Ezetimibe, a Potent Cholesterol Absorption Inhibitor, Normalizes Combined Dyslipidemia in Obese Hyperinsulinemic Hamsters

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Ezetimibe potently and selectively inhibits cholesterol absorption in the intestine, thereby reducing plasma cholesterol in preclinical models of hypercholesterolemia. Clinical trials have demonstrated that ezetimibe lowers LDL cholesterol and raises HDL cholesterol in humans. The effect of ezetimibe on other dyslipidemias, particularly hypertriglyceridemia, is not yet known. In the present studies, we assessed the effect of ezetimibe on combined hypercholesterolemia and hypertriglyceridemia in obese hyperinsulinemic hamsters. Hamsters were fed chow, chow with cholesterol (0.12%), or the same cholesterol diet containing different dietary triglycerides (15%) in the absence or presence of 1 mg/kg ezetimibe (in diet) for up to 84 days. Body weight, serum insulin, leptin, glucose, cholesterol, and triglyceride levels were analyzed. Cholesterol and triglyceride levels were also determined in VLDL+IDL, LDL, and HDL. Hamsters maintained on high-fat diets became obese, hyperinsulinemic, hyperleptinemic, hypercholesterolemic, and hypertriglyceridemic. Ezetimibe did not affect body weight, insulin, or leptin, but ablated the combined hypercholesterolemia and hypertriglyceridemia induced by high-fat diets. Ezetimibe normalized VLDL+IDL cholesterol and triglyceride and significantly decreased LDL cholesterol to below chow-fed levels. The ratio of HDL to LDL cholesterol increased significantly with the addition of ezetimibe. Ezetimibe completely eliminated the accumulation of cholesteryl ester and free cholesterol in liver that was induced under the various dietary conditions in the absence of drug. In conclusion, ezetimibe is very effective in correcting the combined dyslipidemia in diet-induced obese hyperinsulinemic hamsters and may be an effective therapy for ameliorating combined dyslipidemia in obese insulin-resistant and/or type 2 diabetic humans. *Diabetes* 50:1330–1335, 2001

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IDL, intermediate-density lipoprotein.

We have previously described the discovery of a novel cholesterol absorption inhibitor, ezetimibe (Fig. 1) (1–3), that lowers LDL cholesterol and raises HDL cholesterol in hypercholesterolemic humans (4). Evidence is mounting that reducing plasma cholesterol by dietary and/or pharmacological means leads to reductions in the incidence of death from vascular events (5–8). Ezetimibe is rapidly progressing through clinical trials, but it is not yet known what the effect of ezetimibe will be in the obese insulin-resistant and/or type 2 human diabetic population, who often exhibit both hypercholesterolemia and hypertriglyceridemia. Management of combined dyslipidemia is of utmost importance in these populations because these conditions are associated with a much higher incidence of cardiovascular disease (9). Presently, there are very few pharmacological agents that significantly lower both plasma cholesterol and triglyceride levels.

In acute preclinical studies, ezetimibe prevented the transport of ^{14}C-cholesterol from the intestinal lumen through the intestinal wall and into the plasma in rats (1). During chronic preclinical studies, it was determined that ezetimibe inhibited the rise in plasma cholesterol normally observed with cholesterol feeding in the rhesus monkey with an ID_{50} of 0.0005 mg/kg (1) and had comparable potency in dogs, rabbits, rats, and hamsters (10,11). However, no effect on plasma triglycerides was observed in these studies, most likely because all of the species exhibited normotriglyceridemia under the conditions studied. In the present studies, we assessed the effect of ezetimibe on the combined hypercholesterolemia and hypertriglyceridemia in an obese hyperinsulinemic hamster model induced by high-fat diets. The lipid and lipoprotein aspects of this model have been extensively characterized and have been found to closely parallel lipid metabolism in humans (12–16). In the present experiments, this hamster model was chosen to answer several questions: *1*) Does the metabolic profile of this hamster model have similarities to the obese insulin-resistant and/or type 2 diabetic human? *2*) What is the impact of ezetimibe on this metabolic profile? *3*) Can a drug that impacts the absorption of intestinal cholesterol have an effect on the hypercholesterolemia and hypertriglyceridemia induced by the combination of modest dietary cholesterol in the presence of high levels of fat? *4*) If there were changes in the lipid

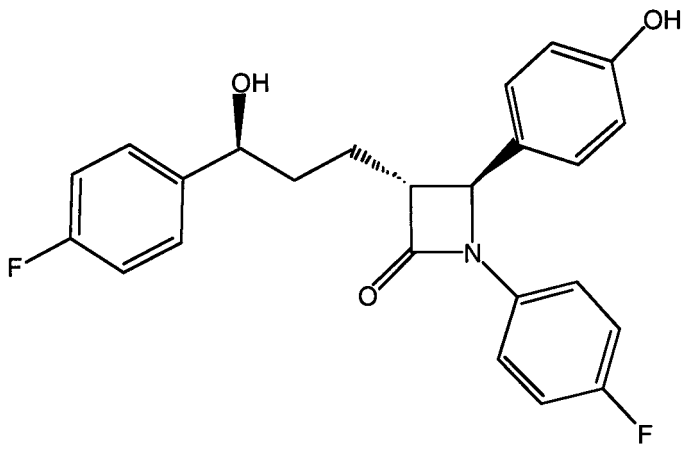

FIG. 1. Structure of ezetimibe, SCH58235 [1-(4-fluorophenyl)-(3R)-[3-(4-fluorophenyl)-(3S)-hydroxypropyl]-(4S)-(4-hydroxyphenyl)-2-azetidinone)].

parameters, which lipoprotein classes would be most affected?

RESEARCH DESIGN AND METHODS

Male golden Syrian hamsters (Charles River Laboratory; Wilmington, MA) were housed individually under 12:12 light:dark (0700–1900) conditions and were allowed free access to chow (Purina Rodent Chow 5001; Ralston Purina, St. Louis, MO) and water during the acclimation before the start of the study. Hamsters (n = 6–7 per group) were assigned to one of the following dietary treatments, so that starting body weights between groups were the same, and were allowed free access to *1*) chow, *2*) chow + 0.12% (wt:wt) cholesterol alone, *3*) chow + 0.12% cholesterol + 15% trimyristin (wt:wt, C14:0), *4*) chow + 0.12% cholesterol + 15% triolein (C18:1), *5*) chow + 0.12% cholesterol + 15% trilinolein (C18:2), or the same diets (diets 1–5) with 1 mg/kg ezetimibe admixed in the chow diet (0.002%; Research Diets, New Brunswick, NJ). Purified triglycerides were supplied by Karlshamns USA (Columbus, OH) and Huls (Piscataway, NJ). Hamsters were weighed weekly, and food intake was measured intermittently throughout the 20- and 84-day feeding periods to ensure that hamsters were thriving on the different dietary regimens and were receiving the appropriate doses of ezetimibe.

After 20 and 84 days, hamsters were killed by CO_2, exsanguinated by heart puncture, and livers were excised, weighed, and frozen at –80°C until further analysis. Serum was separated by low-speed centrifugation, and aliquots were assayed for insulin (Linco Research, St. Charles, MO), leptin (Multispecies Kit; Linco), and glucose (Sigma; St. Louis, MO). Another aliquot of fresh serum was subjected to sequential ultracentrifugation for the separation of the following lipoprotein classes: $d < 1.019$ g/ml (VLDL+IDL), $1.019 < d < 1.055$ g/ml (LDL), and $1.055 < d < 1.21$ g/ml (HDL). Cholesterol (Wako, Osaka, Japan) and triglyceride (Sigma) were determined in whole serum as well as in the lipoprotein fractions. Recovery of cholesterol and triglyceride in the lipoprotein fractions compared with total was consistently >90% and >80%, respectively, and did not differ between groups. Livers were extracted by the method of Folch et al. (17), and cholesteryl ester and free cholesterol content were determined by high-performance liquid chromatography analysis as previously described in detail (18). Liver data are expressed as milligrams of cholesteryl ester or free cholesterol per whole liver.

Data were analyzed for statistical significance using analysis of variance and Student's unpaired t test. Statistical comparisons were performed comparing *1*) the chow-fed control group with the dietary treatment groups and *2*) the dietary treatment groups without ezetimibe to those with ezetimibe.

The results and the conclusions drawn for all the serum values were similar within error when comparing the 20-day to the 84-day data, indicating that steady state in the serum compartment had been reached by 20 days. Liver

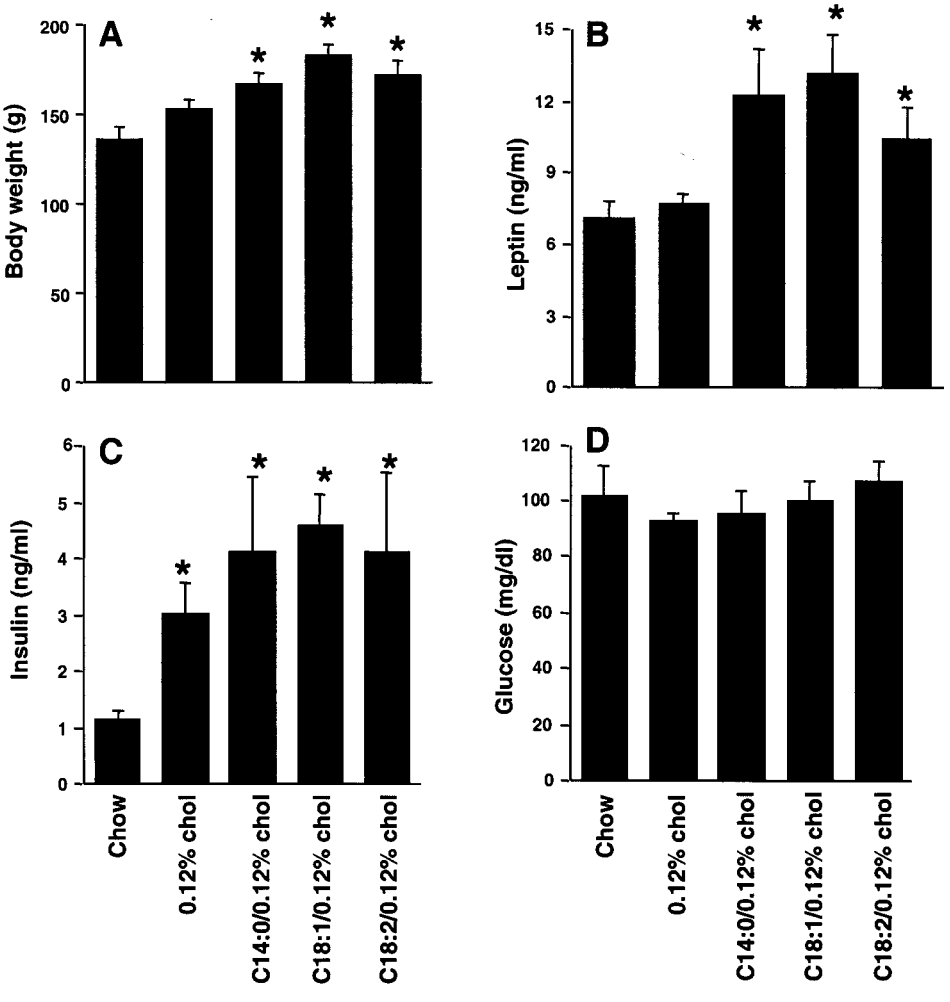

FIG. 2. Body weight, serum leptin, insulin, and glucose. Body weight (*A*), leptin (*B*), insulin (*C*), and glucose (*D*) in hamsters fed chow, chow + 0.12% (wt:wt) cholesterol, or the same cholesterol-containing diet with 15% (wt:wt) trimyristin (C14:0), triolein (C18:1), or trilinolein (C18:2) for 84 days. Values are means ± SE (n = 6–7 per group). *Significantly different from chow-fed controls, $P < 0.05$.

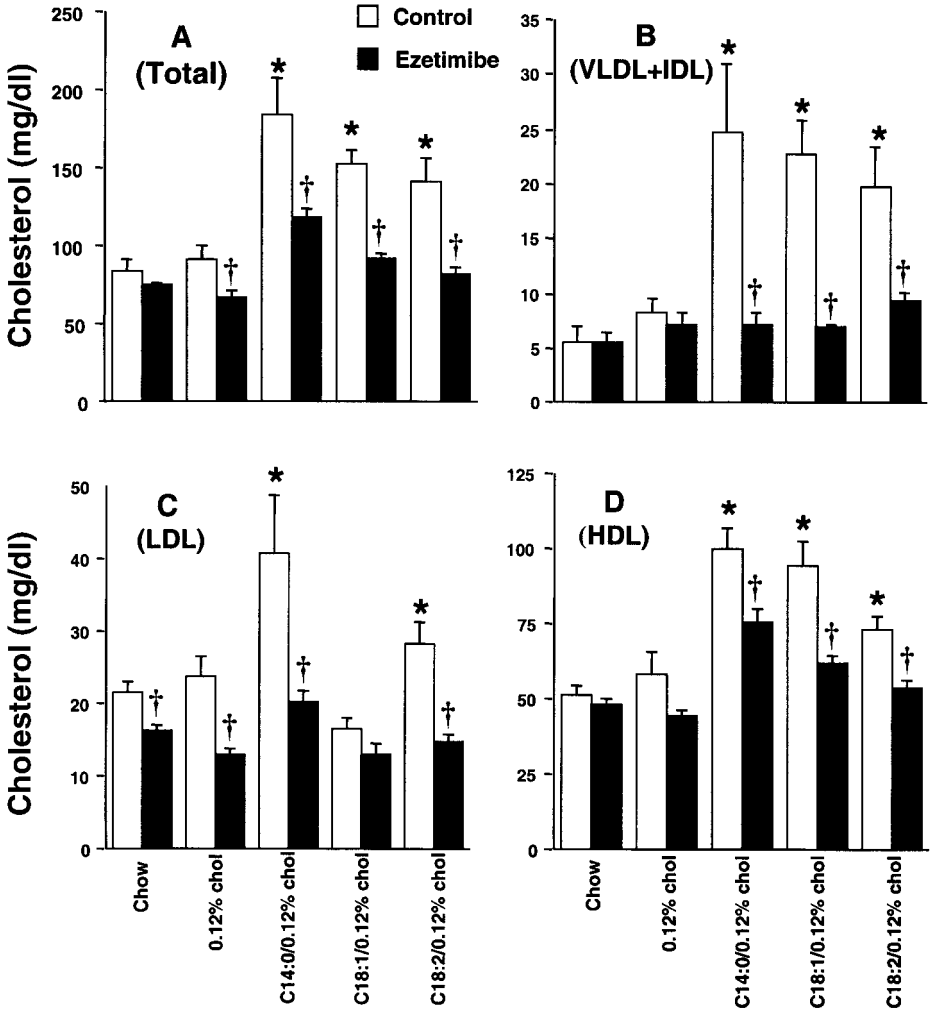

FIG. 3. Total LDL, VLDL+IDL, and HDL cholesterol. Total serum cholesterol (*A*), VLDL+IDL cholesterol (*B*), LDL cholesterol (*C*), and HDL cholesterol (*D*) in hamsters fed chow, chow + 0.12% (wt:wt) cholesterol, or the same cholesterol-containing diet with 15% (wt:wt) trimyristin (C14:0), triolein (C18:1), or trilinolein (C18:2). Data for hamsters fed diets without ezetimibe are in unfilled bars. Data for hamsters fed diets containing 1 mg/kg ezetimibe are in black bars. Values are mean ± SE (n = 6–7 per group). *Significantly different from chow-fed controls, $P < 0.05$; †significantly different from the same dietary group, but without ezetimibe, $P < 0.05$.

cholesterol values were higher in the hamsters treated for 84 days compared with 20 days because liver cholesterol is a cumulative index, but the conclusions drawn from the liver data were also identical between the 20-day and 84-day time point. Therefore, we report only the 84-day data because this longer time period is more representative of what occurs under chronic treatment with ezetimibe and because it also indicates that the hypolipidemic effects of ezetimibe are maintained over time.

All studies were conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care following protocols approved by the Schering-Plough Research Institute's Animal Care and Use Committee. The procedures were performed in accordance with the principles and guidelines established by the National Institutes of Health for the care and use of laboratory animals.

RESULTS

By general appearance, hamsters in all groups appeared to thrive and be healthy. On average, hamsters consumed ~10 g of diet per day; translated to energy consumption, the hamsters on the high-fat diets consumed significantly more kilocalories per day than the chow-fed groups. Accordingly, body weight was significantly greater in the high-fat fed groups compared with the chow-fed groups (Fig. 2*A*). Serum insulin and leptin were significantly higher in the groups fed a high-fat diet, but all groups exhibited normoglycemia (Fig. 2*B–D*). Leptin was highly correlated to body weight (data not shown; $r = 0.72$, $P < 0.0001$), indicating that the increase in body weight was likely attributable to an increase in fat mass. Ezetimibe did not affect body weight, insulin, leptin, or glucose (data not shown). Serum cholesterol in hamsters under chow-fed conditions was ~80 mg/dl (Fig. 3*A*). Addition of a modest amount of dietary cholesterol (0.12% cholesterol) alone did not increase serum cholesterol. However, the addition of 15% fat to the diet significantly increased serum cholesterol above the chow diet and moderate cholesterol-only diet values. Specifically, serum cholesterol increased 2.2, 1.8, and 1.7 times over chow-fed levels under dietary conditions of moderate cholesterol and high levels of saturated (C14:0), monounsaturated (C18:1), or polyunsaturated (C18:2) fat, respectively. The presence of ezetimibe dramatically reduced serum cholesterol in all but the chow-fed hamsters. Ezetimibe in the moderate cholesterol diet significantly reduced cholesterol (−26%) below its own control group as well as below chow-fed levels (−20%). Ezetimibe completely ablated the hypercholesterolemia induced under the monounsaturated (C18:1) and polyunsaturated (C18:2) diet conditions, and it significantly reduced the hypercholesterolemia induced by the saturated fat (C14:0) diet. The cholesterol concentration of VLDL+IDL, LDL, and HDL was not altered under moderate cholesterol-only conditions compared with chow (Fig. 3*B–D*). The cholesterol concentration in VLDL+IDL and HDL increased significantly under all dietary conditions containing high fat. LDL cholesterol increased significantly with trimyristin (C14:0) and trilinolein (C18:2), but was

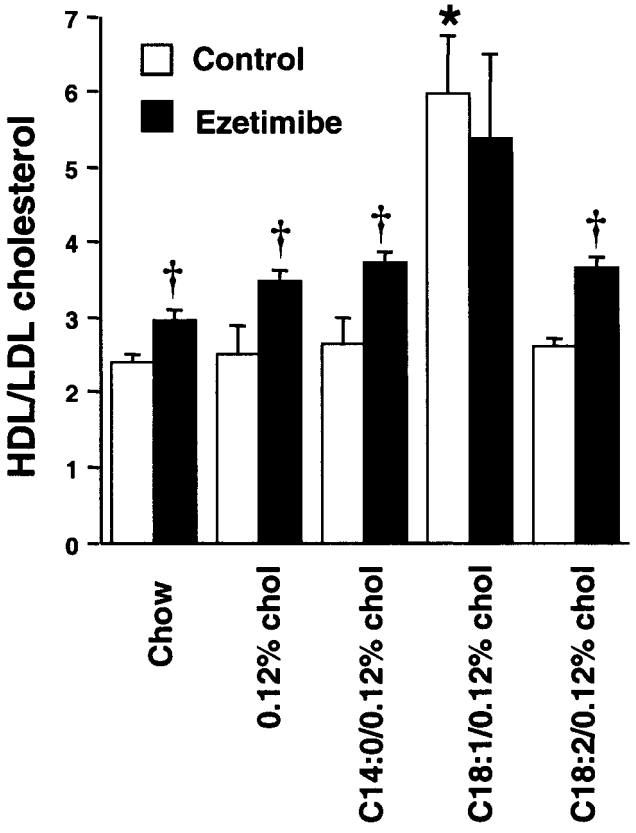

FIG. 4. Ratio of HDL to LDL cholesterol. Ratio of HDL to LDL cholesterol (HDL:LDL) in hamsters fed chow, chow + 0.12% (wt:wt) cholesterol, or the same cholesterol-containing diet with 15% (wt:wt) trimyristin (C14:0), triolein (C18:1), or trilinolein (C18:2). Data for hamsters fed diets without ezetimibe are in unfilled bars. Data for hamsters fed diets containing 1 mg/kg ezetimibe are in black bars. Values are means ± SE (n = 6–7 per group). *Significantly different from chow-fed controls, $P < 0.05$; †significantly different from the same dietary group, but without ezetimibe, $P < 0.05$.

actually lower than chow-fed levels with triolein (C18:1) feeding. Ezetimibe ablated the rise in VLDL+IDL cholesterol completely (Fig. 3*B*). Ezetimibe reduced LDL cholesterol to chow-fed levels or below under all dietary conditions studied (Fig. 3*C*). Ezetimibe reduced HDL cholesterol levels as well, but these HDL levels still remained above chow-fed levels (Fig. 3*D*). Expressing these data as a ratio of HDL to LDL cholesterol (Fig. 4) demonstrated that ezetimibe significantly raised the HDL:LDL cholesterol in every group except the triolein (C18:1) group. In the triolein (C18:1) group without ezetimibe, the HDL:LDL cholesterol was already very high due to the very low LDL cholesterol concentration, and ezetimibe did not affect this high ratio.

Serum triglyceride (Fig. 5*A*) was unaffected by the addition of 0.12% cholesterol to the diet, but hypertriglyceridemia was induced to approximately the same degree under all dietary conditions containing fat (>2 times over chow-fed controls). The induced hypertriglyceridemia was nearly normalized by ezetimibe. The hypertriglyceridemia induced by the high-fat diets was reflected almost exclusively in the VLDL+IDL fraction (Fig. 5*B*). Ezetimibe reduced the VLDL+IDL triglyceride to chow-fed levels. Triglycerides were low in the LDL and HDL fractions, and ezetimibe had no effect on triglyceride in these fractions (data not shown).

In general, liver weights were higher under dietary conditions containing cholesterol or cholesterol plus dietary fat compared with chow-fed controls; liver weights were significantly lower in all groups fed cholesterol plus ezetimibe (Table 1). However, the results and conclusions are the same whether the data are expressed as milligrams of cholesterol per gram of liver or total milligrams of cholesterol per liver; therefore total milligrams of cholesterol per liver is presented in Fig. 6. Inclusion of 0.12% cholesterol

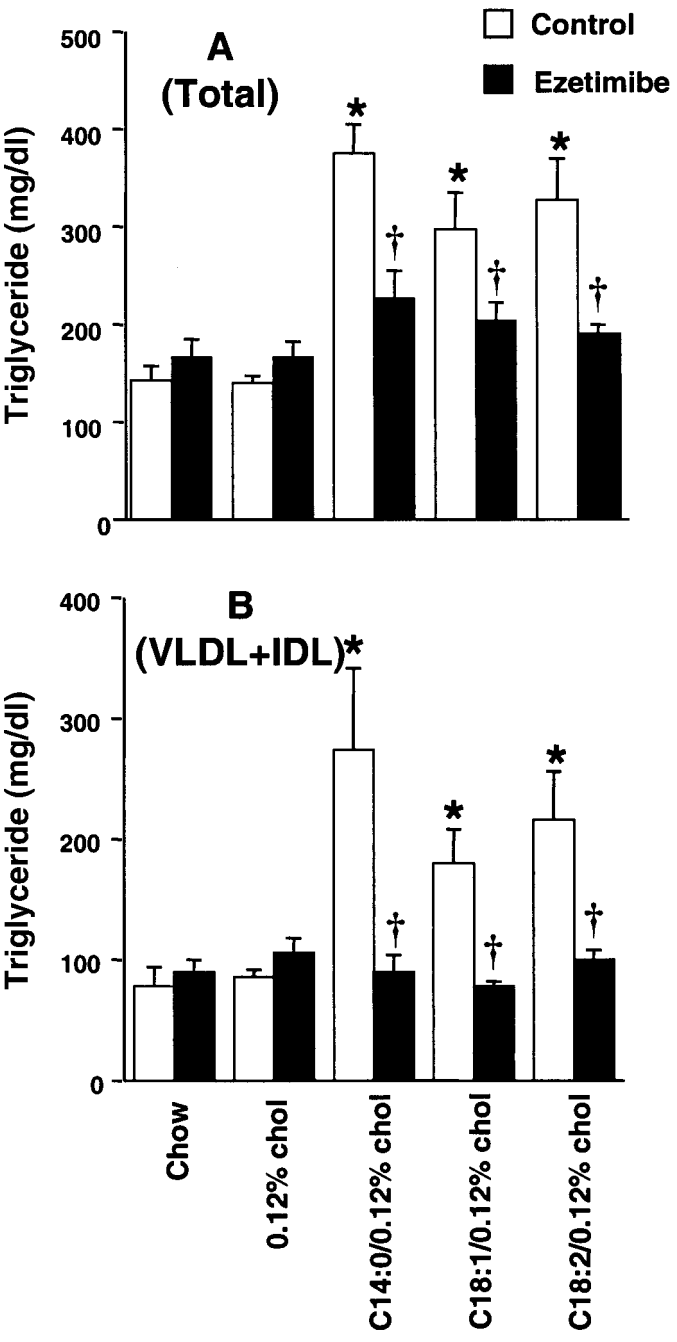

FIG. 5. Total and VLDL+IDL triglyceride. Total serum triglyceride in VLDL+IDL in hamsters fed chow, chow + 0.12% (wt:wt) cholesterol, or the same cholesterol-containing diet with 15% (wt:wt) trimyristin (C14:0), triolein (C18:1), or trilinolein (C18:2). Data for hamsters fed diets without ezetimibe are in unfilled bars. Data for hamsters fed diets containing 1 mg/kg ezetimibe are in black bars. Values are means ± SE (n = 6–7 per group). *Significantly different from chow-fed controls, $P < 0.05$; †significantly different from the same dietary group, but without ezetimibe, $P < 0.05$.

TABLE 1
Liver weights of hamsters under various dietary conditions in the absence and presence of ezetimibe (1 mg/kg)

Dietary treatment	Control liver weight (g)	Ezetimibe liver weight (g)
Chow	4.94 ± 0.26	5.17 ± 0.20
Chow + 0.12% cholesterol	6.26 ± 0.35*	5.28 ± 0.33†
Chow + 0.12% cholesterol + 15% trimyristin (C14:0)	7.30 ± 0.39*	6.15 ± 0.29*†
Chow + 0.12% cholesterol + 15% triolein (C18:1)	7.96 ± 0.5*	5.94 ± 0.42*†
Chow + 0.12% cholesterol + 15% trilinolein (C18:2)	7.38 ± 0.56*	5.78 ± 0.24*†

Data are means ± SE and are for hamsters from the 84-day time point. *Significantly different from the chow-fed controls ($P < 0.05$); †significantly different from the same dietary group, but without ezetimibe ($P < 0.05$).

alone or cholesterol plus the dietary fats in the diet increased the accumulation of cholesteryl ester in liver by 4.5 (cholesterol only), 3.1 (C14:0), 12.6 (C18:1), or 5.0 (C18:2) times over chow-fed controls (Fig. 6*A*). Accumulation of free cholesterol in livers (Fig. 6*B*) followed the same pattern, but to a lesser degree. Ezetimibe completely blocked the accumulation of either cholesteryl ester or free cholesterol that was observed under any dietary condition (Fig. 6*A* and *B*).

DISCUSSION

Ezetimibe is a potent cholesterol absorption inhibitor that lowers LDL cholesterol and raises HDL cholesterol in hypercholesterolemic humans (4) and is now in phase III clinical trials. Preclinical studies have demonstrated that ezetimibe selectively inhibits the transport of radiolabeled cholesterol through the intestinal wall and ultimately into the plasma (1,3). The precise molecular mechanism by which cholesterol is absorbed in the intestine (and how ezetimibe inhibits this absorption) is currently unknown and under intensive investigation.

In the present studies, we demonstrated that feeding high-fat diets containing modest cholesterol to hamsters leads to obesity accompanied by hyperinsulinemia, hyperleptinemia, hypercholesterolemia, and hypertriglyceridemia, which are characteristic of the profile often observed in obese insulin-resistant and/or type 2 diabetic patients (9). In an initial report, we described the potent cholesterol absorption inhibitor ezetimibe, which inhibited the rise in plasma cholesterol normally observed in cholesterol-fed rhesus monkeys with an ID_{50} of 0.0005 mg/kg (1), but we observed no change in plasma triglycerides, most likely because these monkeys were normotriglyceridemic. Here, we report that ezetimibe markedly reduced the combined hyperlipidemia in obese hamsters without affecting body weight, hyperinsulinemia, hyperleptinemia or serum glucose. In the presence of ezetimibe, an improvement in the HDL-to-LDL ratio was observed. Total cholesterol content of the liver was equivalent to that of hamsters fed chow only, indicating a complete inhibition of cholesterol absorption. These observations may have potential scientific and clinical significance for the management of dyslipidemia in humans, particularly in the obese insulin-resistant and/or type 2 diabetic population, who often exhibit combined hyperlipidemia (9).

Numerous studies have shown that feeding dietary fats in the absence of dietary cholesterol does not alter plasma or serum lipids (19,20, rev. in 16). In the present studies, the modest level of dietary cholesterol (0.12%) alone was insufficient to raise serum cholesterol or triglyceride levels. Inclusion of a high level of dietary fat (15%) in combination with the same amount of dietary cholesterol led to hypercholesterolemia and hypertriglyceridemia, particularly with saturated fat, as previously described (12–16). This would initially suggest that the dietary fat component was the more important driving force behind the

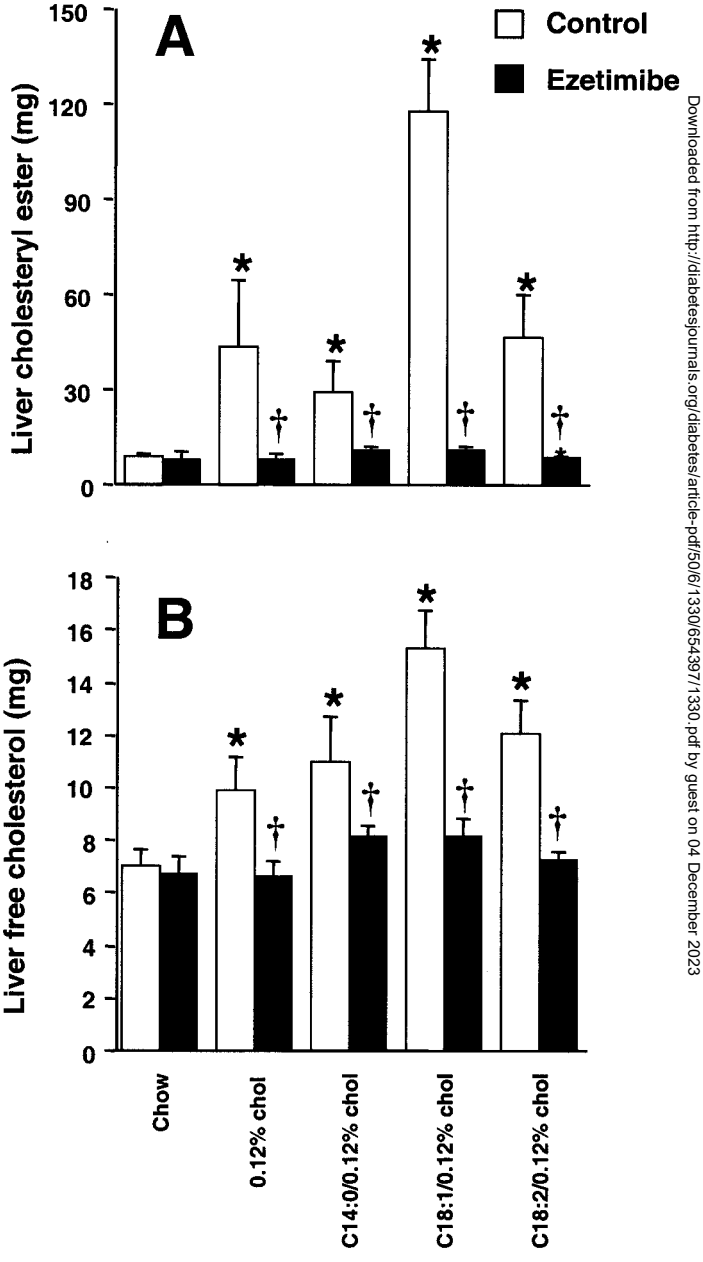

FIG. 6. Accumulation of cholesteryl ester and free cholesterol in liver. Cholesteryl ester (*A*) and free cholesterol (*B*) content in liver from hamsters fed chow, chow + 0.12% (wt:wt) cholesterol, or the same cholesterol-containing diet with 15% (wt:wt) trimyristin (C14:0), triolein (C18:1), or trilinolein (C18:2). Data for hamsters fed diets without ezetimibe are in unfilled bars. Data for hamsters fed diets containing 1 mg/kg ezetimibe are in black bars. Data are expressed as total milligrams per liver. Values are means ± SE (n = 6–7 per group). *Significantly different from chow-fed controls, $P < 0.05$; †significantly different from the same dietary group, but without ezetimibe, $P < 0.05$.

combined hyperlipidemia. Yet, inhibiting absorption of cholesterol from the intestine with ezetimibe completely ameliorated the combined hyperlipidemia induced by the combination of cholesterol and fat, indicating that dietary fat without the accompanying cholesterol absorption did not affect serum lipids. The data thus indicate that the combination of moderate dietary cholesterol and high fat is necessary for combined hyperlipidemia to exist, which is in effect the naturally occurring dietary situation in a large portion of the human population.

Clinically, this has potential implications for humans who exhibit combined hyperlipidemia, which includes the growing population of obese and type 2 diabetic patients. It is well accepted that the high incidence of hyperlipidemia, obesity, and type 2 diabetes in the Western hemisphere is caused by a genetic predisposition compounded by a diet high in cholesterol and fat. Patients are advised to reduce both their dietary cholesterol and fat intake to reduce hyperlipidemia. In a large number of patients, these dietary changes have little or only modest lipid-lowering effect, partially because of patient compliance and partially because of the concomitant increase in cholesterol synthesis that accompanies a decrease in dietary cholesterol consumption. Presently, these patients are then prescribed a hydroxymethyl glutaryl CoA reductase inhibitor ("a statin") to reduce the synthesis of cholesterol, and successful plasma cholesterol lowering is often achieved.

The data in the present report suggest that the hypercholesterolemia and hypertriglyceridemia induced by modest cholesterol and high-fat diets may be largely ameliorated by blocking cholesterol absorption, which would include both dietary and biliary cholesterol. In hypercholesterolemic humans, ezetimibe alone significantly decreased LDL cholesterol (−18.5%) and increased HDL cholesterol (+3.5%) (4). The combination of ezetimibe and the statins has shown significant synergy in lowering cholesterol in preclinical trials (10,11). Recent studies in hypercholesterolemic humans demonstrated that combining simvastatin (10–20 mg/day) and ezetimibe (10 mg/day) led to a 50–60% decrease in LDL cholesterol in 2 weeks of treatment (21,22). The effect of ezetimibe alone and in combination with the statins has not yet been tested in humans with combined hyperlipidemia. Based on the present studies, ezetimibe alone may lead to a reduction in plasma cholesterol and triglyceride in humans with combined hyperlipidemia, such as obese insulin-resistant and/or type 2 diabetic patients. Combining the two pharmacological interventions may lead to a greater decrease than is presently possible in patients with combined hyperlipidemia.

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