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Effects of pravastatin on cholesterol metabolism of cholesterol-fed heterozygous WHHL rabbits

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1 We administered the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor pravastatin at a daily dose of 1 mg kg^{-1} body weight to cholesterol-fed (0.03%) heterozygous Watanabe heritable hyperlipidaemic rabbits, an animal model for heterozygous familial hypercholesterolaemia.

2 After 12 months of cholesterol treatment, immunohistochemistry with the monoclonal antibody 9D9 was used to detect hepatic low density lipoprotein (LDL) receptors, which were quantified by densitometry. In addition we determined LDL receptor mRNA by competitive reverse transcriptase polymerase chain reaction. The cholesterol precursor lathosterol and the plant sterol campesterol were analysed by gas-liquid chromatography.

3 The drug reduced total plasma cholesterol levels by 51% ($P=0.04$), when compared to the control group. Unexpectedly, hepatic LDL receptor density and mRNA showed no significant differences between the groups. Total plasma levels of lathosterol and campesterol also revealed no significant differences between the groups, if expressed relative to plasma cholesterol.

4 The findings suggest that mechanisms other than induced hepatic LDL receptors are responsible for the cholesterol-lowering effect of pravastatin in this animal model. We propose a reduced cholesterol absorption efficiency compatible with similar campesterol levels between both groups observed in our study.

Keywords: 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors; pravastatin; LDL receptor; lathosterol; campesterol; WHHL rabbit; dietary cholesterol

Introduction

Inhibitors of the rate limiting enzyme in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, are efficient drugs used for the treatment of hypercholesterolaemia (Grundy, 1988; Illingworth, 1993). Clinical studies have shown a significant reduction in cardiovascular morbidity and mortality by HMG-CoA reductase inhibitor therapy (The Pravastatin Multinational Study Group for Cardiac Risk Patients, 1993; Pearson & Marx, 1993; Furberg *et al*., 1994; Scandinavian Simvastatin Survival Study Group, 1994), as well as a beneficial effect on progression of coronary atherosclerosis (Pitt *et al*., 1994; MAAS investigators, 1994; Blankenhorn *et al*., 1993). The lipid-lowering effect of these drugs has been mainly attributed to an increased clearance of low density lipoproteins (LDL) from the circulation due to up-regulation of hepatic LDL receptors (Kovanen *et al*., 1981; Bilheimer *et al*., 1983; Brown & Goldstein, 1986; Reihnér *et al*., 1990). However, additional effects, like reduced absorption efficiency of cholesterol from the intestine (Ishida *et al*., 1988; Miettinen, 1991; Vanhanen *et al*., 1992; Nielsen *et al*., 1993) and a lowered production of very low density lipoprotein apoB may also contribute (Grundy & Vega, 1985). The homozygous Watanabe heritable hyperlipidaemic (WHHL) rabbit represents a well accepted model for familial hypercholesterolaemia (FH), characterized by grossly elevated serum lipoproteins and spontaneous development of atherosclerosis (Kondo & Watanabe, 1975; Watanabe, 1980; Havel *et al*., 1982; Buja *et al*., 1983; Goldstein *et al*., 1983). Since heterozygous FH patients are more frequent than the homozygous form, the combination of a genetic disorder and a potentially atherogenic diet may represent the more relevant model in which to study this disease in animals and draw conclusions with regard to the situation in man (Atkinson *et al*., 1985; 1989; Goldstein & Brown, 1989). Therefore, heterozygous WHHL rabbits were fed a cholesterol-enriched diet (300 p.p.m. wt/wt) supplemented with pravastatin in doses comparable to those used for therapy in man. Previous studies have been done on cholesterol-lowering and antiatherosclerotic effects by pravastatin in WHHL rabbits (Tsujita *et al*., 1986; Watanabe *et al*., 1988; Shiomi *et al*., 1990). Increased hepatic LDL receptor activity in WHHL rabbits was considered to be the main cause of the reduction of plasma cholesterol levels by pravastatin treatment (Kume *et al*., 1989; Kuroda *et al*., 1992). The antiatherosclerotic effect by pravastatin treatment in our animal model has been shown previously (Harsch *et al*., 1997). In this study, we aimed to evaluate the long-term effects of treatment with pravastatin on cholesterol metabolism, determined by hepatic LDL receptor density and mRNA, as well as plasma levels of total cholesterol, lathosterol and campesterol. The cholesterol precursor lathosterol in serum has been shown to be an indicator of whole-body cholesterol synthesis and hepatic HMG-CoA reductase activity (Miettinen, 1970; Björkhem *et al*., 1987; Kempen *et al*., 1988; Meijer *et al*., 1992). The serum levels of plant sterols, like campesterol, reflect cholesterol absorption, since they are not endogenously synthesized and are derived entirely from dietary intake (Tilvis & Miettinen, 1986; Miettinen *et al*., 1990).

Methods

Study design

Eight months old heterozygous WHHL rabbits ($n=12$) from two litters, bred at the animal facilities of the Medical

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Hospital of the University of Hamburg, were matched for sex-, sibling- and total plasma cholesterol level and divided into pravastatin and control group, each consisting of 3 females and 3 males. The duration of the study was 18 months. Animals of the pravastatin group were treated with pravastatin at a daily dose, kg^{-1} body weight, of 0.5 mg for the first 9 weeks and then 1 mg to the end of the study. Pravastatin was added to the diet and daily doses periodically adjusted to body weights. All animals were fed daily with 120 g of a standard rabbit diet, containing 10 p.p.m. wt/wt cholesterol (Altromin No. 2120, Lage, Germany) for the first 6 months and then for the following 12 months 100 g day^{-1} of a cholesterol-enriched diet, containing 300 p.p.m. wt/wt cholesterol (Altromin No. 3/463). Remaining food was weighed, but no significant differences in food intake were found between the groups. Water was available *ad libitum*. Blood samples were taken from the marginal ear vein and plasma was stored at $-20^{\circ}C$ for lipid analysis. All animals were killed by injecting an overdose of a barbituric acid derivative (sodium thiamylal, 40 mg kg^{-1}), and then exsanguinated via the portal vein. Parallel blocks of liver tissues from all animals were fixed in 4% buffered formaldehyde and embedded in paraffin or stored at $-80^{\circ}C$ for further analysis. For reference purposes, LDL receptor mRNA analysis was also performed in 4 normal rabbits (mongrel piebald rabbits, fed the standard diet), killed at the age of 3 months and one homozygous WHHL rabbit. The experimental procedure had been approved by the local ethical committee of animal studies.

LDL receptor analysis

Immunohistochemistry was used to detect hepatic LDL receptor protein by use of the monoclonal antibody 9D9 (Huettinger *et al*., 1984), purified from supernatants of the respective hybridoma cell line (ATCC, Rockville, MD, U.S.A.) and a standard ABC kit (Vector Laboratories Inc., Burlingame, CA, U.S.A.) at a dilution of 1:20 (Figure 1). Quantification of staining intensity was performed with a densitometer (Model 301 X, X-Rite Company, Grand Rapids, MI, U.S.A.); 10 measurements per slide were taken at random points and the mean value calculated. Slides of unstained tissue served as blank.

Total RNA was isolated from liver tissues by use of RNAzol (Cinna/Biotecx Laboratories Inc., Houston, TX, U.S.A.) and quantified by measuring the absorbance at 260 nm. Total LDL receptor mRNA was quantified by a competitive reverse transcriptase polymerase chain reaction (cRT–PCR) assay essentially as described previously (Gebhardt *et al*., 1994). An example is given in Figure 2. Due to sequence differences between the human and rabbit LDL receptor within the primer sequence (Yamamoto *et al*., 1984; 1986), a truncated reverse primer was used (reverse: CTCTCACACCAGTTCACTCCTCT, forward: AAGATGCGAAGATATCGATGAGTG) and annealing was performed at 50°C. The relative expression of wildtype and mutant form of the LDL receptor mRNA in liver tissues of heterozygous WHHL rabbits was analysed by RT–PCR. Primers (reverse: AGGAGCTGTTGCACCGGAAGT, forward: AAGACGTGCTCCCAGGACGAGTT) were chosen to amplify a 157 bp sequence (as in wildtype) around the 12 bp deletion found in the mutated form of the receptor (Yamamoto *et al*., 1986). Before RT–PCR, RNA samples were treated with DNase I to digest any genomic DNA carryover. Reaction conditions were essentially as described above.

Sterol analysis

Total plasma cholesterol levels were determined once before and 12 times during pravastatin treatment by use of a commercially available kit (Boehringer Mannheim, Mannheim, Germany). Concentrations of cholesterol, lathosterol and campesterol were measured in nonsaponifiable material of plasma samples by gas-liquid chromatography (g.l.c.), according to the method of Lang and Schliack, Klinge Pharma Munich (personal communication from Lang, G.). These analyses were performed before pravastatin treatment, after 14 weeks of pravastatin treatment and at the end of the study. The analysed sample volume of plasma contained from 30 to 200 μl. Samples were added to 500 mg anhydrous $NaSO_4$ and 120 μg epicoprostanol (Sigma, Deisenhofen, Germany) as internal standard, and total lipids were extracted with 7 ml hexane-isopropanol 1.5:1 (v/v). The extracts were washed with 1.5 ml lipoprotein buffer (154 mM NaCl, 0.27 mM Titriplex III (Merk, Darmstadt,

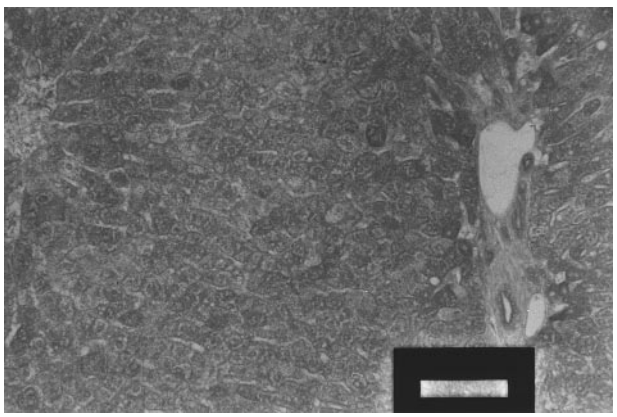

Figure 1 Immunohistochemical detection of the LDL receptor protein in liver tissue from a pravastatin treated heterozygous WHHL rabbit, by use of the monoclonal antibody 9D9, shows slightly granular staining of hepatocytes. Section without staining of cell nuclei to avoid interference with densitometry. (Portal tract in the right and central vein in the upper left). Bar = 100 μm.

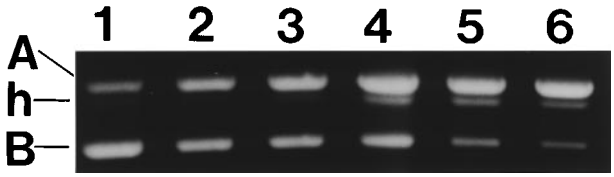

Figure 2 Quantification of total LDL receptor mRNA in the liver of a heterozygous WHHL rabbit from the pravastatin group by use of competetive RT–PCR. Two micrograms of total cellular RNA per reaction were co-reverse transcribed and co-amplified with a titration series of competitor RNA. For competition, we used *in vitro* synthesized RNA, that was homologous to human LDL receptor mRNA, but carries a deletion of 264 bp within the amplified sequence (Gebhardt *et al*., 1994). RT–PCR products of cellular LDL receptor mRNA (957 bp, wildtype and mutant form; A) and competitor RNA (693 bp; B) were analysed on 1.5% agarose gels (10 μl) and stained with ethidium bromide. Bands of equal intensity (lane 2) can be detected at the equivalence point, which defines the amount of specific mRNA in the sample. Lanes 1–6, 2× dilution series of LDL receptor competitor RNA, starting with 1.2×10^7 molecules per reaction. Heteroduplex molecules (h).

Germany), 1.02 mM Natriumazid/NaOH, pH = 7.4) to eliminate polar lipids, evaporated and saponified in methanol-10 M NaOH 10:1 (v/v). The lipophilic components were reextracted with hexane, evaporated and derivatized with 10 μl pyridin (Sigma, Deisenhofen, Germany) and 50 μl N-methyl-N-trimethylsilyl-trifluoracetamide (MSTFA; CS, Langerwehe, Germany). Derivatized sterols were analysed by g.l.c., by use of a Hewlett Packard 5890 Series II chromatograph equipped with a 25 m long, 0.22 mm (i.d.) HT-5 fused silica capillary column (SGE, Weiterstadt, Germany) at 250°C. Hydrogen was used as the carrier gas at a linear velocity of 58 cm s^{-1} and a split ratio of 1:50. Detection was carried out by flame ionization. Since the LDL fraction of serum lipoproteins transports most of the noncholesterol sterols, a reduction in the lipoprotein level could also reduce noncholesterol sterol levels, even without a change in cholesterol synthesis. Therefore, total plasma levels of lathosterol and campesterol are expressed in terms of mg g^{-1} cholesterol (Vanhanen *et al*., 1992).

Total concentrations of cholesterol, lathosterol and campesterol were also determined in samples of the standard diet and the cholesterol-enriched diet by g.l.c. One hundered milligrams of homogenized food samples were preparated and analysed as described for plasma samples above. Results are expressed in parts per million (p.p.m. wt/wt).

Cholesterol concentration of liver tissue was determined as follows: frozen tissues were ground in liquid nitrogen, and then homogenized in 25 μl mg^{-1} tissue of extraction buffer (10 mM Tris/HCl, pH 8.0, 154 mM NaCl, 1 mM EDTA, 1% (v/v) Triton-X 100 (Sigma, Deisenhofen, Germany), 2 mM phenylmethylsulphonylfluoride (PMSF; Sigma, Deisenhofen, Germany) and 10000 KIE aprotinin). After centrifugation at 12000 r.p.m. for 3 min, total cholesterol content of supernatants was determined enzymatically according to Rath *et al.* (1989).

Statistical analysis

Statistical analysis was performed by use of SigmaStat for Windows (Jandel). Data that passed the appropriate constraints of equivalent variances and normal distribution were analysed with unpaired Student's *t* test, while other data were analysed by the Mann–Whitney rank-sum test (two-tailed for LDL receptor expression and total plasma levels of lathosterol and campesterol; one-tailed for total plasma and liver cholesterol levels). *P* values less than 0.05 were considered indicative of a statistically significant difference. Data are presented as means ± s.e.mean.

Results

Effect of pravastatin on plasma sterol levels under standard diet

Initial total plasma levels of cholesterol, lathosterol and campesterol showed no significant differences between the groups. At 14 weeks of pravastatin treatment, total plasma cholesterol levels were slightly lower and total plasma lathosterol levels were lower by 32% ($P = 0.03$) in the pravastatin group, when compared to the control group, whereas total plasma campesterol levels were not significantly different between the groups (Figures 3 and 4). Total plasma campesterol levels in both groups were about 10 fold higher under standard diet than under the cholesterol-enriched diet. Therefore, the total content of campesterol in the different diets was analysed and found to be 5 fold higher in standard diet than in the cholesterol-enriched diet (Figure 4).

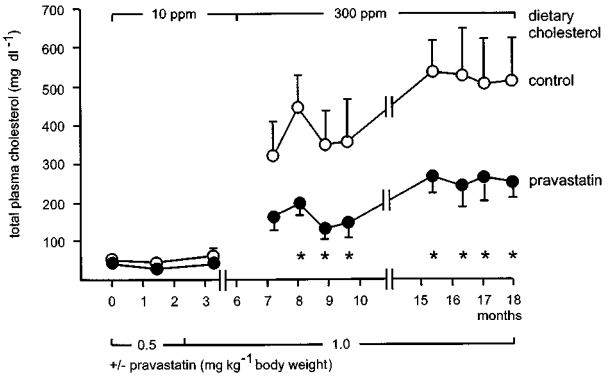

Figure 3 Effect of pravastatin on total plasma cholesterol levels in cholesterol-fed (300 p.p.m.) heterozygous WHHL rabbits. Points represent the mean ($n = 6$ per group) and vertical lines show s.e.mean (included in symbols where not shown). *Denotes significantly lower means, $P < 0.05$, when compared to the control group. Data at 18 months failed equal variance testing and therefore were analysed by Mann–Whitney rank-sum test. Other data were analysed by Student's *t* test.

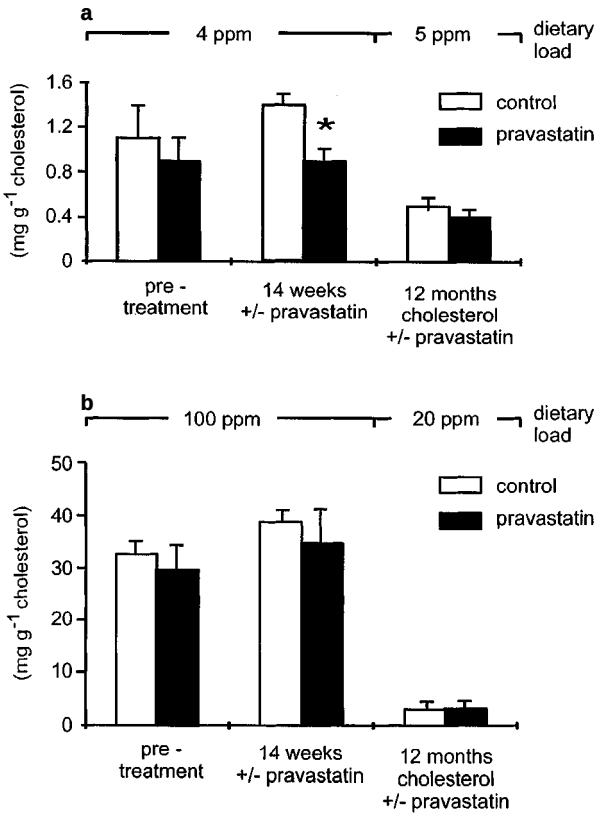

Figure 4 Effect of pravastatin on total plasma lathosterol (a) and campesterol (b) levels in cholesterol-fed (300 p.p.m.) heterozygous WHHL control and pravastatin-treated rabbits. The dietary load of lathosterol (a) or campesterol (b) is indicated above each panel. Columns represent means ($n = 6$ per group) and vertical lines show s.e.means. *Denotes a significant difference, $P < 0.05$, when compared to the control group. Data were analysed by Student's *t* test.

Effect of pravastatin on plasma sterol levels, hepatic LDL receptor density and mRNA under the cholesterol-enriched diet

Total plasma cholesterol levels in the pravastatin group were consistently lower, when compared to the control group (Figure 3). Data on total plasma cholesterol levels of individual animals have been described elsewhere (Harsch *et al*., 1997). After 12 months feeding of the cholesterol-enriched diet, total plasma cholesterol levels and total cholesterol contents of liver tissue in the pravastatin group were lower by 51% ($P=0.04$) and 27% ($P=0.03$), respectively, than in the control group, whereas total plasma lathosterol and campesterol levels were not significantly different between the groups (Figure 4, Table 1). Compared to the standard diet, total plasma lathosterol levels in the groups were lowered by about 55% (control: $P=0.001$, pravastatin: $P=0.003$) under the cholesterol-enriched diet (Figure 4). Analysis of staining intensity of immunohistochemical detection of the LDL receptor protein and total LDL receptor mRNA expression in liver tissues also showed no significant differences between the groups at the end of the study (Table 1). In both groups, both mutant and wildtype form of the LDL receptor mRNA were equally expressed (a typical result is shown in Figure 5). Therefore, we estimated the wildtype LDL receptor mRNA levels in heterozygous WHHL rabbits to be a half of total LDL receptor mRNA levels. The estimated wildtype LDL receptor mRNA levels were lower (2.5 ± 0.4 vs 4.2 ± 0.9 copies pg^{-1} total cellular RNA), when cholesterol-fed heterozygous WHHL control rabbits were compared to normal rabbits under standard diet.

Table 1 Effect of pravastatin on cholesterol levels and LDL receptor expression in cholesterol-fed heterozygous WHHL rabbits

	Control	*Pravastatin*
Total cholesterol		
Plasma (mg dl^{-1})	513 ± 113	$250\pm49^{1,2}$
Liver (mg g^{-1})	9.0 ± 0.4	6.6 ± 1.1^{1}
LDL rceptor protein (density units)	0.11 ± 0.007	0.11 ± 0.012
Total mRNA* (copies pg^{-1} total cellular RNA)	5.0 ± 0.9	4.1 ± 0.7

Values are represented as mean$\pm$s.e.mean ($n=6$ per group). [1]Significantly lower, compared to the control group, $P<0.05$. [2]Data failed equal variance testing and therefore were analysed by Mann–Whitney rank-sum test. Other data were analysed by Student's t test. *To estimate the wildtype mRNA level this value must be divided by two. Values were determined after 12 months feeding the cholesterol-enriched diet (300 p.p.m.).

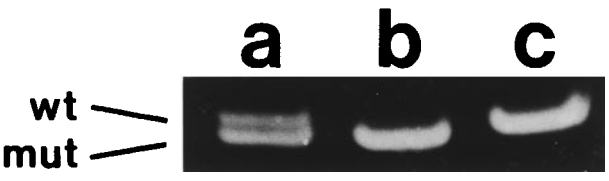

Figure 5 RT–PCR products on a 15% polyacrylamide gel (5 μl) show the expression of wildtype and mutant form of the LDL receptor mRNA, migrating at slightly different levels. Typical result of a cholesterol-fed (300 p.p.m.) heterozygous control WHHL rabbit (a) compared to homozygous WHHL rabbit (b) and normal rabbit (c).

Discussion

The present study showed that total plasma cholesterol levels were lower in pravastatin treated heterozygous WHHL rabbits, when compared to the control group, even on exposure to dietary cholesterol. Despite an increase in the pravastatin dose of animals on a standard diet, the cholesterol-lowering effect remained slight. The increase of total plasma cholesterol levels under cholesterol-enriched diet to the end of the study may reflect the natural age-dependent course of plasma cholesterol levels in heterozygous WHHL rabbits (Esper *et al*., 1993). Previous studies in heterozygous as well as in homozygous WHHL rabbits have shown a cholesterol-lowering effect of pravastatin (Tsujita *et al*., 1986; Watanabe *et al*., 1988; Kume *et al*., 1989; Shiomi *et al*., 1990; Kuroda *et al*., 1992). In these studies much higher doses of up to 50 mg kg^{-1} body weight were given and animals were not fed cholesterol-enriched diets. The cholesterol-lowering effect in these studies has been attributed to the up-regulation of hepatic LDL receptors (Kume *et al*., 1989; Kuroda *et al*., 1992), albeit under the conditions of several weeks. Our data on hepatic LDL receptor density and mRNA at the end of the study did not reveal significant differences between the groups. The findings suggest that in our animal model mechanisms other than induced hepatic LDL receptors are responsible for the cholesterol-lowering effect of pravastatin. Additionally, plasma levels of lathosterol showed no significant differences between the two groups at the end of the study.

Serum contents of cholesterol precursor sterols, like total lathosterol, reflect whole body cholesterol synthesis in man and rabbits (Miettinen, 1970; Kempen *et al*., 1988; Meijer *et al*., 1992). Serum lathosterol levels must be carefully controlled for dietary cholesterol, most likely due to different amounts of lathosterol in the diets (Duane, 1995). In our study, both diets contained nearly equal amounts of lathosterol (Figure 4). In patients with heterozygous FH and in healthy volunteers, serum lathosterol levels have been shown to be reduced during lovastatin and pravastatin treatment (Kempen *et al*., 1988; Miettinen, 1991; Vanhanen *et al*., 1992; Uusituba *et al*., 1992; Vanhanen & Miettinen, 1992; De Cuyper *et al*., 1993). Various reasons may account for the lack of an effect of pravastatin therapy on plasma lathosterol levels at the end of the study. Firstly, whole-body cholesterol synthesis did not reflect inhibition of cholesterol synthesis in this animal model, due to the tissue selectivity of pravastatin, although under standard diet and after drug administration total plasma lathosterol levels were significantly lower. Secondly, during long-term pravastatin treatment the tissues of the body fully compensate for the inhibition of cholesterol synthesis by increasing the expression of HMG-CoA reductase. A lack of effect of long-term lovastatin therapy on whole-body cholesterol synthesis has already been shown (Goldberg *et al*., 1990). Inhibition of HMG-CoA reductase leads to a coordinated induction of a number of genes, including those for the HMG-CoA reductase and the LDL receptor (Brown & Goldstein, 1986). At least, a compensatory mechanism in liver tissue could be excluded, since in a new steady state with increased HMG-CoA reductase activity increased LDL receptor activity would also have been expected to occur. Thirdly, a very low rate of hepatic cholesterol synthesis (Andersen *et al*., 1982; Spady & Ditschy, 1983) and feedback inhibition of both hepatic and extrahepatic cholesterol synthesis, when dietary cholesterol intake is increased, has been shown in rabbits (Andersen *et al*., 1982). A comparison of plasma lathosterol levels under both diets showed that they were lower under the cholesterol-enriched diet, which indicates a lowered cholesterol synthesis. It is

reasonable to assume that in our animal model, a low dose therapy of pravastatin could not decrease cholesterol synthesis further. This explanation is supported by suppressed wildtype LDL receptor mRNA levels under the cholesterol-enriched diet.

In our study, the cholesterol-lowering effect of pravastatin treatment was more prominent during the cholesterol-enriched diet than during the standard diet. Pravastatin might exert its effects not only by inhibiting cholesterol synthesis but also by reducing the efficiency of cholesterol absorption. While the mechanism for reduced cholesterol absorption efficiency by statin treatment has not been elucidated, the latter has previously been described in heterozygous FH patients treated with pravastatin (Miettinen, 1991; Vanhanen *et al*., 1992) and in cholesterol-fed rabbits treated with different HMG-CoA-reductase inhibitors, i.e. simvastatin and lovastatin (Ishida *et al*., 1988; Nielsen *et al*., 1993). Moreover, egg yolk feeding to hypercholesterolaemic subjects treated for 2 years with lovastatin, left total amounts of absorbed cholesterol unchanged (Miettinen, 1991). In contrast, no effect of different HMG-CoA-reductase inhibitors on cholesterol absorption efficiency has been found in normal rabbits and in homozygous WHHL rabbits, fed a standard diet and in non-FH subjects on a diet low in fat and cholesterol (Ishida *et al*., 1988; Amorosa *et al*., 1992; Vanhanen & Miettinen, 1995). The similar campesterol levels between the groups are not in contradiction to a reduced cholesterol absorption efficiency being the most likely explanation for the cholesterol-lowering effect of pravastatin in our study. Serum plant sterols were positively related to cholesterol absorption efficiency, but negatively related to biliary cholesterol secretion (Tilvis & Miettinen, 1986; Gylling & Miettinen, 1988; Miettinen *et al*., 1990). For pravastatin, in addition to a reduced cholesterol absorption efficiency, a markedly reduced biliary cholesterol elimination has been shown in man, which was supposed to be the main cause for the observed small increases of serum plant sterol levels (Miettinen, 1991; Vanhanen *et al*., 1992; Uusituba *et al*., 1992; Vanhanen & Miettinen, 1992).

In conclusion, increased dietary cholesterol intake suppressed cholesterol synthesis and LDL receptors to a degree that could not have been overcome by pravastatin treatment in this animal model, resulting in a lack of effect on whole-body cholesterol synthesis and LDL receptors. Therefore, other mechanisms, such as a reduced cholesterol absorption efficiency, are responsible for the cholesterol-lowering effect of pravastatin in our study.

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