Attenuation of plasma low density lipoprotein cholesterol by select 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors in mice devoid of low density lipoprotein receptors

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Abstract Low density lipoprotein (LDL) reduction independent of LDL receptor regulation was investigated using HMG-CoA reductase inhibitors in LDL receptor-deficient mice. In males, LDL cholesterol dose-dependently decreased with atorvastatin treatment after 1 week. As untreated mice grew older, their LDL cholesterol progressively rose above basal levels, but was quelled with atorvastatin treatment. In females, atorvastatin treatment time-dependently decreased LDL cholesterol levels and induced hepatic HMG-CoA reductase activity. Unlike males, cholesterol-lowering effects of the drug were sustained in females. Lovastatin, simvastatin, and pravastatin also reduced total and LDL cholesterol; however, additional studies in females demonstrated that atorvastatin caused the greatest dose-dependent and sustained effect after 2 weeks. In females, hepatic HMG-CoA reductase mRNA inversely correlated with LDL cholesterol lowering, with atorvastatin showing the greatest increase in mRNA levels (17.2-fold), followed by lovastatin (10.7-fold), simvastatin (4.1-fold), and pravastatin (2.5-fold). Atorvastatin effects on lipoprotein production were determined after acute (1 day) or chronic (2 week) treatment prior to intraperitoneal injection of Triton WR1339. Acute treatment reduced cholesterol (−29%) and apoB (−16%) secretion, with no change in triglyceride secretion. In contrast, chronic treatment elevated cholesterol (+20%), apoB (+31%), and triglyceride (+57%) secretion. Despite increased cholesterol and apoB secretion, plasma levels were reduced by 51% and 46%, respectively. Overall, under acute or chronic conditions, apoB paralleled cholesterol secretion rates, and triglyceride to cholesterol secretion ratios were elevated by 38% and 32%, respectively. We propose that atorvastatin limits cholesterol for lipoprotein assembly, which is compensated for by triglyceride enrichment. In addition, with either acute or chronic atorvastatin treatment, apoB-100 secretion was blocked, and compensated for by an increased secretion of apoB-48. The apoB-48 particles produced are cleared by LDL receptor-independent mechanisms, with an overall effect of reducing LDL production in these mice. These studies support the idea that HMG-CoA reductase inhibitors modulate lipoprotein levels independent of LDL receptors, and suggest they may have utility in hyperlipidemias caused by LDL-receptor disorders.—**Bisgaier, C. L., A. D. Essenburg, B. J. Auerbach, M. E. Pape, C. S. Sekerke, A. Gee, S. Wölle, and R. S. Newton.** Attenuation of plasma low density lipoprotein cholesterol by select 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors in mice devoid of low density lipoprotein receptors. *J. Lipid Res.* 1997. **38:** 2502–2515.

Supplementary key words atorvastatin • lovastatin • pravastatin • simvastatin

Homozygous familial hypercholesterolemia (FH) is a rare vascular disease caused by defective or deficient LDL receptors and is characterized by marked hypercholesterolemia, tendon xanthomas, arcus corneae, and life expectancy of less than 30 years due to myocardial infarction (1–3). Treatments include plasmapheresis, LDL-apheresis, combined liver and heart transplants, and experimental trials of LDL receptor replacement by gene therapy (4–8). As up-regulation of LDL receptors is thought to be the major mechanism of action of HMG-CoA reductase inhibitors, their effectiveness in LDL receptor-deficient subjects would not be anticipated (1, 9, 10). In contrast, however, we recently and unexpectedly discovered that atorvastatin lowered plasma LDL cholesterol in dietary casein-induced pre-

Abbreviations: ANOVA, analysis of variance; HDL, high density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HPGC, high performance gel-filtration chromatography; LDL, low density lipoprotein; TBS, Tris-buffered saline; VLDL, very low density lipoprotein.

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established hypercholesterolemia in normal rabbits through the direct inhibition of LDL production and not enhanced clearance (11). In addition, studies of Grundy and Vega (12), Vega, East, and Grundy (13, 14) and Arad, Ramakrishnan and Ginsburg (15, 16) have suggested that lovastatin can act, in part, by limiting LDL production in specific human hyperlidemias. More recently, Shiomi et al. (17) have shown that fluvastatin sodium modestly reduced VLDL production in the Watanabe heritable hyperlipidemic (WHHL) rabbit. However, the WHHL rabbit expresses about 2% of the normal amount of LDL receptors, and therefore an effect on LDL receptor up-regulation cannot be totally ruled out as partially responsible for cholesterol lowering. Similarly, of the three FH subjects studied by Arad et al. (15) one was heterozygous, another LDL receptor defective, while the third subject was not classified. To directly test the hypothesis that HMG-CoA reductase inhibitors can lower LDL cholesterol independent of functional LDL receptors, we treated LDL receptor-deficient mice with various concentrations of vastatins administered as diet admixtures and then measured changes in their lipoprotein profiles. In addition, the acute and chronic effects of atorvastatin administration on cholesterol, triglyceride, and apoB secretion rates and apoB forms in these mice were also determined. Although this mouse model has allowed us to study effects of the vastatins on LDL cholesterol levels and production independent of the LDL receptor, inherent differences in hepatic apolipoprotein B mRNA editing (18), lipoprotein production as well as differences in lipoprotein metabolism between mice and humans may limit the interpretation of how these compounds act to lower LDL cholesterol in this mouse model and humans.

METHODS

Mice

Breeding pairs of low density lipoprotein receptor-deficient mice originally created by Ishibashi et al. (19, 20) were obtained from Jackson Laboratories and a colony was established and maintained at Parke-Davis. Mice were maintained with up to 5 mice per cage on chow and water ad libitum in temperature-controlled rooms on a 12 h light/12 h dark cycle (lights on at 6 AM) prior to study. Mice were assigned to be either in control groups fed ground rodent chow alone or to groups fed chow such that approximately 10, 30, 100, or 300 mg/kg vastatins were consumed daily as a diet admixture.

Vastatins

Atorvastain was prepared at Parke-Davis (21). Lovastatin (Merck, Sharp & Dohme) was prepared by extracting formulated capsules of Mevacor (22). Pravastatin was provided by Bristol-Meyers Squibb and simvastatin was provided by Merck, Sharpe & Dohme.

Blood sampling

Blood samples (100–200 μl) prior to and after drug treatment were collected at 8 AM in 0.04% EDTA and 0.01% sodium azide from mice while under Metofane (Pitman-Moore, Mundelein, IL) anesthesia after an 8–10-h fast. For production studies, non-fasting blood samples were collected before (at 9 PM) and after (9 AM) Triton WR 1339 treatment as described below.

Determination of plasma lipids

Plasma total cholesterol was determined enzymatically by the method of Allain et al. (23) and plasma triglycerides were determined with a commercially available kit (Triglyceride G Kit #997-69801 Wako Pure Chemical Industries, Ltd., Richmond, VA) using a microtiter plate method (24). The reagent kit measures plasma free glycerol after hydrolysis of triglycerides. Values were not corrected for the presence of plasma free glycerol. Distribution of cholesterol among plasma lipoproteins was determined by on-line continuous monitoring of post-column eluant after Superose 6 high performance gel-filtration chromatography (HPGC) of 5 μl individual or 5 μl pooled plasma on a high pressure liquid chromatography system (Rainin Instrument Co., Inc., Woburn, MA) as previously described (25, 26). Peaks were collected and areas were integrated and averaged using Dynamax and Compare Module Software (Rainin Instrument Co., Inc., Woburn, MA) developed for Macintosh computers. Lipoprotein cholesterol was determined from independent total cholesterol determinations and percent area distribution of cholesterol determined by HPGC. In studies where samples were pooled for HPGC, plasma total cholesterol was determined on both the pools as well as the individual samples. In these studies, the pooled plasma total cholesterol determinations (which were essentially identical to those obtained from the average of the individual determinations) were used to determine cholesterol in the lipoprotein fractions. To verify that the two major plasma cholesterol peaks separated by the HPGC procedure corresponded to LDL and HDL, a pooled plasma sample from untreated female LDL receptor-deficient mice was gel-filtered, and individual fractions were collected and analyzed for apoB, apoA-I (27), and total cholesterol. Essentially all the apoB eluted in the first major cholesterol peak, while

all the apoA-I eluted in the second major peak (data not shown).

Determination of plasma apoB

Plasma apoB was determined by an immunoturbidometric assay as previously described (27). The difference in plasma turbidity (Δ340) measured immediately after treatment and after overnight incubation with sheep anti-mouse apoB antiserum is directly linear to the amount of mouse plasma added to the assay. For each sample, turbidity change was assessed at multiple plasma volumes (1–4 μl) to assure detection in the linear portion of the response curve. ApoB data are arbitrarily expressed as change in OD340.

Determination of hepatic HMG-CoA reductase activity

Hepatic HMG-CoA reductase activity was determined in microsomes prepared from livers of control and atorvastatin-treated mice (28). Briefly, to prepare microsomes, mice were killed and livers were excised and then homogenized in 5 mL of cold 0.3 M sucrose, 5 mM EGTA, 5 mM dithiothreitol, 53 mM leupeptin, pH 7.4, with 10 strokes of a tight-fitting Potter-Elvehjem homogenizer. The homogenates were centrifuged for 12,000 *g* for 15 min; decanted supernatants were respun at 12,000 *g* for 15 min, and supernatants were centrifuged at 100,000 *g* for 1 h to prepare microsomal-rich pellets. The microsomal pellets were resuspended in 200 mM phosphate-buffered saline, pH 7.4, and frozen immediately in liquid nitrogen, and assayed within 1 month. HMG-CoA reductase activities were determined as previously described by Kita, Brown, and Goldstein (28).

Determination of hepatic HMG-CoA reductase mRNA levels

Hepatic RNA was isolated with RNAzol (Cinna/Biotecx, Inc., Houston, TX) and assessed for any degradation by agarose electrophoresis (29). A partial cDNA clone for mouse HMG-CoA reductase (30) was isolated by PCR. The amplified product of approximately 250 base pairs was obtained by performing PCR with mouse liver cDNA (Clontech, Palo Alto, CA) as template and conserved primers for HMG-CoA reductase which were described previously (31). The reaction buffer contained 60 mM Tris-HCl, 15 mM ammonium sulfate, 2 mM $MgCl_2$, pH 8.5, and the 35 cycles were run at 94°C for 15 sec and 60°C for 30 sec. The amplified product was cloned into pCRII (Invitrogen, San Diego, CA) and sequenced. The cDNA was found to be 100% identical to mouse HMG-CoA. A specific antisense riboprobe and an internal standard RNA for mouse HMG-CoA reductase were synthesized and mRNA levels were determined by an internal standard/RNAse protection assay essentially as described previously in detail (31, 32). The protection assay contained 30 μg of total liver RNA and 10 pg of internal standard RNA. Autoradiographic images were analyzed using the Molecular Dynamics 400E PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Lipoprotein production studies in female LDL receptor-deficient mice

For lipoprotein production studies, Triton WR 1339 was used to block plasma lipoprotein clearance, thereby allowing measurement of cholesterol, triglyceride, and apoB accumulation (i.e., secretion rate) in plasma (33, 34). Pilot studies demonstrated linear accumulation of triglycerides in these mice after intraperitoneal administration of 0.9 mg Triton WR 1339/g body wt up to 12 h (not shown). Female LDL receptor-deficient mice were acclimated to eating ground rodent chow prior to the study. Studies were carried out to test the acute (1 day) and chronic (2 week) effects of atorvastatin. Prior to the initiation of the studies, a baseline blood sample (under Metofane) was obtained at 9 PM in the fed state. To determine the acute effects of atorvastatin, ground chow alone or chow containing the equivalent of 300 mg/kg per day atorvastatin was placed in cages in the morning (9 AM). At 9 PM that same day (e.g., 3 h after dark cycle start), a blood sample (under Metofane) was obtained, and mice were then administered intraperitoneal Triton WR 1339 (0.9 mg/g body wt) under Metofane. Mice were returned to their cages without removal of food. Additional blood samples were obtained at 9 AM the following day (under Metofane). For the chronic effects of atorvastatin, mice had access to ground chow alone or chow containing the equivalent of 300 mg/kg per day atorvastatin for 2 weeks, when blood samples were obtained before (at 9 PM) or 12 h post-Triton WR 1339 administration (at 9 AM).

In addition, immunoblot analysis (35, 36) of apoB-100 and apoB-48 in acute atorvastatin, chronic atorvastatin, or control treatment groups was carried out before and after Triton WR-1339 injections. Briefly, pooled plasma (0.7 μl) proteins were separated on a 4–12% sodium dodecyl sulfate polyacrylamide gel (Novex, San Diego, CA) and transferred to a nitrocellulose membrane. After blocking with 3% gelatin in Tris-buffered saline, pH 7.4 (TBS), membranes were incubated with a 1:5000 dilution of polyclonal sheep anti-mouse apoB serum in 10% fecal calf serum in TBS, washed with TBS, and then incubated with a 1:5000 dilution donkey anti-sheep IgG conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) in 10% fetal calf serum in TBS. After TBS washing, the membrane was developed using a bromochloroindolyl phosphate/nitro blue tetrazolium substrate.

Statistical analysis

Statview (Abacus Concepts, Inc., Berkeley, CA) developed for Macintosh computers was used to statistically analyze data. Data were analyzed by *t*-tests, where P values <0.05 were considered statistically significant. For multiple group comparisons, analysis of variance (ANOVA) and post-hoc Fisher's protected least significance difference analysis was used to analyze data.

RESULTS

Baseline cholesterol values from age-matched, fasted chow-fed female (89 ± 1 days old, n = 50) and male (90 ± 2 days old, n = 50) LDLr−/− mice are shown in **Fig. 1**. Female mice had significantly higher total plasma cholesterol (219 ± 7 mg/dl) than males (192 ± 6 mg/dl), which was largely reflected by significantly elevated LDL cholesterol (149 ± 6 mg/dl versus 91 ± 4 mg/dl) and reduced HDL cholesterol (59 ± 2 mg/dl versus 91 ± 2 mg/dl). This marked difference between females and males under basal conditions can also be appreciated as the significantly reduced ratio of HDL cholesterol to VLDL plus LDL cholesterol in the females (0.404 ± 0.016) compared to the males (0.995 ± 0.039). No differencs in VLDL cholesterol was observed between the genders; however, triglycerides were significantly reduced in females (67 ± 2 mg/dl) compared to males (99 ± 4 mg/dl).

To investigate the role atorvastatin might have on plasma lipoprotein cholesterol levels in these mice, males were treated for 3 weeks with various doses of atorvastatin and bled weekly for determination of plasma lipoprotein cholesterol levels and distribution (**Table 1**). In control mice fed chow without drug, total plasma cholesterol and specifically LDL cholesterol progressively rose over 3 weeks compared to the basal bleed (Table 1). A similar effect on plasma lipoprotein cholesterol was observed at the lowest dose of atorvastatin (10 mg/kg per day) tested. At 30 mg/kg per day, atorvastatin neutralized the time dependent rise in plasma cholesterol. At the two highest atorvastatin doses used (100 and 300 mg/kg per day), both total and LDL cholesterol were reduced. A reduction in HDL cholesterol was also observed at these higher doses. At 300 mg/kg per day, both total and LDL cholesterol were markedly reduced by 36% at 1 week compared to the level observed at 2 (13 and 9% reduction for total and LDL cholesterol, respectively) and 3 weeks (10% reduction for total and a 1% increase for LDL cholesterol, respectively). Thus, the mice were able to compensate for the drug effect, possibly by increased drug inactivation or induction of HMG-CoA reductase.

Studies were initially performed in male mice (Table 1); however, because of potential compensatory mechanisms to inactivate HMG-CoA reductase inhibitors, as demonstrated for simvastatin in male rodents (37), we sought to determine atorvastatin's effectiveness in females. We chose the 300 mg/kg dose as this appeared to be initially most effective in the males despite its decreased effectiveness with time. At the time of killing, liver biopsies were removed for isolation of microsomes for determination of HMG-CoA reductase activity. In addition, single male mice were also treated for 1, 2, or 3 weeks with 300 mg/kg per day atorvastatin for determination of hepatic microsomal HMG-CoA activity. Prior to atorvastatin treatment, the plasma lipoprotein cholesterol levels (**Table 2**) were typical of female mice (Fig. 1) having more LDL than HDL. Similarly, as was previously observed in the males at 1 week, atorvastatin treatment at 300 mg/kg per day reduced total and LDL cholesterol. However, unlike males, this reduction was more pronounced and sustained at 2 and 3 weeks in

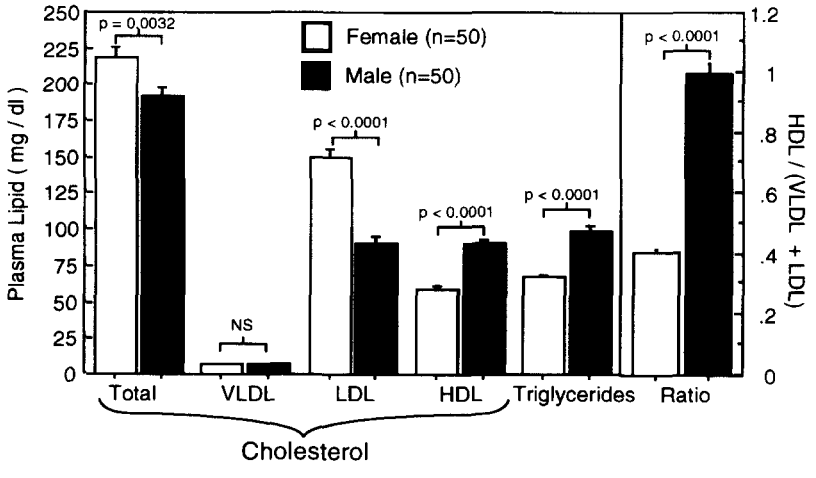

Fig. 1. Fasting plasma lipid levels in female and male LDL receptor-deficient mice maintained on chow diets. Fifty male (90 ± 2 day old) and 50 female (89 ± 1 day old) LDL receptor-deficient mice maintained on chow were bled after an 8-h fast to determine baseline lipid values. Lipid values representing mean ± standard error of the mean were determined as described in Materials and Methods. Data were analyzed by a two-tail unpaired *t*-test.

TABLE 1. Effect of atorvastatin on lipoprotein cholesterol and triglycerides in male LDL receptor-deficient mice

Atorvastatin	Basal	1 Week	2 Weeks	3 Weeks
mg/kg/day		*mg/dL*		
Total plasma cholesterol				
0	142 ± 11	138 ± 8 (−3)	159 ± 19 (+12)	201 ± 11 (+42)[c]
10	197 ± 13	202 ± 8 (+3)	242 ± 16 (+23)[a]	255 ± 13 (+29)[c]
30	164 ± 13	159 ± 6 (−3)	181 ± 9 (+10)	166 ± 9 (+1)
100	158 ± 4	130 ± 9 (−18)	129 ± 9 (−18)	124 ± 13 (−22)[a]
300	164 ± 5	105 ± 11 (−36)[c]	143 ± 8 (−13)	147 ± 18 (−10)
VLDL cholesterol				
0	3.9 ± 0.8	4.0 ± 0.5 (+1)	6.1 ± 1.7 (+57)	8.4 ± 2.3 (+115)[c]
10	6.2 ± 0.2	6.4 ± 0.3 (+4)	6.2 ± 0.6 (+1)	10.4 ± 0.6 (+67)[a]
30	3.5 ± 0.3	4.1 ± 0.4 (+15)	4.6 ± 0.4 (+30)	5.6 ± 0.7 (+60)
100	4.0 ± 0.3	5.0 ± 0.5 (+23)	5.4 ± 0.9 (+33)	5.7 ± 0.5 (+41)
300	4.1 ± 0.5	9.6 ± 1.9 (+123)[d]	5.2 ± 1.5 (+27)	14.1 ± 1.8 (+242)[c]
LDL cholesterol				
0	68 ± 9	67 ± 9 (−1)	79 ± 11 (+16)	121 ± 11 (+78)[c]
10	88 ± 8	94 ± 4 (+7)	118 ± 9 (+34)[a]	142 ± 8 (+62)[c]
30	82 ± 4	78 ± 7 (−5)	90 ± 7 (+10)	86 ± 4 (+5)
100	67 ± 3	56 ± 7 (−17)	58 ± 6 (−14)	55 ± 8 (−18)
300	75 ± 5	48 ± 7 (−36)[a]	68 ± 6 (−9)	76 ± 9 (+1)
HDL cholesterol				
0	68 ± 7	64 ± 2 (−6)	72 ± 8 (+6)	67 ± 1 (−1)
10	99 ± 9	99 ± 10 (0)	115 ± 11 (+16)	99 ± 11 (0)
30	78 ± 9	75 ± 7 (−4)	84 ± 5 (+7)	71 ± 6 (−10)
100	84 ± 5	67 ± 7 (−20)[a]	64 ± 7 (−24)[b]	61 ± 8 (−28)[c]
300	84 ± 2	46 ± 5 (−45)[c]	69 ± 5 (−18)	56 ± 8 (−33)[c]
HDL/(VLDL + LDL)				
0	0.98 ± 0.14	0.94 ± 0.13 (−4)	0.87 ± 0.08 (−11)	0.53 ± 0.06 (−45)[c]
10	1.07 ± 0.11	1.01 ± 0.13 (−6)	0.94 ± 0.10 (−12)	0.65 ± 0.06 (−39)[c]
30	0.91 ± 0.10	0.96 ± 0.15 (+5)	0.91 ± 0.10 (−1)	0.77 ± 0.05 (−16)
100	1.19 ± 0.10	1.17 ± 0.16 (−2)	1.07 ± 0.16 (−11)	1.03 ± 0.15 (−14)
300	1.07 ± 0.08	0.82 ± 0.08 (−24)[a]	0.97 ± 0.12 (−10)	0.62 ± 0.03 (−42)[c]
Plasma triglycerides				
0	75 ± 7	67 ± 7 (−11)	120 ± 17 (+60)[c]	65 ± 4 (−13)
10	94 ± 7	76 ± 5 (−19)	83 ± 10 (−12)	76 ± 1 (−19)
30	63 ± 6	57 ± 4 (−10)	65 ± 5 (+3)	59 ± 4 (−6)
100	72 ± 3	58 ± 2 (−19)	73 ± 6 (+1)	75 ± 6 (+4)
300	74 ± 5	82 ± 9 (+11)	62 ± 9 (−16)	78 ± 2 (+5)

Dose-dependent effects of atorvastatin in male LDL receptor-deficient mice. Mice were fed chow alone (n = 4) or chow containing atorvastatin such that approximately 10 (n = 5), 30 (n = 5), 100 (n = 5), or 300 (n = 4) mg drug/kg body weight was consumed daily. Values represent mg/dL ± SEM. Numbers in parenthesis represent percent change from basal level. Data were analyzed by ANOVA of the percent changes from pretreatment (basal) values

[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.005$; [d] $P < 0.0005$; [e] $P < 0.0001$, compared to basal value.

the females (Table 2) compared to males (Table 1). Determination of hepatic microsomal HMG-CoA reductase activity in the females demonstrated a marked induction of the enzyme as early as 1 week, reaching levels greater than 10 times that of untreated females by 3 weeks (**Fig. 2**). Hepatic microsomes prepared from single male mice after 1, 2, and 3 weeks of 300 mg/kg per day atorvastatin treatment also suggested a time-dependent rise in HMG-CoA reductase activity (not shown).

The observation that atorvastatin reduced total and LDL cholesterol in LDL receptor-deficient mice implies that LDL production might be impeded by HMG-CoA reductase inhibitors. LDL lowering by an HMG-CoA reductase inhibitor through a mechanism unrelated to LDL receptor elevation is not unique. Indeed, kinetic studies, albeit in LDL receptor-competent animals, have suggested a potential block in LDL production by HMG-CoA reductase inhibitors (11–16). To determine whether LDL reduction is a class specific effect, LDL receptor-deficient mice were treated for 1 week with 300 mg/kg per day with various HMG-CoA reductase inhibitors, and their plasma lipoprotein cholesterol distributions were compared to pretreatment levels (**Fig. 3**). All treatment groups consisted of three males and three females, except the pravastatin group in which there were two males and three females. At

TABLE 2. Effect of atorvastatin on lipoprotein cholesterol in female LDL receptor-deficient mice

Atorvastatin	Duration	n	Total Cholesterol		VLDL Cholesterol		LDL Cholesterol		HDL Cholesterol		HDL/(VLDL + LDL)	
			Before	After	Before	After	Before	After	Before	After	Before	After
mg/kg/day	*weeks*											
0	3	4	262 ± 24	248 ± 16 (−5)	8 ± 2	12 ± 1 (+50)	172 ± 22	144 ± 10 (−16)	82 ± 5	92 ± 5 (+12)	0.48 ± 0.08	0.60 ± 0.02 (+24)
300	1	4	282 ± 17	235 ± 25 (−17)	10 ± 2	22 ± 5 (+120)	171 ± 6	120 ± 15 (−30)	102 ± 10	93 ± 15 (−9)	0.56 ± 0.04	0.65 ± 0.08 (+16)
300	2	4	261 ± 17	164 ± 11 (−37)[a]	6 ± 1	10 ± 1 (+67)	165 ± 8	56 ± 2 (−66)[d]	90 ± 16	99 ± 13 (+10)	0.53 ± 0.10	1.53 ± 0.23 (+188)[c]
300	3	4	270 ± 4	165 ± 23 (−39)[b]	6 ± 2	21 ± 6 (+250)	163 ± 11	70 ± 11 (−57)[b]	100 ± 11	74 ± 12 (−26)[b]	0.62 ± 0.10	0.92 ± 0.27 (+50)

Effects of 300 mg/day atorvastatin in female LDL receptor-deficient mice. Values represent mg/dL ± SEM. Numbers in parentheses represent percent change from before treatment values. Data were analyzed by ANOVA of the percent changes from before to after values. Comparisons were made to percent change in the untreated mice.
[a] $P < 0.01$; [b] $P < 0.005$; [c] $P < 0.001$; [d] $P < 0.0005$.

Fig. 2. Hepatic HMG-CoA reductase activity in atorvastatin-treated LDL receptor-deficient female mice. LDL receptor-deficient mice were fed chow alone (n = 3) or chow + atorvastatin diet admixtures approximating 300 mg/kg per day drug for 1 week (n = 3), 2 weeks (n = 4), or 3 weeks (n = 4). Data were analyzed by ANOVA. Comparison to untreated control; $*P < 0.05$, $***P < 0.0005$.

this dose and treatment duration, total and LDL cholesterol were reduced by 38 and 44% by atorvastatin, 35 and 41% by lovastatin, 20 and 25% by pravastatin, and 31 and 40% by simvastatin, respectively. All the vastatins also reduced HDL cholesterol.

Based on the above observations, a comparative vastatin efficacy study was performed in female LDL receptor-deficient mice fed chow alone or admixtures containing the equivalent of 10, 30, 100, or 300 mg/kg per day of the HMG-CoA reductase inhibitor, atorvastatin, lovastatin, pravastatin, or simvastatin for 2 weeks. Plasma was pooled from treatment groups before and after vastatin treatments for lipoprotein cholesterol profile analysis by HPGC (**Fig. 4**). Each plasma pool was derived from equal volumes of plasma from seven mice per group with the exception of the 300 mg/kg per day post simvastatin group where only one animal survived. Essentially no changes were observed in the control female LDL receptor-deficient mice maintained on chow for 2 weeks (Fig. 4, panel A). Panel A shows the profiles before (open profile), after 2 weeks on chow (shaded profile), and also superimposed (overlaid). Similarly, for the 16 vastatin treatment groups (Fig. 4, panel B) the pooled post treatment profiles (shaded profiles) are superimposed on the pretreatment profiles. For atorvastatin, a dose-dependent reduction in total and LDL cholesterol is apparent at all doses (Fig. 4, panel B). For lovastatin, a slight reduction in total and LDL cholesterol was observed at the 100 mg/kg dose. At 300 mg/kg lovastatin, cholesterol was redistributed whereby marked VLDL cholesterol elevation and reduced LDL and HDL cholesterol were observed. At this dose, four of seven mice in this group

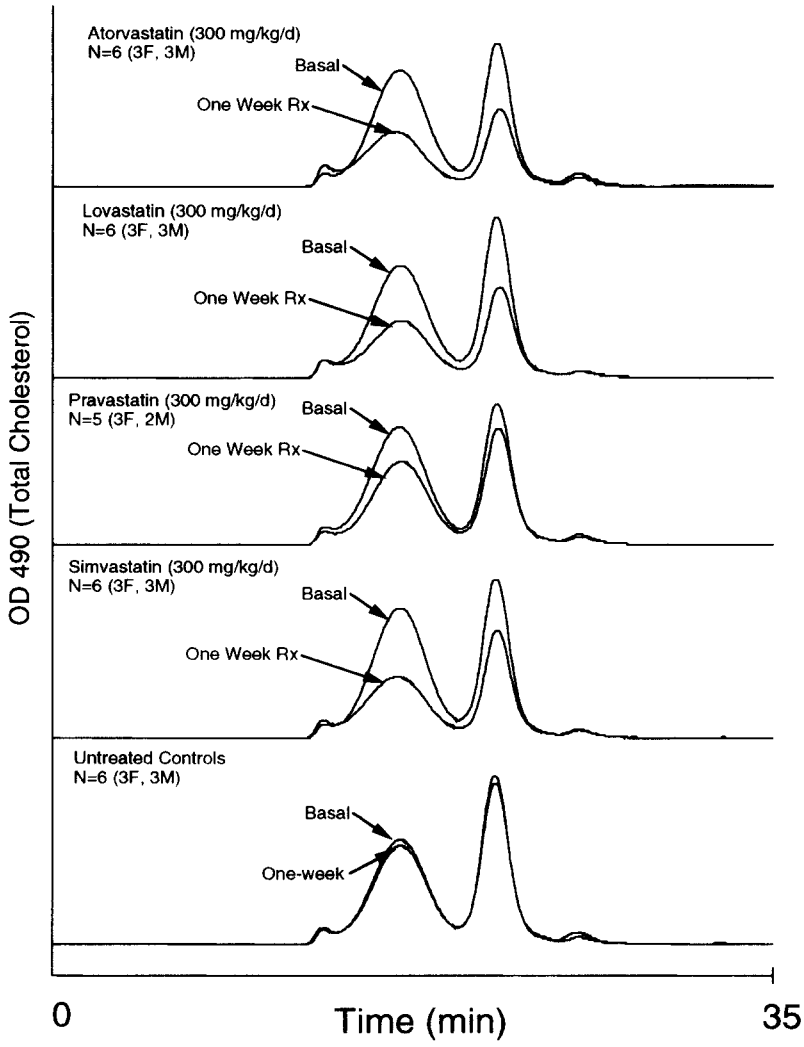

Fig. 3. Comparative effects of HMG-CoA reductase inhibitors in LDL receptor-deficient mice. Female and male mice (number of each indicated on figure) were fed chow alone (Untreated Controls) or the indicated vastatin for 1 week as a diet admixtures approximating 300 mg/kg per day drug.

were lethargic at the time of the 2 week post treatment bleed. For pravastatin, essentially no reduction in total or LDL cholesterol was observed at any dose. For simvastatin, essentially no reduction in total or LDL cholesterol was observed at the 10 or 30 mg/kg dose. At 100 mg/kg simvastatin, cholesterol began to redistribute from LDL and HDL to VLDL. In the 300 mg/kg simvastatin treatment group, the lipoprotein cholesterol profile of the single surviving morbid mouse showed reduced total, LDL, and HDL cholesterol and elevation of VLDL cholesterol. The independent total cholesterol determination of the plasma pools (**Table 3**) and the percent distribution of total cholesterol from the profiles (Fig. 4) were used to determine the average amount of cholesterol in each lipoprotein fraction for the various treatments and are shown in Table 3. In addition, cholesterol of the combined VLDL plus LDL fraction, the ratio of HDL cholesterol to VLDL plus LDL cholesterol, and plasma triglycerides are shown (Table 3). For atorvastatin, the dose-dependent reduction in total cholesterol is largely reflected by reduced LDL cholesterol (Table 3) and to a lesser extent HDL, and therefore an elevation of the ratio of HDL cholesterol to VLDL plus LDL cholesterol becomes apparent (Table 3). The pooled total cholesterol determinations (Table 3) were essentially identical to those obtained from the average of individual total cholesterol determinations. Statistical analysis to determine whether vastatin treatment results in reduction of the individually determined total cholesterol (ANOVA of percent change from pretreatment values) demonstrated significant lowering at the two highest atorvastatin doses used (no treatment, +6%; 10 mg/kg, −11%; 30 mg/kg, −12%; 100 mg/kg, −15%, $P < 0.05$; 300 mg/kg, −45%, $P < 0.0001$). No other vastatin tested significantly lowered total plasma cholesterol in this experiment.

To determine whether differences in the degree of total and LDL cholesterol lowering between the va-

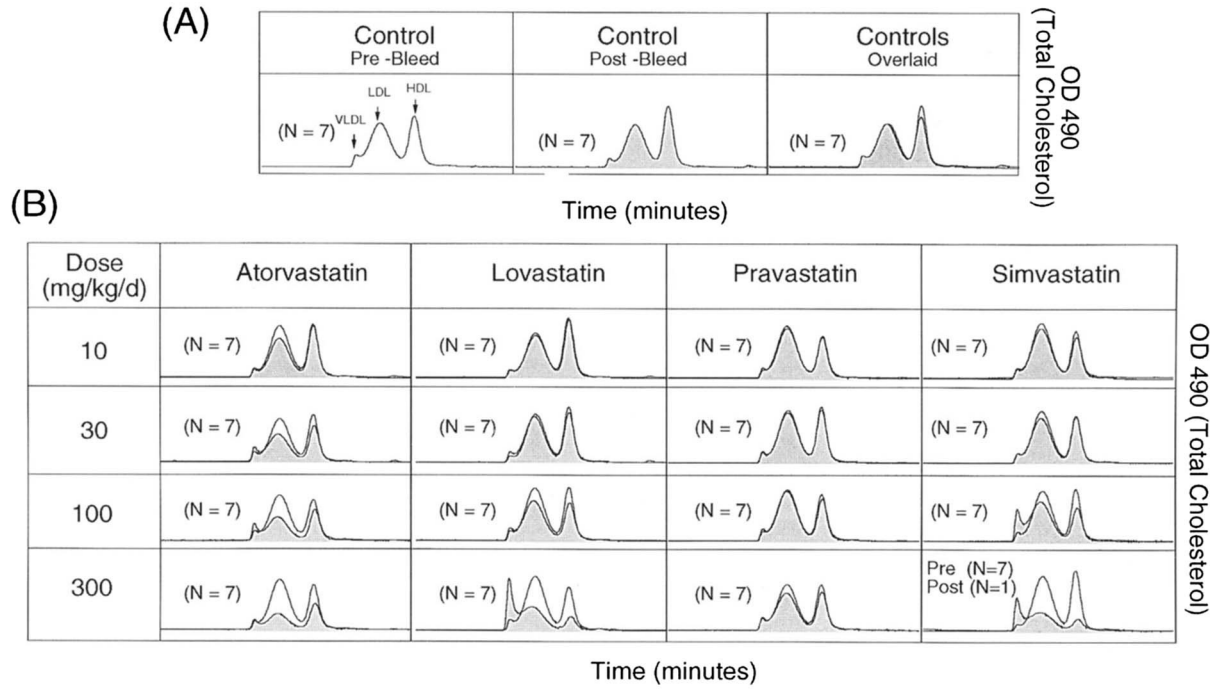

Fig. 4. Dose-dependent lipoprotein cholesterol profiles in female LDL receptor-deficient mice treated with various vastatins for 2 weeks (group plasma pools). Blood samples from chow-fed mice were obtained after an 8-h fast before (open profiles) and after 2 weeks on chow alone or chow + vastatin diet admixtures approximating 10, 30, 100, and 300 mg/kg per day drug (shaded profiles). Panel A displays the before (open profile), after (shaded profile), and these profiles overlaid from mice fed chow alone for 2 weeks. Panel B displays the before (open profiles) overlaid on the treatment profile (shaded profiles) for each treatment group. In some cases the baseline values were essentially identical to treatment values and therefore the overlay of the open profile on the shaded profile is not apparent. Each profile was generated from a pool containing equivalent amounts of plasma from each mouse in the group.

statins used in this model were related to the induction level of HMG-CoA reductase, hepatic mRNA levels were determined by a sensitive internal standard/RNAse protection assay. Female LDL receptor-deficient mice were bled prior to and after 2 weeks treatment with vastatins at the 100 mg/kg per day treatment. In this experiment, LDL and total plasma cholesterol were significantly lowered with atorvastatin (−41 and −27%), lovastatin (−27 and −21%) and simvastatin (−22 and −15%), but not with control (+8 and +11%) or pravastatin (+8 and +12%) treatment, respectively. After 2 weeks of treatment, hepatic HMG-CoA reductase mRNA levels were elevated 17.2-fold with atorvastatin, 10.7-fold with lovastatin, 2.5-fold with pravastatin, and 4.1-fold with simvastatin treatment (**Fig. 5A**). These values were inversely related to the plasma LDL cholesterol levels (Fig. 5B). An almost identical relationship was observed with total plasma cholesterol (not shown). Interestingly, a positive relation between the ratio of HDL to VLDL plus LDL cholesterol with that of HMG-CoA reductase was also observed (Fig. 5C).

To explore a possible mechanism of action responsible for reduced total and LDL cholesterol, especially in the atorvastatin-treated LDL receptor-deficient mice, cholesterol, triglyceride, and apoB secretion rates were assessed after acute (1 day) and chronic (2 weeks) dosing in females by utilizing Triton WR 1339 to block lipoprotein clearance. We observed a significant 29% reduction in cholesterol secretion in plasma after 1 day of 300 mg/kg atorvastatin treatment (**Fig. 6A**). No significant difference in triglyceride or apoB secretion rates was observed (Fig. 6B and 6C). After 2 weeks treatment, the total plasma cholesterol and apoB levels were reduced by 51% ($P < 0.0001$) and 46% ($P < 0.0001$) in the atorvastatin group, respectively, and not changed in the control group (**Fig. 7**). However, with 2 weeks atorvastatin treatment, cholesterol (+20%), triglyceride (+57%), and apoB (+31%) secretion rates were significantly elevated (Fig. 6A, 6B, and 6C). Therefore, the secretion ratio of triglyceride to cholesterol was significantly elevated whether the mice were treated acutely or chronically with atorvastatin (Fig. 6D).

To determine whether atorvastatin treatment had effects on the molecular forms of apoB secreted, plasma samples before or after Triton WR 1339 treatment were analyzed by apoB immunoblot analysis (**Fig. 8**). In untreated mice, apoB-100 was predominantly secreted, whereas during acute atorvastatin treatment only apoB-

TABLE 3. Dose-dependent plasma lipid changes in female LDL receptor-deficient mice treated with various vastatins for 2 weeks (group plasma pools)

		Total Cholesterol			VLDL Cholesterol			LDL Cholesterol			VLDL + LDL Cholesterol			HDL Cholesterol			HDL/(VLDL + LDL)			Triglycerides		
Group	Dose	Pre	Post		Pre	Post		Pre	Post		Pre	Post		Pre	Post		Pre	Post		Pre	Post	
Control	0	224	217	(−3)	9	7	(−23)	131	118	(−10)	140	125	(−11)	84	92	(10)	0.60	0.74	(24)	83	80	(−4)
Atorvastatin	10	239	206	(−14)	7	6	(−12)	149	117	(−21)	155	123	(−21)	84	83	(−1)	0.54	0.68	(26)	81	69	(−15)
	30	205	172	(−16)	8	13	(64)	124	95	(−23)	132	108	(−18)	73	64	(−13)	0.55	0.59	(7)	94	57	(−39)
	100	204	159	(−22)	7	17	(144)	130	84	(−35)	137	102	(−26)	67	57	(−15)	0.49	0.57	(15)	72	69	(−3)
	300	211	107	(−49)	6	9	(68)	138	51	(−63)	143	61	(−58)	68	46	(−32)	0.47	0.76	(62)	68	72	(6)
Lovastatin	10	218	219	(0)	6	5	(−18)	121	126	(4)	128	131	(3)	90	88	(−3)	0.71	0.67	(−6)	82	66	(−20)
	30	216	224	(4)	4	8	(76)	131	136	(4)	136	144	(6)	80	80	(−1)	0.59	0.55	(−7)	78	70	(−10)
	100	236	197	(−17)	7	11	(64)	149	123	(−17)	156	134	(−14)	80	63	(−22)	0.52	0.47	(−9)	69	56	(−18)
	300	214	220	(3)	8	71	(816)	143	113	(−21)	151	184	(22)	63	36	(−44)	0.42	0.19	(−54)	85	117	(38)
Pravastatin	10	212	219	(3)	8	4	(−41)	142	150	(5)	150	154	(3)	62	65	(4)	0.41	0.42	(1)	78	52	(−34)
	30	228	226	(−1)	7	8	(11)	141	139	(−2)	149	147	(−1)	79	79	(0)	0.53	0.54	(1)	80	63	(−22)
	100	224	221	(−1)	9	6	(−33)	145	149	(3)	154	155	(1)	70	66	(−6)	0.46	0.43	(−7)	77	53	(−31)
	300	202	196	(−3)	9	11	(20)	125	120	(−4)	134	131	(−2)	68	65	(−4)	0.51	0.50	(−2)	95	61	(−36)
Simvastatin	10	232	213	(−8)	7	6	(−6)	155	143	(−8)	162	150	(−8)	70	63	(−9)	0.43	0.42	(−2)	77	56	(−27)
	30	227	215	(−5)	7	7	(−10)	148	135	(−9)	156	142	(−9)	71	73	(2)	0.46	0.51	(12)	78	53	(−32)
	100	217	242	(12)	7	34	(411)	137	146	(7)	143	180	(26)	74	62	(−16)	0.51	0.34	(−33)	77	73	(−5)
	300	234	147	(−37)	5	39	(700)	146	82	(−44)	151	120	(−20)	83	27	(−68)	0.55	0.22	(−60)	65	45	(−31)

Dose-dependent plasma lipid changes in female LDL receptor-deficient mice treated with vastatin for 2 weeks (group plasma pools). All treatment groups consisted of seven female mice. Mice were fed chow alone or chow containing the indicated vastatin such that approximately 10, 30, 100, or 300 mg drug/kg body weight was consumed daily. Values represent mg/dL of plasma pools from each group and are derived from the total cholesterol determination and the lipoprotein distribution of the pools (i.e., Fig. 5). Numbers in parentheses represent percent change from basal level. For simvastatin, only one animal survived at the 300 mg/kg per day dose and the comparison for this single mouse is made to its pretreatment group pool.

48 was detected. With chronic atorvastatin treatment, both apoB-100 and apoB-48 were secreted; however, apoB-48 was the major form detected.

DISCUSSION

In the current study, the effects of various vastatins on plasma lipoprotein cholesterol in the LDL receptor-deficient mouse were determined. It is generally accepted that HMG-CoA reductase inhibitors act primarily to lower plasma LDL cholesterol by up-regulation of LDL receptors (1, 9, 10). However, lipoprotein kinetic studies, albeit in LDL receptor-competent models, have suggested that HMG-CoA reductase inhibitors could, in addition, act by blocking lipoprotein production (11–16). Therefore, we chose to directly test the hypothesis that HMG-CoA reductase inhibitors could lower plasma and LDL cholesterol in the LDL receptor-deficient mouse. As prior studies in both mice and rats have suggested marked inactivation of HMG-CoA reductase inhibitors caused by robust P450 enzyme induction (37–40) and elevation of HMG-CoA reductase levels (28), it was necessary to use a relatively high dose (e.g., in comparison to doses used in humans) of the HMG-CoA reductase inhibitors to supersede compensatory mechanisms and to observe a biological effect. Indeed, when levels of atorvastatin were equal to or greater than 30 mg/kg per day, the rise in LDL cholesterol observed with time in male mice was quelled, and furthermore, reduced at the 100 and 300 mg/kg per day doses. Thus, these studies demonstrated a direct effect by an HMG-CoA reductase inhibitor on lowering LDL cholesterol in the absence of LDL receptors. Even though atorvastatin dose-dependently lowered plasma total and LDL cholesterol in male LDL receptor-deficient mice, with time the effectiveness of the drug waned, suggestive of compensatory mechanisms to maintain hepatic cholesterol homeostasis. Possibly, P450 induction (37–40) or HMG-CoA reductase overproduction (28) could reverse the effectiveness of the compound. Indeed, HMG-CoA reductase activity and mRNA levels were markedly induced in atorvastatin-treated mice.

Interestingly, we observed a marked gender-specific difference in both total and lipoprotein cholesterol in these mice. Females appeared to have more atherogenic profiles, having markedly more LDL and less HDL cholesterol than males. Therefore, we initially tested a single dose of atorvastatin (300 mg/kg per day) in females, and observed that their response to treatment was more profound and longer lasting than observed in males treated with the same dose. Females also had markedly elevated hepatic HMG-CoA reduc-

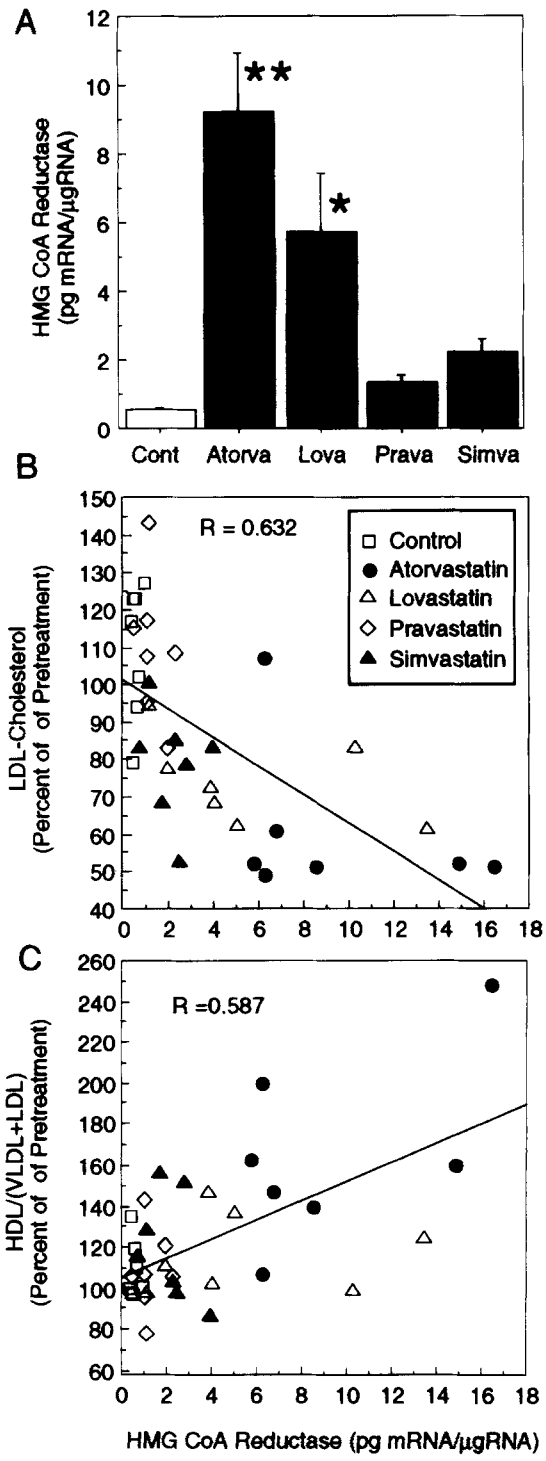

Fig. 5. Hepatic HMG-CoA reductase mRNA levels in female LDL receptor-deficient mice treated with various vastatins for 2 weeks. Mice were fed diet admixtures approximating 100 mg/kg per day of each statin for 2 weeks. Hepatic HMG-CoA reductase levels were determined by an internal standard/RNAse protection assay as described in Materials and Methods. Blood samples were taken before and after treatment and assessed for total and lipoprotein cholesterol as described in Materials and Methods. (A) Hepatic HMG-CoA reductase mRNA levels; (B) correlation between hepatic HMG-CoA reductase mRNA levels and percent change in LDL cholesterol separated by treatment; and (C) correlation between hepatic HMG-CoA reductase mRNA levels and the percent change in the ratio of HDL cholesterol to VLDL plus LDL cholesterol separated by treatment (indicated on panel B). Data in panel A were analyzed by ANOVA. Comparison to control; $*P < 0.005$, $**P < 0.0001$.

tase activity, as did a few males treated with the drug, and this raises the possibility that females are less efficient than males in inactivating atorvastatin. Measurement of plasma drug and metabolite levels, although not determined in the current study, may also shed light on the response differences between males and females. In this regard, studies of Ohtawa and Uchiyama (37) have shown that hepatic microsomes isolated from male rats were markedly more active than those isolated from female rats for yielding simvastatin metabolic products. Although not directly determined in the current studies, perhaps the female mice are less capable of atorvastatin inactivation than males.

We were curious as to whether the hypercholesterolemic effect in these mice could be observed with other HMG-CoA reductase inhibitors, indicating a class specific effect. In a 1-week study at the 300 mg/kg per day dose, we observed a significant total and LDL cholesterol reduction for atorvastatin, lovastatin, and simvastatin, indicating a class specific effect. Therefore, the relative effectiveness of select vastatins was compared. Atorvastatin treatment for 2 weeks resulted in significant and dose-dependent lowering of total cholesterol in female LDL receptor-deficient mice. Total cholesterol lowering was largely due to reduction of LDL cholesterol. Taken together with observations in males, these reductions are more profound and sustained in the females. In contrast to the 1-week experiment at the highest dose, under these experimental conditions (female LDL receptor-deficient mice vastatin-treated for 2 weeks) and at similar doses, pravastatin, lovastatin, and simvastatin were less effective for lowering total and non-HDL cholesterol than atorvastatin and suggest induction of compensation mechanisms or lower efficacies. Interestingly, the efficacy of the vastatins for lowering total and LDL cholesterol was inversely related to the level of induction of HMG-CoA reductase mRNA. In that the only major source of cholesterol in this model is de novo synthesis and maintenance of cholesterol homeostasis is tightly regulated, the level of HMG-CoA reductase mRNA may therefore reflect the relative effectiveness of these compounds.

Lipoprotein cholesterol production was identified as the initial mechanism by which atorvastatin lowered

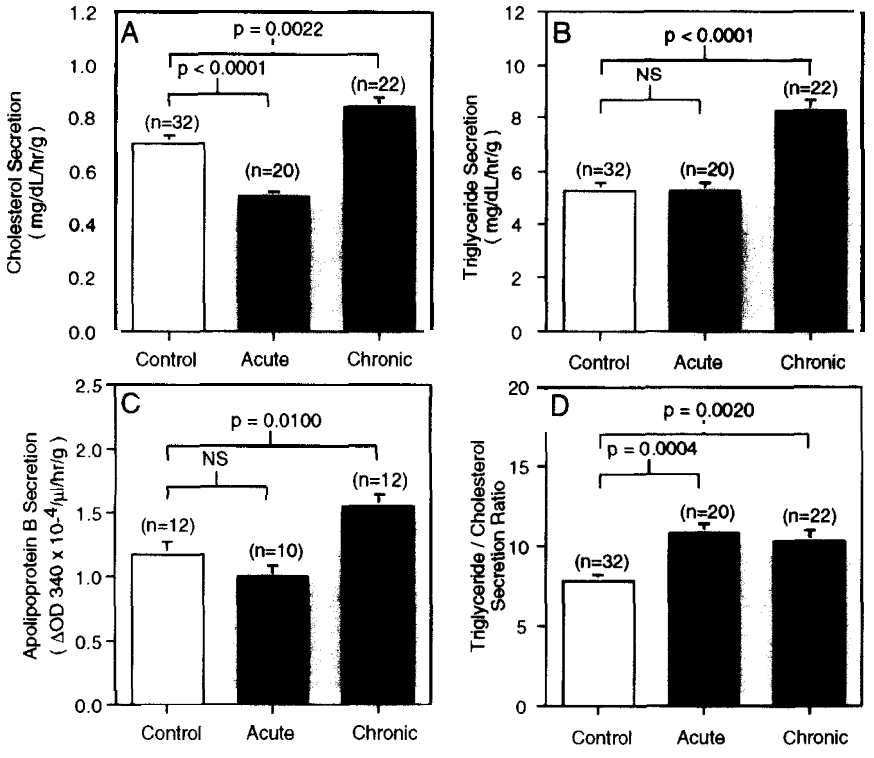

Fig. 6. (A) Cholesterol, (B) triglyceride, (C) apoB secretion rates and the ratio of the triglyceride to cholesterol secretion rates (D) in atorvastatin-treated female LDL receptor-deficient mice. Mice were fed a diet of admixture approximating 300 mg/kg per day atorvastatin acutely (1 day) or chronically (14 days). Blood samples were obtained before (9 PM) and 12 h post-Triton WR1339 administration. In the chronic study, blood samples were also obtained before initiation of atorvastatin treatment. Data were derived from two separate experiments in which the cholesterol and triglyceride secretion rates were measured in both experiments, and in which the apoB secretion rate was determined in the second experiment. The secretion rates of cholesterol, triglycerides, and apoB were determined by the difference in accumulated lipid over 12 h as described in Materials and Methods. Data were analyzed by ANOVA.

total plasma cholesterol in female LDL receptor-deficient mice. Acute atorvastatin treatment resulted in decreased cholesterol production and perhaps contributed to the reduced levels of LDL cholesterol. However, with chronic treatment, mechanisms to compensate for inhibition of cholesterol de novo synthesis, and possibly drug inactivation, subsequently led to increased cholesterol production by 2 weeks. Surpris-

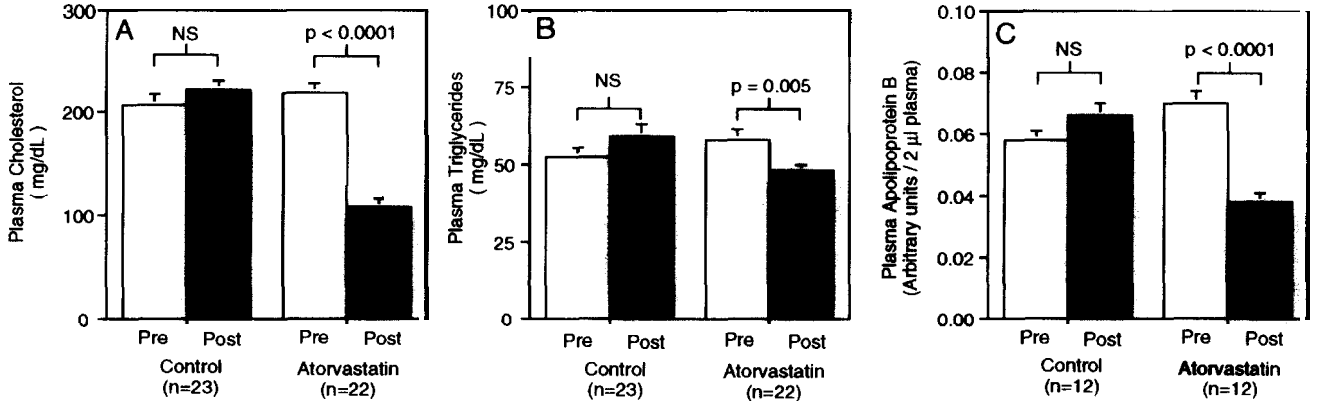

Fig. 7. Total plasma cholesterol (A), triglycerides (B), and apoB (C) in the chronic study before and 2 weeks after atorvastatin treatment (i.e., prior to Triton WR1339 administration). Data were analyzed by one-tailed paired *t*-tests.

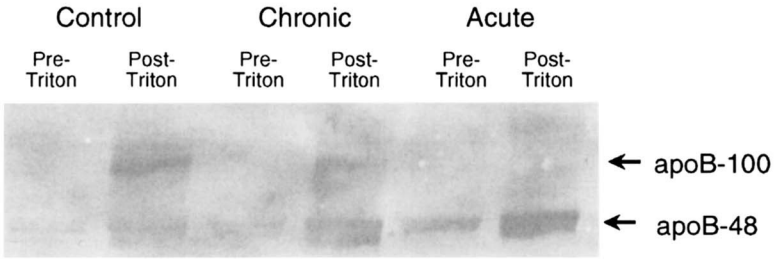

Fig. 8. ApoB immunoblot analysis of pooled plasma from control (n = 12), chronic atorvastatin-treated (n = 12), and acute atorvastatin-treated (n = 10) mice before and after Triton WR 1339 treatment. Plasma proteins (0.7 μl plasma/lane) were separated on 4–12% sodium dodecyl sulfate polyacrylamide gel. High molecular weight apoB-100 and low molecular weight apoB-48 were detected with a polyclonal sheep and anti-mouse apoB serum as described in Materials and Methods.

ingly, the reestablished increased cholesterol synthetic rate appeared to maintain the newly derived plasma cholesterol level rather than induce an elevation. Thus, it appears that a new steady state plasma cholesterol level was achieved. In both the acute and chronic studies, the ratio of triglyceride to cholesterol secretion was elevated with atorvastatin treatment, and raises the possibility that production of triglyceride-rich cholesterol-poor VLDL may also contribute to the LDL cholesterol-lowering effects observed.

The observation that both acute and chronic atorvastatin treatment resulted in a greater proportion of apoB-48 secreted relative to apoB-100 may also contribute to the reduced production of LDL. ApoB-48 particles produced in the mice are cleared efficiently through a non-LDL receptor-mediated mechanism (41). Similarly, clearance of apoB-48-containing lipoproteins occurred normally in familial hypercholesterolemic humans (42) and WHHL rabbits (43). The reduced secretion of apoB-100 may result from increased apoB mRNA editing (44), an enhanced intracellular degradation (45), or decreased synthesis when cholesterol becomes limiting for lipoprotein production. Alternatively, the apoB-48-containing lipoproteins that accumulate with Triton WR 1339 treatment may represent a disproportionate contribution of particles from the intestine. During chronic atorvastatin treatment, when cholesterol secretion increased above control levels, the apoB-48 still represented a significant portion of the secreted apoB. Interestingly, in studies in rabbits (46, 47) and mice (48), when apoB mRNA editing was increased by adenovirus gene therapy, a reduced LDL production was observed. Under conditions where only apoB-100 is made, such as in mice deficient in apoB editing, LDL levels increase (49). It should be noted that in humans, unlike mice, apoB-100 is the only apoB produced in the liver (18), and perhaps a direct effect of a vastatin on apoB-100 production would also lead to reduced LDL levels.

Overall these data suggest that vastatins lower total and LDL cholesterol in LDL receptor-deficient mice. For the vastatins tested, atorvastatin caused a sustained and greatest plasma cholesterol-lowering effect in females. With treatment, males, unlike females, appeared to compensate for the drug effects for lowering total and LDL cholesterol with time. Therefore, the greater response in females may also reflect an inability to inactivate the drug as efficiently as males. In a human homozygous familial hypercholesterolemia subject with true receptor negative phenotype, Feher et al. (50) demonstrated that simvastatin monotherapy could sustain and reduce plasma cholesterol by 30% over approximately 1 month of treatment. Although the cholesterol-lowering effect in this subject was sustained over the following 20 months, combination therapies were added during this time. In a preliminary report, Naoumova et al. (51) showed that atorvastatin augmentation to usual therapy in homozygous familial hypercholesterolemic humans (bi-weekly LDL apheresis in seven subjects, no other treatment in three subjects) over 2 months reduced LDL cholesterol by 31%. Furthermore, these investigators (51) reported that using a combined therapy consisting of LDL apheresis, bile acid sequestration, and atorvastatin achieved a 70% reduction in LDL cholesterol. Despite the limited data regarding vastatin treatment of humans with homozygous familial hypercholesterolemia, the above observations, taken together with the data presented in the current studies, suggest that pharmacological inhibition of cholesterol production can lead to a reduction of plasma cholesterol in the absence of LDL receptors.

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