Pravastatin Decreases Serum Lipids and Vascular Cholesterol Deposition in Watanabe Heritable Hyperlipidemic (WHHL) Rabbits

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SUMMARY

The effects of long term administration of pravastatin (a competitive inhibitor of hydroxymethylglutaryl CoA reductase) were assessed by measuring serum lipids and aortic and coronary atherosclerosis in Watanabe Heritable Hyperlipidemic (WHHL) rabbits. Six-month-old WHHL rabbits were given either 50 mg/kg/day of the drug or vehicle. The rabbits were sacrificed following 6 or 12 months of treatment and serum cholesterol and triglycerides and aortic cholesterol and hydroxyproline were measured. Atherosclerotic plaques in the aorta and coronary arteries were quantified with morphometric methods.

Mean serum cholesterol $\pm$ SEM(n) in the control vs. pravastatin groups after 6 months were: 535 ± 34 (11) vs. 411 ± 22 (12) ($p < 0.005$) and after 12 months 458 ± 43 (9) vs. 309 ± 29 mg/dl (12) ($p < 0.005$). In the pravastatin group, percent aortic area covered with plaque and aortic cholesterol content were reduced 35% (ns) and 55% ($p < 0.05$) at 6 months, and 26% (ns) and 44% (ns) at 12 months, respectively. Little difference was found in serum triglycerides and aortic hydroxyproline in the 2 groups. There was strong correlation of serum cholesterol with aortic cholesterol content ($r = 0.61$, $p < 0.003$) and with the percent aortic plaque area ($r = 0.67$, $p < 0.001$), at 12 months. Morphometric analysis of wall thickness and lumen area of major coronary arteries revealed no significant differences in the 2 groups. In conclusion, pravastatin effectively lowered the serum cholesterol level in an animal model defective in low density lipoprotein receptors; this reduction was strongly correlated with amelioration of such atherosclerotic processes as lipid deposition and plaque formation.

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ELEVATED serum cholesterol levels play a major role in the development of atherosclerosis.^{1),2)} A strategy used recently to lower serum cholesterol is the inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (mevalonate: NADP+oxireductase [CoA acylating], EC 1.1.1.34), the enzyme catalyzing the conversion of HMG-CoA to mevalonic acid. Inhibition of this enzyme produces an increase in mRNA for the low density lipoprotein (LDL) receptor in the liver³⁾ which results in increased receptor mediated clearance of LDL,⁴⁾ the major cholesterol transport lipoprotein in human plasma. Endo and coworkers first reported that mevastatin, a fungal metabolite of *Penicillium citrinum*, was a strong competitive inhibitor of HMG-CoA reductase.^{5),6)} This compound was shown to reduce plasma cholesterol levels in several species including humans.⁷⁾ Subsequently, other naturally occurring HMG-CoA reductase inhibitors were isolated, including lovastatin and simvastatin. These compounds are inactive prodrugs that are enzymatically transformed *in vivo* to their metabolically active form.⁸⁾ Pravastatin (SQ 31,000) is a competitive inhibitor of HMG-CoA reductase derived from *Nocardia autotrophica*.⁹⁾ As opposed to mevastatin, lovastatin, and simvastatin, this drug does not require *in vivo* transformation to exert its action, and it is more water soluble than the other analogues.¹⁰⁾

Familial hypercholesterolemia (FH) is an inherited disease in which the LDL receptor pathway is defective. In its homozygous state, FH is characterized by very high levels of serum cholesterol and accelerated atherosclerosis.^{11),12)} An animal model of this disease is the Watanabe Heritable Hyperlipidemic (WHHL) rabbit which develops hyperlipidemia due to a severe deficiency of functional LDL receptors.^{13)–16)} The lack of receptor is responsible for both a reduced uptake of LDL and an overproduction of LDL from intermediate density lipoproteins (IDL), which are also cleared by the LDL receptor.^{11),17)} Thus, WHHL rabbits may also over-produce very low density lipoproteins (VLDL), the precursor of IDL, because hepatic cholesterol synthesis is not suppressed by LDL-receptor mediated events. In the arterial wall, the accumulation of lipids seems to be independent of cellular LDL receptors¹⁸⁾ and WHHL rabbits develop marked aortic atherosclerosis at a premature age.^{13),19),20)} The purpose of these studies was to determine the effects of pravastatin on serum lipids and the atherosclerotic process in an animal model that lacks the LDL receptor.

MATERIALS AND METHODS

Experimental Protocol

Six-month-old homozygous WHHL rabbits weighing between 2.7 and 3.4 kg with serum cholesterol levels equal or higher than 400 mg/dl, were maintained on a reverse light cycle in an environmentally controlled room in individual metal cages and fed a commercial rabbit chow (Lab Rabbit Chow HF 5326, Purina-Ralston, Chicago, IL). All animal procedures conformed to the guidelines of NIH and the American Association for Accreditation of Laboratory Animal Care.

Baseline levels of the variables studied (see below) were obtained in 6 rabbits who were sacrificed before the initiation of the study. In 48 additional rabbits, serum cholesterol levels were measured after an overnight fast. They were then divided into 4 groups of 12 animals each matched for sex and baseline cholesterol levels. Two control groups were given 1 ml saline (pH 7.5) daily by gavage, and 2 experimental groups were given a single daily dose of pravastatin (50 mg/kg) dissolved in saline (pH 7.5) between 9:00 and 10:00 a.m. The dose was adjusted based on monthly weighing. One control and one experimental group were treated for 6 months, while the other control and experimental groups were treated for 1 year. In all groups, serum cholesterol was monitored monthly from blood drawn from the ear vein after an overnight fast. One rabbit in the 6 month and 3 rabbits in the 12 month control groups died prior to completion of the studies. At the end of 6 months or 1 year, animals were fasted overnight and anesthetized with pentobarbital (35 mg/kg body wt) given into the ear vein. Blood was then collected from the abdominal aorta or inferior vena cava. Immediately after exsanguination the thorax was opened and the aorta was excised from the ascending aorta to the iliac artery and rinsed in cold saline. The perivascular fat was then dissected and the aorta opened longitudinally along the anterior wall and kept in saline at 4°C for planimetry.

Serum Cholesterol and Triglycerides Determinations

Serum cholesterol and triglycerides were determined spectrophotometrically utilizing a commercial kit (Ciba Corning kit for cholesterol, Gilford Systems, Oberlin, Ohio, and Roche kit for triglycerides, Roche Diagnostic Systems, Nutley, New Jersey).

Planimetry of Aortic Lesions

Within 3 hours of excision, color slides were taken of the interior surface of the aorta. The photograph was digitized and the proportion of the surface area covered by plaque was determined with the aid of a software package developed at the Laurie Imaging Center of the UMDNJ-Robert Wood John-

son Medical School. This system was developed by Dr. Reuben Mezrich for use with a McIntosh II PC computer interfaced with a video monitor. All determinations were performed by the same investigator.

Determination of Aortic Cholesterol

The aorta was finely minced and subjected to saponification and extraction procedures.²¹⁾ Cholesterol was determined by gas chromatography on a glass column packed with 3% OV-17 on 100–200 mesh gas chrome Q. Temperatures were set at 260°C for the column, 290° for the injection port, and 300°C for the detector. Addition of 5- α -cholestane to the tubes prior to saponification showed that the average recovery of cholesterol was greater than 90%.

Determinations of Aortic Hydroxyproline

After saponification and petroleum ether extraction, the protein in the aqueous phase was hydrolyzed by addition of KOH (3N final concentration) and heated at 110°C for 3 hours in a pressure cooker. The hydrolyzate was adjusted to pH 8–9 with concentrated HCl and aliquots were taken for hydroxyproline assay. Hydroxyproline was determined by the method of Juva and Prockop²²⁾ as modified by Berg²³⁾ using a colorimetric reaction.

Morphometric Determination of Extent of Coronary Artery Atherosclerosis

Hearts were removed from the animals and fixed in 10% formaldehyde solution. Three 3-mm thick cross-sections were made starting just below the atrioventricular junction. The cross-sections were embedded in paraffin and 5 μ m sections were prepared and stained with hematoxylin and eosin and the Verhoeff-Van Gieson technique for elastic fibers.²⁴⁾ The four main coronary arteries (the left anterior descending, circumflex, and septal and the right) were examined histopathologically for atherosclerotic lesions at proximal, middle and distal levels, with a Zeiss Universal Photomicroscope III. The image was then sent through a black and white video camera to a computer coupled with a Quantex QX-7 Image Processing and Analysis System (Quantex Corp., Sunnyvale, CA), digitized, and displayed on a Sony Trinitron video monitor. For each individual coronary artery, the outlines of the external elastic lumina and the lumen were traced (Fig. 1), and the total cross sectional areas (TA) of the artery and its lumen (LA) were quantitated in pixels. The ratio (R) of LA to TA, and the difference between TA and LA, which represents arterial wall cross-sectional area (WA), were calculated to provide a measure of the extent of atherosclerosis for each coronary artery.

Statistical Analysis

All results are expressed as mean $\pm$ SEM(n). Data was compared between groups by analysis of variance or as appropriate, by independent

Fig. 1. Line A traces the external elastic lamina and L indicates the actual luminal outline. The areas enclosed by line A and boundary L represent total arterial and luminal cross-sectional areas and are designated TA and LA, respectively, in Table II. Modest mononuclear cell infiltrate is seen in the adventitial surface exterior to line A. The observed focal intimal thickening is included in the calculation of arterial wall thickness. Verhoeff-Van Gieson elastic stain $\times 186$.

samples t-test. All analyses were performed using a SAS statistical software package (SAS Institute, Gary, Indiana).²⁵⁾

RESULTS

The data for serum cholesterol and triglycerides from the 6 month and 1 year treatment groups were combined and are shown in Fig. 2. Both serum cholesterol and triglycerides decreased with age. Treatment with pravastatin significantly decreased serum cholesterol when compared to control animals ($p < 0.001$, Fig. 2a). At the end of the first 6 months mean serum cholesterol levels (the average of all monthly determinations) in the control vs. the experimental group were 535 ± 34 (11) and 411 ± 22 (12) mg/dl, respectively, $p < 0.005$. The corresponding values after 1 year of treatment were 458 ± 43 (9) and 309 ± 24 (12), $p < 0.005$. Serum triglyceride levels in treated and untreated groups did not differ significantly (Fig. 2b). There were no differences in rabbit weights between the control and treated groups after 6 and 12 months of the protocol.

The percent of the aortic surface area covered with plaques (planimetry) increased with age in control WHHL rabbits (Fig. 3a). Pravastatin de-

Fig. 2. Effect of pravastatin on serum cholesterol (a) and triglycerides (b). WHHL rabbits were given an oral daily dose of pravastatin (50 mg/kg) or carrier for 6 months or 1 year. At the specified times, serum cholesterol and triglycerides were determined. In each treatment group, 24 animals were studied for 6 months and 12 animals for 24 months. Values are expressed as percent of baseline value which were determined before initiation of the treatment. Baseline values were 662 ± 25 mg/dl for cholesterol and 287 ± 19 mg/dl for triglycerides ($m \pm SEM$). Vertical bars represent SEM. Statistical analysis was done by ANOVA with repeated measurements. Differences between groups were statistically significant in the case of cholesterol ($p < 0.01$). NS = not significant.

Fig. 3. Effect of pravastatin on percent of aortic surface with plaque as measured by planimetry (a) and on aortic cholesterol (b) in WHHL rabbits. Control animals were given carrier alone. Baseline values were obtained from animals sacrificed before the initiation of treatment. Bars represent mean $\pm$ SEM. Numbers in parentheses represent number of animals. $^{\circ} P < 0.05$ with respect to control. $^{*} P < 0.01$ with respect to baseline. $^{\dagger} P < 0.001$ with respect to baseline.

creased the plaque area by 35% at 6 months and 26% after 1 year but the differences did not reach statistical significance. Aortic cholesterol increased markedly with age in the control group (Fig. 3b). Pravastatin significantly ($p < 0.05$) reduced aortic cholesterol by 55% at the end of the 6-month study. The drug also reduced aortic cholesterol in the 1-year study by 45%, but the decrease failed to reach statistical significance. Aortic hydroxyproline levels were 7.9 ± 0.8 mg/g (6) at baseline; 6.6 ± 0.82 (11) and 5.7 ± 0.63 (12) at 6 months and 9.0 ± 1.0 (9) and 8.2 ± 0.6 (12) at 12 months for control and

Table I. Correlation of Aortic Cholesterol and Planimetry with Serum Cholesterol over 1 Year

Vessel Parameter	Mean	Serum Cholesterol		
		mg/dl	r	p
Aortic cholesterol (mg/g wet wt)	7.6± 6.7%	380±39 ¹	0.62	<0.003
		288±39 ²	0.52	<0.03
Planimetry (% aorta covered by plaque)	36.6±31.4%	380±39 ¹	0.67	<0.001
		288±35 ²	0.62	<0.01

¹ Mean of monthly averages. ² Mean of final value.

Table II. Morphometric Analysis of Coronary Atherosclerosis in Left Circumflex Artery

Arterial Areas	Proximal Section		Distal Section	
	Control	Pravastatin	Control	Pravastatin
LA	29.5±4.3	27.0±5.0	18.1±6.2	11.2±2.1
TA	57.2±5.1	62.2±8.2	33.7±9.4	29.1±4.9
R	0.5±0.1	0.4±0.1	0.5±0.1	0.4±0.0
WA	27.7±2.5	35.1±5.6	15.5±3.7	17.8±3.0

LA and TA are the luminal and total arterial cross-sectional areas, respectively, measured in pixels ($m \pm SEM \times 10^{-3}$). R is the ratio of LA/TA. WA is arterial wall area (TA-LA). Number is 8 for the control group and 12 for the pravastatin group. See text for details.

pravastatin groups, respectively. None of these differences were significant.

There were significant positive correlations in the 1-year study between serum cholesterol levels and either aortic cholesterol levels or the proportion of the aortic surface covered by plaque [Table I]. These correlations were found in both control and treated animals, suggesting that these factors are mechanistically related. Microscopic inspection of the four coronary arteries from each heart examined at 3 levels showed atherosclerotic plaques in only 2 of 8 controls and 4 of 12 of the treated group. There was wide variation in the severity of the lesions and no qualitative differences were apparent between groups. Cross-sectional area analysis confirmed this marked variation and lack of differences as shown by the representative results in Table II from the left circumflex coronary artery.

DISCUSSION

WHHL rabbits lack functional LDL receptors and are animal models of homozygous FH.¹³⁾⁻¹⁶⁾ These animals develop atherosclerosis which is histologically similar to that in humans.¹⁹⁾ The hyperlipidemia has been ascribed to deficient removal of LDL from the circulation²⁰⁾ and an increased

conversion of VLDL and IDL to LDL. Inhibition of cholesterol synthesis by lovastatin has been shown to stimulate LDL receptor activity in normal animals,^{3),26),27)} which is preceded by an increase in receptor mRNA.⁴⁾ Whether pravastatin will similarly influence LDL receptor activity of WHHL rabbits thereby modulating clearance of LDL has not been definitely shown. This problem was not the scope of the present study.

Though the hypolipidemic effects of bile chelating resins have been ascribed to the induction of LDL receptors,^{26),27)} treatment of patients with homozygous FH with these agents or nicotinic acid results in regression of xanthomatous lesions.^{28),29)} These observations suggest that mechanisms other than the up regulation of LDL receptors could be involved. Thus, we undertook the present studies to determine the effects of a competitive inhibitor of cholesterol synthesis on serum cholesterol and atherosclerosis in the animal model of homozygous FH.

Cholesterol levels in treated and untreated animals decreased with age. This age-related effect has been shown previously.^{30),31)} Pravastatin consistently lowered serum cholesterol levels ($p < 0.001$, Fig. 1a) when given in a single, daily oral dose of 50 mg/kg body weight. A dosage of 5 mg/kg of the HMG-CoA reductase inhibitor, lovastatin, has been shown to significantly reduce plasma cholesterol ($p < 0.005$) in WHHL rabbits but not the extent of aortic atherosclerosis.³²⁾ By comparison, the clinically effective dosage of these agents to lower plasma cholesterol in humans varies from 10–50 mg/day.

Concurrent studies in our laboratory have shown that hepatic cholesterol synthesis *in vivo* in WHHL rabbits is significantly reduced by pravastatin for up to 24 hours at the same dose utilized in these experiments.³³⁾ Recently, Arad et al showed that lovastatin decreases synthesis of apolipoprotein B.³⁴⁾ Wyne et al³⁵⁾ reported that lovastatin reduced apolipoprotein E secretion from rat granulosa cells. Considered together, these studies suggest that inhibition of HMG-CoA reductase may alter apolipoprotein B & E regulation resulting in decreased formation of cholesterol rich lipoproteins. This may account for the pravastatin effect in these animals.

Although triglyceride levels were not significantly reduced by pravastatin treatment, levels in the treated animals were on the average lower than in controls (Fig. 2b). Tsujita et al⁹⁾ suggested that treatment with the drug produces a substitution of cholesterol by triglycerides in VLDL, a hypothesis that may explain the lack of significant effects of pravastatin on serum triglyceride levels.

Of the three parameters for aortic atherosclerosis that we monitored, aortic cholesterol showed the steepest rise over the period of the study. The effect of pravastatin treatment was most apparent for this parameter. Our

data do not preclude a direct inhibitory effect of pravastatin on aortic cholesterol synthesis. However, the correlations found between serum cholesterol and aortic cholesterol and between serum cholesterol and the percent of the aortic surface area showing plaque indicate that serum cholesterol is the determinant of aortic atherosclerosis in these animals. This is consistent with the demonstration by Carew et al³²⁾ that modified LDL cholesterol is degraded by macrophages in the wall of the aorta of WHHL rabbits, resulting in the formation of foam cells laden with cholesterol esters. This process is attenuated by drugs which reduce plasma cholesterol, but a further reduction in aortic atherosclerosis may require agents which prevent oxidative modification of LDL cholesterol.

Watanabe et al³¹⁾ reported that pravastatin decreased coronary atherosclerosis and xanthoma formation, but had no significant effect on aortic atherosclerosis in WHHL rabbits. These inbred rabbits had much higher baseline levels of cholesterol than ours and were treated earlier (at the age of 2–3 months) and for only 24 weeks. Our control rabbits showed minimal evidence of coronary atherosclerosis, indicating a marked variability in the severity and distribution of lesions in this animal model. However, the studies are in general agreement and indicate that inhibition of cholesterol synthesis by HMG-CoA reductase inhibitors is effective in decreasing serum lipids and vascular cholesterol deposition, even in animals that lack LDL receptors.

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