Effect of *Pravastatin* on Intermediate-Density and Low-Density Lipoproteins Containing Apolipoprotein CIII in Patients With Diabetes Mellitus

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The apolipoprotein (apo) B lipoproteins, intermediate-density lipoproteins (IDL) and low-density lipoproteins (LDL) that contain apo-CIII are associated with coronary heart disease in patients with diabetes mellitus. Apo-CIII is prominent in diabetic dyslipidemia. We studied whether these apo-B lipoprotein types containing apo-CIII in diabetics are reduced by 1 year of pravastatin treatment. We randomly selected 45 age- and gender-matched placebo/pravastatin pairs from diabetic patients in the Cholesterol and Recurrent Events trial, a randomized, double-blinded trial of pravastatin 40 mg monotherapy. Very-low-density lipoproteins (VLDL) and IDL + LDL particles were subdivided based on the presence of apo-E and apo-CIII to yield 3 particle types: E+CIII+, E−CIII+, and E−CIII−. Compared with placebo, pravastatin reduced IDL + LDL apo-B concentrations for E+CIII+, E-CIII+, and E−CIII− by 42% ($p = 0.02$), 17% ($p = 0.7$), and 29% ($p = 0.002$), respectively, commensurate with IDL + LDL cholesterol concentration reductions in the particle types of 29% ($p = 0.002$), 25% ($p = 0.2$), and 36% ($p < 0.0001$), respectively. These IDL + LDL CIII+ particles are rich in triglycerides and cholesterol and are likely to be remnant particles of VLDL. Thus, pravastatin reduced potentially atherogenic remnant particles, a prominent component of diabetic dyslipidemia associated with coronary events; these results may contribute to its demonstrated effectiveness in reducing coronary heart disease in diabetics. ©2003 by Excerpta Medica, Inc.

(Am J Cardiol 2003;92:121–124)

Patients with diabetes mellitus often have triglyceride-rich lipoproteins in plasma.[1,2] Apolipoprotein CIII (apo-CIII) is a small protein on the surface of apo-B lipoproteins (very-low-density lipoprotein [VLDL] + intermediate-density lipoprotein [IDL] + low-density lipoprotein [LDL]) that strongly affects their metabolism. Apo-CIII inhibits the activity of lipoprotein lipase,[3–5] which metabolizes triglycerides in VLDL and facilitates their clearance from plasma. Apo-CIII also obstructs the clearance of VLDL and LDL from plasma by interfering with their interaction with hepatic lipoprotein receptors.[6,7] The plasma concentration of apo-CIII in apo-B lipoproteins is associated with coronary heart disease,[8–11] independent of triglycerides.[8,10] Patients with noninsulin dependent diabetes mellitus with coronary heart disease had higher apo-CIII levels than diabetic patients who did not have coronary heart disease.[12] We recently found that apo-B lipoproteins have diverse effects on coronary events in diabetics. IDL + LDL with apo-CIII is a strong predictor of recurrent events, whereas VLDL with apo-CIII and plasma triglycerides are not a predictor.[13] In contrast, apo-E functions as a ligand for cell surface receptors that take up VLDL and LDL from plasma.[14] Surprisingly, however, the plasma level of apo-E in apo-B lipoproteins is also higher in coronary heart disease survivors.[10,11] This may be because most apo-E in apo-B lipoproteins co-exists with apo-CIII. Recently, we reported that pravastatin treatment decreases the level of apo-B in VLDL, and apo-CIII in VLDL + IDL + LDL.[15] That study examined apo-CIII in apo-B lipoproteins together (VLDL, IDL, and LDL); these are heterogenous in composition, metabolic pathways, and in relation to coronary events. Therefore, we determined the effects of pravastatin on VLDL and IDL + LDL particle types separately, according to their apo-CIII and apo-E contents.

METHODS

Subjects and blood samples: The Cholesterol and Recurrent Events trial was a randomized controlled trial of pravastatin in 4,159 patients who had a myocardial infarction 3 to 20 months before enrollment.[16] Lipid and glucose level inclusion criteria were a cholesterol level of <240 mg/dl, a LDL cholesterol level of 115 to 174 mg/dl, a fasting triglyceride level of <350 mg/dl, and a fasting glucose level of <220 mg/dl. Institutional review boards at each center approved the study, all patients gave informed consent, and the procedures were carried out in accordance with institutional guidelines. Patients were randomly assigned to treatment with pravastatin sodium (Pravachol, Bristol-Myers Squibb, Princeton, New Jersey) 40 mg/day or to placebo. The average duration of

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0002-9149/03/$–see front matter
doi:10.1016/S0002-9149(03)00524-1

TABLE 1 Baseline Characteristics of the Placebo and Pravastatin Groups With Diabetes Mellitus

Variable	Placebo (n = 45)	Pravastatin (n = 45)	p Value
Age (yrs)	60 ± 9	62 ± 8	0.3
Caucasian	39 (87%)	39 (87%)	1.0
Men	35 (78%)	35 (78%)	1.0
Current smoker	6 (13%)	6 (13%)	1.0
Coronary bypass	11 (24%)	14 (31%)	0.5
Coronary angioplasty	13 (29%)	10 (22%)	0.5
Hypertension	24 (53%)	22 (49%)	0.7
Exercise (>3 times/wk)	23 (51%)	25 (56%)	0.7
Blood pressure (mm Hg)			
Systolic	129 ± 19	134 ± 25	0.4
Diastolic	76 ± 10	79 ± 11	0.3
Blood glucose (mg/dl)	148 ± 44	147 ± 49	0.9
Body mass index (kg/m^2)	29 ± 5	31 ± 6	0.16
Waist circumference (cm)	102 ± 13	104 ± 14	0.5
Oral hypoglycemic	23 (51%)	14 (31%)	0.06
Insulin	12 (27%)	7 (16%)	0.2
β blockers	19 (42%)	15 (33%)	0.4

Data are presented as number of patients (percent) in the population and mean ± SD. p Values were calculated from paired *t* tests.

follow-up was 5 years. A total of 589 subjects in the whole cohort had diabetes.

Forty-five pairs of diabetic patients, taking pravastatin or placebo, matched for age (15-year intervals) and gender, comprised this study. At baseline and at each visit after randomization, blood samples were obtained after >8 hours of fasting, and were collected in glass tubes containing ethylenediaminetetraacetic acid. Plasma samples were separated by centrifugation and sent on dry ice by overnight delivery to the core laboratory at Washington University of School (St. Louis, Missouri). Baseline laboratory measurements were conducted on fresh plasma, and 1 ml of each plasma sample was placed in a polypropylene vial and stored at −80°C. Vials containing frozen plasma were shipped to the Harvard School of Public Health and stored at −80°C until lipoprotein types were analyzed.

Immunoaffinity chromatography: Anti–apo-CIII and anti–apo-E affinity chromatography was conducted to separate plasma into 3 fractions using 2-step incubation as previously described.[13] Briefly, after thawing at 37°C for 5 minutes, plasma samples (1 ml) were incubated on anti–apo-E immunoaffinity columns overnight at 4°C. Unbound lipoproteins that did not contain apo-E (E−) were collected by gravity flow and were subsequently washed with phosphate-buffered saline solution containing edetic acid. Bound lipoproteins containing apo-E (E+) were recovered after incubation with 3 ml of 3M sodium thiocyanate for 3 minutes. The elution was repeated 3 times, and the sodium thiocyanate in E+ was rapidly removed by gel filtration on a PD-10 column. Because a previous study of the Cholesterol and Recurrent Events trial diabetic population showed that >95% of E+ particles were E+CIII+,[17] we did not further incubate E+ fractions with anti–apo-CIII; for the present study, we denote the E+ fraction as E+CIII+. The E− fractions were further incubated on anti–apo-CIII immunoaffinity columns at 4°C overnight to isolate E−CIII+ and E−CIII−. Bound (E−CIII+) and unbound (E−CIII−) fractions were recovered using the previously described procedure. Recovery after the 2 immunoaffinity chromatographies ranged from 83% to 87%.

Very fast ultracentrifugation and laboratory measurements: VLDL (density <1.006) and IDL + LDL (1.006 < density <1.050) were isolated from the 3 immunofractions (E+CIII+, E−CIII+, and E−CIII−) by very fast ultracentrifugation using a previously described method[18,19] with modification.[13] Ultracentrifugation was performed using a Beckman Optima TLX ultracentrifuge (Beckman, Palto Alto, California), a Beckman TLA 120.2 fixed-angle rotor (r_{max} = 38.9 mm, r_{min} = 24.5 mm), and thick-walled polycarbonate tubes (11 × 35 mm, No. 343778, without cap; Beckman). Densities were adjusted and verified on an optical densitometer (Bausch & Lomb, Rochester, New York). The run times for VLDL and IDL + LDL separation were 1 hour, 17 minutes (15°C, 625,000 *g*) and 2 hours, 30 minutes (15°C, 625,000 *g*), respec-tively. The supernatants containing VLDL and IDL + LDL (400 μl) were collected by aspiration. The run time and speeds were standardized by comparison with the classic Lindgren method using an SW41 swinging bucket rotor (Beckman). We used d <1.050 for LDL to isolate relatively pure IDL + LDL, minimizing contamination with lipoprotein (a) and large high-density lipoprotein (HDL) particles. We found nearly complete recovery of VLDL + IDL + LDL with the restricted cutpoint for LDL, 1.050 g/ml: 98% for cholesterol, 99% for triglyceride, and >95% for apo-B. This computation uses for standards non-HDL lipoproteins for cholesterol and triglyceride recovery, and the d >1.050 fraction for apo-B loss.[13] After immunoaffinity chromatography and ultracentrifugation, 6 lipoprotein subtypes were isolated from a sample from 1 patient: VLDL E+CIII+, VLDL E−CIII+, VLDL E−CIII−, IDL + LDL E+CIII+, IDL + LDL E−CIII+, and IDL + LDL E−CIII−. Cholesterol and triglycerides (Roche Diagnostics Systems, New Jersey) were measured in each subtype by enzymatic methods using the Cobas MIRA plus system (Roche Diagnostic Systems, Inc.), and apo-B was measured by an enzyme-linked immunosorbent assay using standards calibrated to reference plasma provided by the Centers for Disease Control and Prevention (Atlanta, Georgia) as previously described.[13] The detection limit was ~0.005 mg/dl. The intra-assay coefficients of variation for cholesterol, triglycerides, and apo-B were 2%, 3%, and 6%, respectively, and the interassay coefficients of variation for measurements were 4%, 6%, and 8%, respectively.

TABLE 2 Plasma Lipid Concentrations at Baseline and After One Year of Treatment

	Placebo Group (n = 45)			Pravastatin Group (n = 45)				
	Mean ± SD			Mean ± SD			Pravastatin/Placebo	
Variable	Baseline	1-Yr	p Value	Baseline	1-Yr	p Value	% Change†	p Value
Total cholesterol (mg/dl)	206 ± 18	207 ± 56	0.58	208 ± 18	172 ± 34	0.0002	−18%	0.006
LDL cholesterol (mg/dl)	135 ± 16	134 ± 31	0.70	137 ± 14	93 ± 28	<0.0001	−31%	0.01
HDL cholesterol (mg/dl)	38 ± 9	39 ± 10	0.23	37 ± 9	38 ± 8	0.94	−2%	0.21
Triglyceride* (mg/dl)	148 ± 44	150 ± 54	0.55	159 ± 50	166 ± 55	0.69	−1%	0.62

*Triglyceride values are geometric mean ± SD.
†Percent change means the percentage difference between the 1-year baseline difference in the placebo and the 1-year baseline difference in the pravastatin group.

TABLE 3 Particle (apo-B) Concentration and Cholesterol and Triglyceride Levels in Apo-B Lipoproteins Containing Apo-CIII or Apo-E at Baseline and After One Year of Treatment in Placebo and Pravastatin Groups

	Placebo (n = 45)			Pravastatin (n = 45)			Pravastatin/Placebo	
	Baseline	1 Yr	p Value	Baseline	1 Yr	p Value	% Change*	p Value
VLDL E+CIII+								
Apo-B	1.9 ± 1.4	2.1 ± 1.3	0.3	2.0 ± 1.5	2.3 ± 1.5	0.3	1%	1.0
Cholesterol	12 ± 9	13 ± 10	0.5	13 ± 8	14 ± 12	0.4	3%	0.8
Triglycerides	67 ± 56	79 ± 63	0.3	64 ± 46	79 ± 57	0.09	6%	0.8
VLDL E−CIII+								
Apo-B	0.8 ± 0.6	1.1 ± 0.8	0.002	1.0 ± 1.1	1.4 ± 1.7	0.04	−9%	0.9
Cholesterol	3.6 ± 3.0	4.5 ± 3.4	0.11	4.4 ± 4.2	6.2 ± 8.0	0.09	17%	0.5
Triglycerides	20 ± 17	28 ± 22	0.03	23 ± 22	37 ± 48	0.02	26%	0.3
VLDL E−CIII−								
Apo-B	3.2 ± 1.4	3.4 ± 1.6	0.6	3.6 ± 1.8	3.5 ± 3.8	0.9	−8%	0.7
Cholesterol	6.3 ± 3.2	7.3 ± 4.0	0.09	7.3 ± 3.4	6.9 ± 6.9	0.8	−21%	0.3
Triglycerides	20 ± 10	22 ± 12	0.17	21 ± 11	20 ± 18	0.8	−16%	0.4
IDL+LDL E+CIII+								
Apo-B	4.2 ± 1.6	3.3 ± 1.2	<0.0001	6.5 ± 3.6	2.3 ± 1.2	<0.0001	−42%	0.02
Cholesterol	20 ± 6	14 ± 7	<0.001	25 ± 11	10 ± 7	<0.001	−29%	0.002
Triglycerides	12 ± 7	11 ± 9	0.8	12 ± 7	9 ± 5	0.003	−28%	0.5
IDL+LDL E−CIII+								
Apo-B	5.6 ± 3.2	4.5 ± 3.2	0.01	5.7 ± 3.6	3.6 ± 2.6	0.0001	−17%	0.7
Cholesterol	21 ± 11	16 ± 11	<0.001	23 ± 14	11 ± 7	<0.001	−25%	0.2
Triglycerides	7.9 ± 3.8	10 ± 9	0.12	7 ± 3	7 ± 6	0.9	−24%	0.7
IDL+LDL E−CIII−								
Apo-B	86 ± 29	92 ± 24	0.3	92 ± 29	72 ± 22	0.0002	−29%	0.002
Cholesterol	111 ± 25	114 ± 35	0.7	129 ± 43	85 ± 31	<0.001	−36%	<0.0001
Triglycerides	18 ± 5	19 ± 4	0.07	18 ± 5	16 ± 6	0.02	−22%	0.003

Values (mg/dl) are mean ± SD.
*Percent change means the percentage difference between the 1-year baseline difference in the placebo and the 1-year baseline difference in the pravastatin group.

Statistical analysis: Statistical analyses were performed at the Harvard School of Public Health. The differences between baseline and after 1 year of treatment were computed with a paired *t* test procedure. The significance of baseline/1-year differences between the groups that received pravastatin treatment and placebo treatment was calculated with an unpaired *t* test. Results were regarded as statistically significant if p values were <0.05 (2-sided).

RESULTS

The baseline characteristics of the 90 patients with diabetes are shown in Table 1. Age and gender were matching factors. At baseline, other coronary heart disease risk factors, including alcohol consumption, smoking, exercise, obesity indexes, blood pressure, and history of hypertension were similar between the placebo and the pravastatin groups, as was heart disease status. Medications that might have affected lipid metabolism were similar, although a few more subjects in the placebo group used oral hypoglycemic agents (p = 0.06). Pravastatin significantly lowered total cholesterol by 18% and LDL cholesterol by 31% compared with the changes in the placebo group (Table 2). There were no significant effects on HDL cholesterol and triglyceride concentrations.

Baseline/1-year differences in lipid and apo-B of the specific particle types in the pravastatin group were compared with those in the placebo group (Table 3). There were minor, nonsignificant changes in VLDL particles, but IDL + LDL particles were significantly reduced. Pravastatin significantly reduced the concentrations of apo-B and cholesterol in IDL + LDL E+CIII+ by 42% and 29%, respectively. The concentrations of IDL + LDL E−CIII+ were not significantly affected by prav-

astatin, although the changes were in the same direction as IDL + LDL E+CIII+. IDL + LDL E−CIII− particles, the predominant IDL + LDL type, were reduced significantly by pravastatin by 29% for apo-B, 36% for cholesterol, and 22% for triglycerides. Overall, the cholesterol/apo-B and triglyceride/apo-B ratios, which indicate the cholesterol and triglyceride content per particle, did not change during treatment (ratios are not shown, but they can be estimated from the data in Table 3).

DISCUSSION

Pravastatin specifically lowers LDL cholesterol in diabetic patients, as do other statins. Consequently, its use as a hypolipidemic drug in the Cholesterol and Recurrent Events trial provided a unique opportunity to examine whether it had differing effects on 3 IDL + LDL subtypes in diabetic patients. In a previous ancillary study of the Cholesterol and Recurrent Events trial, apo-CIII but not apo-E in apo-B lipoproteins (VLDL + IDL + LDL) was an independent predictor of recurrent coronary events.[10] Subsequently, it was reported that pravastatin reduced apo-CIII in apo-B lipoproteins, and that the drug's effects were greater in patients with a baseline triglyceride greater than the median.[15] Diabetics were a relatively small percentage of the total population in that study and were not analyzed separately.

In the present study, we extended our lipoprotein subfraction studies to diabetic patients and to specific apo-B lipoproteins containing apo-E and apo-CIII, apo-CIII but not apo-E, or neither apolipoprotein. Our results showed that pravastatin affects apo–B-containing lipoproteins in a selective manner. Pravastatin had little effect on the concentrations of triglyceride-rich VLDL particle types, those with apo-CIII. We recently found in diabetics in the Cholesterol and Recurrent Events trial that these VLDL particles were not associated with coronary events.[13] However, pravastatin markedly reduced the concentrations of the common cholesterol-rich IDL + LDL type, E−CIII−, and also of the triglyceride- and cholesterol-rich IDL + LDL type E+CIII+, which is believed to be a remnant of triglyceride-rich VLDL, and is strongly predictive of coronary events in diabetics.[13] IDL + LDL E−CIII+ particles were not significantly reduced; the percent differences were 17% for apo-B and 25% for cholesterol concentrations. This lack of a significant effect could be due to these particles lacking apo-E as a ligand for LDL receptors, or simply chance. Clearly, there is no significant difference in the changes among the 3 IDL + LDL particle types.

These findings suggest that pravastatin lowers the risk of coronary heart disease by reducing atherogenic IDL + LDL CIII+ particles, as well as by lowering IDL + LDL E−CIII−, the major IDL + LDL type. The largest absolute reduction was for IDL + LDL E−CIII−, and the largest percentage reduction was found for IDL+LDL E+CIII+. Both of these particle types were predictive of coronary events.[13] Reduction of IDL + LDL particles containing apo-CIII may be an additional protective mechanism of statin therapy, which is especially relevant to diabetic dyslipidemia.

Acknowledgment: We are thankful to Gordon H. Williams, MD, Hannia Campos, PhD, and Eric Rimm, DSc, for their contributions to the early development of this research. We thank Cirrana Bird for her skillful technical assistance. The investigators also thank Eugene Braunwald, MD, Chair of the Steering Committee of Cholesterol and Recurrent Events trial, for his encouragement and vision in establishing the ancillary studies program in the trial.

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