Enhanced Apolipoprotein E Production with Normal Hepatic mRNA Levels in the Watanabe Heritable Hyperlipidemic Rabbit

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The Watanabe heritable hyperlipidemic rabbit (WHHL) provides an experimental animal model for the low density lipoprotein (LDL) receptor defect present in patients homozygous for familial hypercholesterolemia (FH). Both WHHL rabbits and FH patients have a four- to sevenfold increase in plasma levels of apolipoprotein E (apo E). To determine the etiology for the elevated apo E concentrations, kinetic studies of radiolabeled apo E were conducted in WHHL and control New Zealand White (NZW) rabbits. The sites of apo E synthesis in the WHHL rabbit were evaluated by quantitating apo E mRNA levels in 12 tissues by dot-blot analysis of total RNA from each tissue with an apo E cDNA probe. Compared to the NZW rabbit, the WHHL rabbit had a twofold increase in the plasma apo E residence time, a fourfold increase in apo E production rate, and normal apo E mRNA levels in the liver and all other major apo E synthetic tissues. However, a fivefold increase in WHHL aortic apo E mRNA levels was observed. The elevated level of aortic apo E mRNA indicated a potential role for apo E in modulating atherogenesis in the WHHL rabbit. These results established that the increased plasma apo E in the WHHL rabbit was due to increased synthesis and delayed catabolism. Moreover, the fourfold increase in apo E synthesis with normal tissue apo E mRNA levels may reflect a translational or posttranslational regulation of apo E synthesis.

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The Watanabe heritable hyperlipidemic rabbit (WHHL) serves as an animal model for the human disease familial hypercholesterolemia (FH). Analogous to homozygous FH patients, all homozygous WHHL rabbits have histopathologic evidence of spontaneous aortic atherosclerosis and tendinous xanthomata as well as elevated plasma levels of total cholesterol, low density lipoprotein (LDL) cholesterol, and apolipoproteins B-100 and E. Since this inbred variant of the Japanese White rabbit was first developed by Watanabe in the early 1970s, this animal model has been studied extensively. LDL receptor deficiency in WHHL cultured fibroblasts, cultured hepatocytes, and membrane preparations of adrenal and liver cells parallel the LDL receptor deficiency in homozygous familial hypercholesterolemia. Lipoprotein kinetic studies demonstrated normal clearance of chylomicrons and delayed removal of very low density lipoproteins (VLDL), intermediate density lipoprotein (LDL), and LDL with enhanced conversion of VLDL to LDL leading to elevated levels of LDL. The defect in the LDL receptor in the WHHL rabbit is a twelve nucleotide in-frame deletion in the third cysteine-rich ligand binding domain. This defect is also associated with increased HMG-CoA reductase activity, as well as with depressed cholesterol esterification in fibroblasts.

Both WHHL rabbits and homozygous FH subjects have elevated plasma levels of apolipoprotein E (apo E). Previous studies from our laboratory investigated the kinetic mechanisms of elevated plasma apo E levels in FH homozygotes. Apo E kinetic studies demonstrated a normal rate of apo E catabolism and a threefold increase in the apo E production rate in homozygous FH subjects.

To directly address the mechanism for the increased plasma levels of apo E in the WHHL rabbit, apo E kinetic studies and tissue apo E mRNA levels were assessed to determine if enhanced apo E gene expression brought about the increased apo E production. An apo E kinetic study, analogous to the previous apo E studies, was performed in both WHHL and control New Zealand White (NZW) rabbits to directly compare apo E metabolism between rabbit and man. Quantitation of tissue apo E mRNA levels from these rabbits by dot-blot hybridization was performed to evaluate the role of apo E gene expression in the development of increased plasma apo E concentrations in the WHHL rabbit.

Methods

Reagents

Aprotinin and cesium chloride were obtained from Boehringer-Mannheim (West Germany), guanidine hydrochloride and Tris-HCl (ultrapure) from BRL (Gaithersburg, MD), and β-mercaptoethanol from Calbiochem (La...
Guaniidine thiocyanate was obtained from Fluka Chemie (Buchs, Switzerland). Agarose (ME) was procured from FMC Bioproducts (Rockland, ME), and sodium-N-lauroylsarcosinate from ICN Pharmaceuticals (Plainview, NY). 125 I (sodium iodide) was purchased from NEN (Boston, MA).

**Isolation and Analysis of RNA**

Total tissue RNA was isolated from three male NZW rabbits (2 months old) and three male homozygous WHHL rabbits (11 months old) obtained from the National Institutes of Health colony where they had been maintained on standard rabbit chow ad libitum. The rabbits were euthanized with intravenous Nembutal (100 to 250 mg/rabbit). The adrenal, aorta, brain, heart, jejunum, kidney, liver, lung, left quadriceps muscle, full thickness skin, spleen, and testis were harvested and immediately frozen in liquid nitrogen. The total tissue RNA was isolated by the guanidinium thiocyanate/cesium chloride centrifugation method.24 Northern blot analysis was performed in a 6% formaldehyde, 1% agarose denaturing gel and the material was transferred to a Gene Screen Plus.25 Dot-blot analyses were conducted by using 3 μg of total RNA in sterile, doubly distilled water. The RNA samples were denatured at 65°C for 10 minutes, and were then applied in quadruplicate to the Gene Screen Plus by using a 96-well manifold (Schleicher & Schuell, West Germany). The filters were baked for 2 hours at 80°C and then were prehybridized at 50°C for 4 hours in 10 ml per filter of 5× standard saline citrate (SSC; 1× = 0.15 M NaCl, 0.015 M sodium citrate, pH 7), 5× Denhardt's solution (1× = 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin), 0.1% NaDodSO4, 5 mM NaPO4 (pH 7.4), and denatured single-stranded salmon sperm DNA (100 μg/ml). The filters were hybridized with 10^7 CPM of a 32P-labeled nick translated 460 bp Apa I/Ava I restriction fragment of the human apo E cDNA26 in the above solution for 36 hours at 50°C. The filters were then washed in 2x SSC, 0.1% NaDodSO4 at 25°C for four 1-minute periods followed by one 30-minute period. The final wash was in 1× SSC, 0.1% NaDodSO4 at 65°C for 1 hour. The filters were autoradiographed for 48 hours at −20°C between double intensifying screens by using Kodak XAR-2 film. These hybridization conditions led to specific recognition of the rabbit apo E mRNA with the Apa I/Ava I restriction fragment of human apo E (Figure 1). The size of this band corresponds to that of the human apo E mRNA.27 The dot-blot autoradiographs were quantitated by laser scanning densitometric analyses with an LKB Ultrascan XL Laser Scanning Densitometer (Bromma, Sweden).

**New Zealand White Apoprotein E Isolation and Iodination**

Three male 11-month old NZW rabbits were made hyperlipidemic by supplementing their standard rabbit chow with 1% cholesterol for 3 weeks.27 VLDL and LDL (<1.019 g/ml) were isolated from the plasma by ultracentrifugation in a Beckman 50 Ti rotor (50,000 rpm for 18 hours). Aprotinin (200 U/ml) and 0.05% NaN3 were added to the supernatant, which was dialyzed extensively against 0.01 M NH4HCO3 (pH 8.2). Protein (30 mg) was delipidated by three extractions with chloroform/methanol, 3:1 (vol/vol).28 A 10 mg aliquot of protein was dissolved in 0.15 M NaCl, 0.05 M Tris-HCl, 8 M urea (pH 8.2) and was subjected to 1-dimensional SDS (15% acrylamide) and 8 M urea (pH 8.4) PAGE. The apo E band was cut from the gel, electroeluted, dialyzed, lyophilized, solubilized in 600 μl 5 M urea, and then desalted over a Sephadex G-100 (0.9×94 cm) column in a 0.01 M NH4HCO3, pH 8.2 buffer. Apro E (947 μg), which provided a single band on NaDodSO4 PAGE, was recovered and 100 μg of this apo E was iodinated by the iodine-monochloride method.29 The radiolabeled apo E was re-associated with plasma
from either NZW or WHHL rabbits for 20 minutes at 37°C, after which the plasma was adjusted to 1.21 g/ml with solid KBr and the supernatant was isolated by ultracentrifugation (Beckman 50 Ti, 50 000 rpm for 24 hours). The lipoproteins were dialyzed against magnesium and calcium-free phosphate-buffered saline (pH 7.4) followed by sterilization with a 0.22 μm Millipore filter (Millipore, Bedford, MA).

**Kinetics Study**

Five days before the kinetics study, central venous lines were placed subcutaneously in three male 11-month-old NZW rabbits and three male 11-month-old homozygous WHHL rabbits. The catheters were maintained daily by: 1) withdrawing 1 ml of blood to ensure patency, 2) sampling, 3) flushing with 2 ml of 0.9% NaCl, and 4) instilling one catheter volume (0.6 ml) of heparin solution (100 U/ml). Radiolabeled apo E (100 μCi 125I), associated with autologous lipoproteins (i.e., was injected) and blood samples were collected in EDTA tubes at 5 minutes, 30 minutes, 1, 2, 4, 8, 12, 18, and 24 hours, and then daily for 7 days. Radioactivity was quantitated in whole plasma in a model 5260 Auto- gamma Spectrometer (Packard Instruments, CA). Calculation of the residence time (RT = 1/fractional catabolic rate) was made by computer-assisted curve fitting by using the SAAM 29 program on a VAX 11/780 computer.

**Cholesterol, Triglyceride, and Apoprotein E Quantitation**

Cholesterol and triglyceride concentrations were determined on a Gilford 3500 automated system (Gilford, Oberlin, OH). Apo E was quantitated in the supernatant of the plasma samples, whose densities were adjusted to 1.21 g/ml with solid KBr, this was centrifuged in a Beckman 50 Ti at 50 000 rpm for 24 hours. Samples were electrophoresed in parallel with known amounts of isolated NZW apo E on a NaDodSO4 (15% acrylamide) 8 M urea (pH 8.4) PAGE. Laser scanning densitometric analysis of the Coomassie blue-stained lanes of known quantities of apo E standards permitted construction of a standard curve from which the sample apo E concentrations could be determined.

**Results**

**Kinetics of Apoprotein E Metabolism in WHHL and NZW Rabbits**

The characteristics of the rabbits used for the apo E kinetics study are summarized in Table 1. The age-matched male WHHL rabbits, as compared to the NZW rabbits, had a 14-fold elevation in total plasma cholesterol, a fourfold increase in total plasma triglycerides, and a sevenfold elevation in plasma apo E concentration. To investigate the kinetic etiology of the increased plasma apo E level in the WHHL rabbit, NZW rabbit 125I apo E, reassociated with autologous lipoproteins, was injected into three NZW and three WHHL rabbits. Representative multi-exponential plasma 125I-apo E decay curves in both WHHL and NZW rabbits are shown in Figure 2. A decreased fractional catabolic rate of apo E in the WHHL rabbit is evident. The kinetic parameters of apo E metabolism in WHHL and NZW rabbits are shown in Figure 2. A decreased fractional catabolic rate of apo E in the WHHL rabbit is evident. The kinetic parameters of apo E metabolism in WHHL and NZW rabbits are summarized in Table 1. There was a twofold increase in apo E residence time and a fourfold increase in apo E production rate in WHHL rabbits compared to NZW rabbits. Hence, the sevenfold increase in WHHL plasma apo E concentration was due primarily to an overproduction of apo E, although there was also a delayed removal of apo E.

**Dot-Blot Analysis of Tissue RNA**

Once hybridization and posthybridization washing conditions were established for use of the heterologous cDNA probe, we investigated the fourfold increased apo E production rate by determining tissue apo E mRNA levels. To establish the linearity of the affinity of the human apo E cDNA probe for rabbit total RNA, 1.0, 2.5, 5.0, 7.5, and 10.0 μg of NZW total hepatic RNA were applied to the dot-blot manifold. Laser scanning densitometric analysis of the autoradiographed hybridized filter revealed a linear range up to 5 μg of total hepatic RNA. Total RNA (3 μg) from each of the twelve tissues for each of the three WHHL and three NZW rabbits was dot-blotted in quadruplicate. All filters were prehybridized, hybridized, washed, and autoradiographed simultaneously. The results of the

**Table 1. Summary of Rabbit Characteristics and Apo E Kinetic Parameters of Apo E Metabolism in WHHL and NZW Rabbits**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NZW</th>
<th>WHHL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>3194±374</td>
<td>3138±86</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>31±6</td>
<td>442±196*</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>124±63</td>
<td>517±112*</td>
</tr>
<tr>
<td>Apo E (mg/dl)</td>
<td>0.40±0.2</td>
<td>2.90±0.6*</td>
</tr>
<tr>
<td>Residence time (days)</td>
<td>0.37±0.2</td>
<td>0.71±0.09*</td>
</tr>
<tr>
<td>Production rate (mg/kg/day)</td>
<td>0.50±0.2</td>
<td>1.80±0.6*</td>
</tr>
</tbody>
</table>

Values represent the mean±SD.

* A significant difference from NZW rabbits (p<0.05). NZW = New Zealand White rabbits, WHHL = Watanabe hyperlipidemic rabbits.

**Figure 2. Plasma catabolism of 125I-apo E in New Zealand White (NZW) and Watanabe heritable hyperlipidemic (WHHL) rabbits. WHHL (A—A) and NZW (D--D) rabbits were injected with 100 μCi of autologous re-associated NZW 125I-apo E. Plasma samples were drawn at the indicated time intervals and the radioactivity in the plasma was quantitated by scintillation counting. Plasma decay curves were fitted to the data using the SAAM 29 program.**
densitometric analyses of the autoradiographs are displayed graphically in Figure 3. Of particular importance, due to the central role of the liver in lipoprotein metabolism, is that there was no statistically significant difference in the hepatic apo E mRNA contents between the WHHL and NZW rabbits (WHHL, 0.41±0.05; NZW, 0.45±0.01 absorbance units; \( p>0.2 \)). However, statistically significant differences in the apo E mRNA levels between the WHHL and NZW rabbits were observed in the aortic apo E mRNA levels. There was a four- to fivefold increase in the WHHL apo E aortic mRNA levels as compared to the NZW rabbit (WHHL, 0.34±0.13; NZW, 0.06±0.006 absorbance units; \( p<0.05 \)). In the remaining ten tissues studied, there were no statistically significant differences between the mRNA levels in the WHHL and NZW rabbits.

**Discussion**

The LDL receptor is important in cellular cholesterol transport, and attention has been focused upon its role in lipoprotein metabolism.\(^{30}\) Mutations in this receptor lead to a variety of alterations in the steady-state concentrations of circulating plasma lipoproteins, as well as altered intracellular cholesterol homeostasis. Since WHHL rabbits and FH homozygotes lack normally functioning LDL receptors, it is possible to evaluate the impact of the LDL receptor pathway on different components within the lipoprotein transport system.

Apo E is a protein that avidly binds to the LDL receptor.\(^{31}\) Therefore, the increased concentrations of this protein in the blood of both WHHL and homozygous FH individuals has been ascribed to the inability of tissues to remove apo E from the circulation by the LDL receptor pathway.\(^{4}\) The WHHL rabbits (Figure 2) and FH homozygotes\(^{22}\) have similar parameters of apo E kinetics. Both have comparable plasma residence times (WHHL, 0.71±0.09 days; FH, 0.76±0.12 days; mean±SEM) and both have elevated apo E production rates (WHHL, fourfold; FH, twofold, when compared to normal controls). There is a greater difference between normal humans and normal control NZW rabbits. The NZW rabbit catabolized the apo E twice as fast as the normal humans, thus suggesting interspecies differences in apo E metabolism. The further differences between rabbits and humans are hypertriglyceridemia and delayed remnant clearance, which are present in WHHL rabbits but not in FH subjects. These observations may reflect differences between rabbits and humans in hepatic and lipoprotein lipase activities or may indicate that the chylomicron remnant pathway in rabbits is more closely linked to the LDL receptor than in humans.

Analysis of hepatic apo E production in WHHL rabbits has been previously assessed by liver perfusion studies.\(^{32}\) The rate of apo E accumulation was 57% higher in the WHHL rabbit as compared to the normal control NZW rabbit. Although this study could not differentiate between increased hepatic apo E synthesis or reduced nascent VLDL uptake as the mechanism for the apparent elevated rate of apo E accumulation, these data coincide with the findings of the present study. Taken together, the data indicate that increased apo E levels in WHHL rabbits are due to both a reduced clearance from the blood and an increased synthesis of this apolipoprotein.

More significant than the apparent apo E catabolic defect in the WHHL rabbit was the fourfold increased apo E production rate in the face of normal apo E mRNA levels in the major synthetic tissues of the WHHL rabbit. The only tissue in the WHHL rabbit that had significantly elevated apo E mRNA levels was the aorta (Figure 3). There is in vivo evidence that macrophages secrete apo E.
in response to stimulation with cholesteryl ester. Although the current study does not establish the role of the aorta in the contribution of plasma apo E concentrations, the elevated apo E mRNA could represent an enhanced macrophage contribution to the concentration of plasma apo E in WHHL rabbits. With the majority of apo E biosynthesis occurring in the liver, it is surprising that there was no elevation in apo E mRNA levels in the WHHL rabbit liver. Normal apo E mRNA levels in major synthetic tissue reduce the possibility of both increased transcriptional rate, as well as the likelihood of apo E mRNA stabilization, as etiologies of the increased apo E biosynthesis. Therefore, the enhanced apo E production may come about by processes subsequent to transcription. There is in vitro evidence for posttranscriptional regulation in eukaryotes. However, the specific site of action has yet to be elucidated. Regulation at the level of translation, intracellular protein transport, intracellular proteolytic processing, covalent protein modification or secretion could account for the increased circulating plasma levels of apo E.

These results combining in vivo apolipoprotein kinetic analysis with assessment of tissue mRNA levels provide the first direct evidence for enhanced apolipoprotein biosynthesis with normal levels of mRNA as a mechanism for the production of elevated levels of plasma apolipoproteins.

References

2. Rosenfeld ME, Tsukada T, Gown AM, Ross R. Fatty streak initiation in Watanabe heritable hyperlipidemic and comparably hypercholesterolemic fat-fed rabbits. Arteriosclerosis 1987;7:24-34
3. Rosenfeld ME, Tsukada T, Chait A, Bierman EL, Gown AM, Ross R. Fatty streak expansion and maturation in Watanabe heritable hyperlipidemic and comparably hypercholesterolemic fat-fed rabbits. Arteriosclerosis 1987;7:24-34
16. Kita T, Brown MS, Bilheimer DW, Goldstein JL. Delayed clearance of very low density and intermediate density lipoproteins with enhanced conversion to low density lipoprotein in WHHL rabbits. Proc Natl Acad Sci USA 1982;79:5693-5697
31. Innerarity TL, Pittas RE, Mahley RW. Binding of arginine-rich (E) apoprotein after recombination with phospholipid
vesicles to the low density lipoprotein receptors of fibroblasts. J Biol Chem 1979;254:4186-4190
32. Hornick CA, Kita T, Hamilton RL, Kane JP, Havel RJ. Secretion of lipoproteins from the liver of normal and Watanabe heritable hyperlipidemic rabbits. Proc Natl Acad Sci USA 1983;80:6096-6100

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