotes was poor in apo E and contained apo B-100 almost exclusively. In comparison with human VLDL, less apo C was present in all rabbits, irrespective of treatment (Figure 4).

Ethinyl Estradiol Influences Plasma and Tissue Lipolytic Activity

Postheparin plasma HL activity increased in both homozygous and heterozygous WHHL rabbits treated with EE (Figure 5). This difference in HL activity between treated and untreated animals was most pronounced after 28 days. A similar change in HL activity occurred in liver biopsies taken after 28 days of EE treatment (Figure 6). Postheparin plasma LPL activity increased more than threefold in EE-treated homozygous rabbits, whereas LPL activity remained stable in EE-treated heterozygous rabbits (Figure 5). Biopsies of adipose tissue from EE-treated homozygous rabbits showed a fourfold increase in adipose tissue LPL activity versus control homozygotes; LPL activities in biopsies of hearts, skeletal, and buttock muscle in these rabbits were low and did not differ in control and EE-treated animals (Figure 6).

Ethinyl Estradiol Influences Liver Hepatic Lipase, Low Density Lipoprotein Receptor, and Adipose Tissue Lipoprotein Lipase mRNA Levels

HL mRNA levels increased after EE in homozygous as well as heterozygous WHHL rabbits (Figure 7A). This increase, however, was more pronounced in heterozygous compared with homozygous rabbits. Treatment with EE increased LDL receptor mRNA in both homozygous and heterozygous rabbits (Figure 7A). Liver β-actin levels, however, remained constant. Similar changes in liver mRNA levels as determined by dot-blot analysis were observed after Northern blot analysis (Figure 7B).

LPL mRNA levels in adipose tissue of EE-treated homozygotes increased substantially versus those of
controls (Figure 8A). No change in adipose tissue β-actin mRNA was detected under these conditions. In contrast, heart muscle LPL and β-actin mRNA levels remained unchanged in homozygous WHHL rabbits treated with EE (92±2 versus 100±6 relative absorbance units for LPL and 92±4 versus 100±6 relative absorbance units for β-actin in treated and control rabbits, respectively).

Discussion

The present study confirms our previous result that EE treatment is effective in decreasing β-VLDL concentrations in WHHL rabbits. In both studies EE treatment was found to be associated with increased activities of postheparin plasma HL and LPL activities. However, the changes in these enzyme activities after EE are larger in the present study than in the previous one. This effect may be due to biologic variation because the lipoprotein concentrations in homozygous WHHL rabbits showed a high interindividual and intraindividual variation.

Previously, it was shown that VLDL production rates remained constant after EE treatment of WHHL rabbits. Consequently, the decreased β-VLDL levels must be attributed to an increase in the removal of β-VLDL. The cholesterol ester and apo E–rich β-VLDL particle is classically thought to be removed from the circulation by a receptor involving apo E as a ligand. The increase in LDL receptor mRNA levels after EE in the WHHL homozygotes indicates that the LDL receptor may be involved in the removal of β-VLDL. A similar induction of LDL receptor mRNA levels has been observed by Ma et al in normal rabbits treated with EE. The functional LDL receptor deficiency in homozygous WHHL rabbits is caused by an inframere deletion of a portion of the LDL receptor structural gene. Despite the presence of this LDL receptor gene defect, transcriptional control of the LDL receptor seems intact because in addition to EE, mevinolin has also been shown to enhance LDL receptor mRNA levels in WHHL animals. In the present study EE increased LDL receptor mRNA levels even in the absence of a reduction in plasma LDL cholesterol, suggesting that EE acts directly on the control mechanisms governing LDL receptor mRNA levels and not indirectly via an effect on LDL cholesterol. Although LDL receptor mRNA is increased after EE, it is questionable whether this nonfunctional LDL receptor (no lowering of LDL cholesterol after EE) can explain the complete disappearance of β-VLDL and the strong decrease in VLDL and IDL.
steroid hormones such as EE in several mammalian species. The activity of these enzymes appears to be regulated by steroid hormones such as EE in several mammalian species. In the present study, EE activity increased threefold in EE-treated WHHL rabbits, whereas in a previous study, a twofold increase was observed in the basal situation. This increase in EE activity may be caused by a transcriptional induction of the HL gene because it was paralleled by an increase in EE mRNA steady-state levels in the rabbit liver. This coordinated induction of the LDL receptor and HL in rabbit liver contrasts sharply to the situation in the rat, where EE treatment has opposite effects on the LDL receptor and cholesterol in EE-treated WHHL rabbits. It is therefore likely that in addition to this effect, other factors must be invoked to explain the reduction in β-VLDL and cholesterol in the d<1.019 g/ml fraction.

The lipolytic enzymes are potential candidates, as both HL and LPL are involved in the metabolism of apo B-containing lipoproteins. The activity of the enzymes appears to be regulated by steroid hormones such as EE in several mammalian species. In the present study, EE activity increased threefold in EE-treated WHHL rabbits, whereas in a previous study, a twofold increase was observed in the basal situation. This increase in EE activity may be caused by a transcriptional induction of the HL gene because it was paralleled by an increase in EE mRNA steady-state levels in the rabbit liver. This coordinated induction of the LDL receptor and HL in rabbit liver contrasts sharply to the situation in the rat, where EE treatment has opposite effects on the LDL receptor (i.e., EE induces mRNA levels) and HL (i.e., EE suppresses mRNA levels). The activity of the other important lipolytic enzyme LPL also increases markedly after EE treatment. This increase in LPL activity could be detected in postheparin plasma as well as in adipose tissue but not in heart or muscle tissue biopsies. EE treatment also caused a substantial increase in adipose tissue LPL mRNA levels, suggesting that EE has a direct effect on LPL gene expression. Although both LPL and HL are involved in the metabolism of apo B-containing lipoproteins, their differential function is unclear at present, especially in rabbits. In humans, the presence of β-VLDL seems to be more closely associated with aberrant HL activity than with abnormalities of LPL. Subjects with LPL deficiency hardly ever have increased levels of β-VLDL (reviewed in Reference 28). On the contrary, the presence of β-VLDL seems to be a hallmark of HL deficiency. Rabbits generally have low levels of HL relative to other mammals (References 24 and 31 and J.E. Hokanson, personal communication). Therefore, it is tempting to speculate that the increased HL activity after EE might at least partially be involved in the reduction of β-VLDL levels in the EE-treated WHHL rabbits. The higher values for HL activity in postheparin plasma and liver tissue of WHHL homozygotes relative to heterozygotes are not in disagreement with this hypothesis if it is regarded as a compensational mechanism to overcome the defective LDL receptor. In the basal situation, however, this increase in enzyme activity is apparently insufficient to overcome the defective removal of β-VLDL by the LDL receptor. The increased LPL activity may contribute to the observed changes in plasma lipoprotein levels. Also, the apo E content or its conformation within the β-VLDL particle may be an important factor in determining its clearance rate. Our data point to a preferential uptake of apo E-rich β-VLDL in EE-treated homozygotes, either due to an increased amount of apo E per particle or to an increased lipase-associated expression of apo E on the β-VLDL, thereby improving its ability for receptor-mediated uptake by the hepatocyte. Indeed, additional apo E infused into the circulation appeared to stimulate the removal of chylomicron remnants in WHHL rabbits. Whether the decrease in serum
cholesterol observed after apo E infusion was due exclusively to a decrease of particles of exogenous origin or partly to a decrease of particles of endogenous origin was not studied, however. Given the fact that the removal process of chylomicrons is not delayed in these rabbits, it seems likely that intravenous infusion of apo E also stimulates the removal of other cholesterol-containing lipoproteins as $\beta$-VLDL.

If the chylomicron remnant receptor is functional in WHHL rabbits,\textsuperscript{36} it seems likely that it also mediates the removal of VLDL remnants, especially when LPL and HL are activated. However, as discussed above, it seems more likely that the nonfunctional LDL receptor still contributes to the removal of VLDL remnants. Then activation of HL and LPL seems indispensable to partially overcome the molecular defect in the LDL receptor. This stresses the important role of both lipases in determining the fate of VLDL remnants in rabbits.

In conclusion, this study identifies several factors involved in the metabolism of $\beta$-VLDL that are influenced by treatment with EE. These results should be interpreted in the light of species differences and the relatively large amount of EE that evidently overrules the specific sex hormone profile. In the present study we did not observe a differential function for LPL and HL. Furthermore, the defective LDL receptor seems to actively participate in the uptake of $\beta$-VLDL. Our data raise questions concerning a differential uptake mechanism for chylomicrons and lipoproteins in the $d<1.019$ g/ml fraction. Further studies of this topic in the animal model used here seem warranted. Probably, these studies also shed more light on the lipoprotein abnormality of type III hyperlipoproteinemia, a disease state characterized by elevated levels of $\beta$-VLDL, for which estrogen therapy has a beneficial effect.\textsuperscript{37,38}

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**KEY WORDS**  •  Watanabe heritable hyperlipidemic rabbits  •  low density lipoprotein receptor  •  hepatic lipase  •  lipoprotein lipase  •  β-very low density lipoprotein clearance
Increased removal of beta-very low density lipoproteins after ethinyl estradiol is associated with increased mRNA levels for hepatic lipase, lipoprotein lipase, and the low density lipoprotein receptor in Watanabe heritable hyperlipidemic rabbits.

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