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Assessing Lipid Lowering and Plasma Cholesteryl Ester Transfer Protein Activity of Simvastatin Following Administration to Rabbits Fed a High Fat/Cholesterol Diet

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ABSTRACT **Purpose**: The purpose of this study was to assess the lipid lowering and plasma cholesteryl ester transfer protein (CETP) activity following administration of simvastatin to rabbits fed a high fat/cholesterol diet. **Methods**: Male New Zealand white rabbits were housed in individual cages and fed a standard diet for 7 days. After 7 days, animals were fed 10 g of a regular chow diet plus 100 g of the same diet supplemented with 0.5% (w/v) cholesterol and 14.0% (w/v) coconut oil for 28 days. Following 28 days on this diet, the animals were randomized based on plasma cholesterol and triglyceride levels, into a group of control animals and a group ($n = 6$) of animals fed 100 g of cholesterol/coconut diet plus 10 g regular chow diet containing simvastatin (3 mg/kg/day) for an additional 28 days. Blood samples were taken from the marginal ear vein prior to and 28 days after the initiation of drug treatment. Plasma was harvested and stored at 4°C prior to lipid analysis. Plasma total cholesterol and triglyceride levels were quantified using enzymatic kits. HDL (high-density lipoproteins) cholesterol levels were determined using the dextran sulfate-Mg^{2+} precipitation method. ApoB cholesterol levels were determined by subtracting total cholesterol from HDL cholesterol. Cholesteryl ester transfer protein (CETP) activity was determined by standard assay methods. **Results**: We observed that simvastatin significantly reduced total plasma cholesterol, triglyceride, and apoB cholesterol compared to non-treated controls. Simvastatin treatment did not alter serum CETP activity compared to non-treated controls. **Conclusions**: These findings suggest that decreasing plasma lipid levels by treatment with simvastatin is not due to changes in serum CETP activity in rabbits fed a high fat/cholesterol diet.

KEYWORDS Cholesteryl Ester Transfer Activity, Simvastatin, High fat/cholesterol rabbit model, Hypercholesterolemia

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INTRODUCTION

Also known as "statins," 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (i.e., simvastatin) have been accepted as the first choice for lowering low-density lipoprotein (LDL) cholesterol levels (Denke, 2004). These inhibitors act primarily by inhibiting the cellular biosynthesis of cholesterol in the liver. The reduction of intracellular cholesterol content is associated with an increased expression of hepatic LDL receptors, which in turn leads to marked reduction in plasma LDL-cholesterol levels. Recent studies have shown that statin therapy equally induces a reduction in plasma cholesteryl ester transfer protein (CETP) activity in both normolipidemic (Ahnadi et al., 1993) and dyslipidemic subjects (Bagdade et al., 1990; Ahnadi et al., 1993; Guerin et al., 1995; Hunninghake et al., 1990; The Pravastatin Multinational Study Group for Cardiac Risk Patients, 1993).

Cholesteryl ester transfer protein (CETP) facilitates the transfer of cholesteryl esters from HDL to ApoB-containing lipoproteins (VLDL & LDL) with a reciprocal transfer of triglyceride (Tall et al., 1983; Morton & Zilversmit, 1982). Cholesteryl ester transfer protein (CETP) plays an important role in the metabolism and remodeling of plasma lipoproteins (Morton, 1990). Cholesteryl ester transfer protein (CETP) may also play a role in certain disease processes such as atherosclerosis by redistributing cholesterol from the anti-atherogenic HDL particles to the pro-atherogenic LDL particles, although the impact of CETP-mediated lipid transfer reactions on atherogensis remains controversial (McTavish & Sorkin, 1991). For instance, animal studies have suggested opposite actions of this protein. On the one hand, expression of CETP in animals lacking CETP can exert marked atherogenic activity; however, on the other hand inhibition of CETP can reduce the progression of atherosclerosis (McTavish & Sorkin, 1991). This later observation was observed in the hyperlipidemic rabbit (Meijer et al., 1998).

However, such a reduction in cholesteryl ester transfer (CET) results principally from marked reduction in particle numbers of apoB-containing lipoproteins but equally in part from statin-induced reduction in plasma CETP concentration (Ahnadi et al., 1993). The action of statins on CETP mass appears to be dependent on the dose used and on apoE phenotype. By inhibiting cholesterol biosynthesis, statins might also reduce hepatic CETP gene expression via reduction in intracellular oxysterol concentrations thereby reducing the transcriptional activation of the CETP gene by the liver X receptor (LXR).

Recent studies have reported, however, that postprandial lipid state transiently increases CET (Tall et al., 1986; Castro & Fielding, 1985) and CETP activity (Tall et al., 1986; Castro & Fielding, 1985). Thus, purpose of this study was to assess the lipid lowering and plasma CETP activity following administration of simvastatin to rabbits fed a high fat/cholesterol diet.

MATERIALS AND METHODS

Chemicals and Research Diet

Simvastatin lactone was generously donated by Merck Inc. USA (Whitehouse Station, NJ, USA). The regular Purina rabbit and high fat/cholesterol diets (which contains coconut oil) were purchased from Research Diets Inc. (New Brunswick, NJ, USA) (Verd et al., 1999). Diet preparation was carried out at Research Diets and simvastatin was incorporated into the diet. Radiolabeled CE ([1, 2 alpha n-^{3}H] Cholesteryl oleate; Specific Activity, 71.9 mCi/mg) was purchased from Amersham Life Science (Buckinghamshire, England). Sodium bromide was purchased from Sigma Chemical Company (St. Louis, MO, USA).

Cholesterol-Fed Rabbit Model

Rabbits are appropriate animals for the study of the effects of lipid-lowering agents since diet can be manipulated to induce hypercholesterolaemia (Kroon et al., 1982; Prior et al., 1961; Kolodgie et al., 1996). The cholesterol-fed rabbit model has previously been used to demonstrate the lipid-lowering effects of statins (Nielsen et al., 1993). In this study, New Zealand white male rabbits (2.0 to 2.5 kg; Jeo-Bet Rabbits LTD, Aldon B.C., Canada) that exhibit hypercholesterolemia induced by a fat/cholesterol-enriched diet as previously published by Verd et al. (1999) were used. The fat/cholesterol-enriched diet consistent of Purina rabbit chow supplemented with 14.0% (weight/volume) coconut oil and 0.50% (weight/volume) cholesterol.

Animal Care

The Animal Care Committee of the University of British Columbia approved the study. Food and water intakes and body weight were recorded for all animals

on a daily basis. None of the animals refused the diet at any point throughout the duration of the study.

Experimental Design and Lipid Analysis

Male New Zealand white rabbits ($n = 12$) were housed in individual cages in a room with constant humidity and temperature (22°C) under a 12 h light-dark cycle, and fed a standard diet for 7 days. After 7 days, animals were fed 10 g of a regular chow diet plus 100 g of the same diet supplemented with 0.5% (w/v) cholesterol and 14.0% (w/v) coconut oil for 28 days. The final diet administered to these rabbits (following dilution of 100 g of high fat/cholesterol diet with 10 g of regular chow) consistent of 0.45% (w/v) cholesterol and 12.75% (w/v) coconut oil. Following 28 days on this diet, the animals were assigned based on their 16 h post-meal plasma cholesterol and triglyceride concentrations (Table 1; day 28), into two groups of six animals each with no statistically significant differences between total plasma cholesterol (Table 2), and triglyceride concentrati ons (Table 3).

For an additional 28 days (day 28 to day 56 of the study), a group of control animals ($n = 6$) were maintained on the same diet (10 g regular chow diet plus 100 g of cholesterol/coconut diet) and a group of animals were fed 100 g of cholesterol/coconut diet plus 10 g regular chow diet containing simvastatin (3 mg/kg/day; $n = 6$, see Fig. 1). Whole blood was collected (3 mL) prior to and 28 days after initiation of drug treatment for plasma lipid determinations.

Plasma total cholesterol and triglyceride concentrations were directly quantified using enzymatic kits (Sigma Aldrich). HDL cholesterol concentrations were determined using the dextran sulfate-Mg^{2+} precipitation method as developed by Warnick et al. (1982). The Friedewald equation for determining LDL cholesterol can not be used in this rabbit fed model (Friedewald et al., 1972). However, measurement of apoB cholesterol (which takes into account LDL cholesterol) concentrations were determined by subtracting HDL cholesterol concentrations from total cholesterol concentrations.

The 3 mg/kg/day simvastatin dose selected in this study was based on results (i.e., demonstrating significant decrease in total plasma and apoB cholesterol levels) completed in the same animal model by Verd et al. (1999).

CETP Analysis

The plasma was separated into its HDL, LDL, VLDL, and lipoprotein deficient plasma (LPDP) fractions by ultracentrifugation (Ramaswamy et al., 1997; Havel et al., 1955). Rabbit LDLs were labeled by the lipid dispersion technique as previously described (Morton & Zilversmit, 1982; Pattnaik & Zilversmit, 1979). Low-density lipoproteins labeled with ^{3}H-CE had a specific activity of 1.6×10^{-3} uCi/ug LDL cholesterol.

Lipid (CE) transfers were performed within lipoprotein-deficient plasma as has been previously described (Morton & Zilversmit, 1982; Pattnaik & Zilversmit, 1979). Typically, 10 μg (total cholesterol) of radiolabeled donor and unlabeled acceptor are incubated ± CETP (1.0 ug protein/mL; concentration was determined from a dose response curve; data not shown) in delipidated human plasma (delipidated human plasma was used as a CETP source with a concentration of 1.0 ug protein/mL as determined by ELISA), pH 7.4 for 90 min (time was determined from a time response curve; data not shown) at 37°C. Lipid transfer between donor and acceptor lipoprotein is then quantitated by scintillation counting. The fraction of lipid transferred (kt) is calculated as described by Pattnaik & Zilversmit (1979):

$$kt = -ln\,(1 - A_t/D_o)$$

TABLE 1 **Effect of Coconut Oil/Cholesterol Feeding* on Plasma Cholesterol, HDL Cholesterol, and Triglyceride Concentrations**

	Total cholesterol (mg/dL)	HDL cholesterol (mg/dL)	Total triglyceride (mg/dL)
Day 0	47 ± 3	23 ± 2	176 ± 39
Day 28	726 ± 102[a]	79 ± 23[a]	63 ± 10[a]

Data presented as mean ± standard error of the mean ($n = 12$).
[a]$p < 0.05$ vs. Day 0. HDL, high-density lipoproteins.
*High cholesterol diet consists of 0.45% w/v cholesterol plus 12.75% w/v coconut oil.

TABLE 2 **Total Plasma and HDL Cholesterol Concentrations in New Zealand Male Rabbits Fed a High Cholesterol Diet* at the Beginning (Day 28) and at the End (Day 56) of Drug Treatment**

	Total Cholesterol (mg/dL)		HDL-Cholesterol (mg/dL)	
Day	28	56	28	56
CON	725 ± 161	1624 ± 174**	90 ± 45	43 ± 17
SVT	727 ± 141	1127 ± 157	67 ± 19	26 ± 3

Data is presented as mean ± SEM; $n = 6$ in each treatment group.
*High cholesterol diet consists of 0.45% w/w cholesterol plus 12.75% coconut oil.
**$p < 0.05$ vs. Day 28.
HDL, high-density lipoproteins; CON, control; SVT, simvastatin 3 mg/kg/day.

TABLE 3 **Total Triglycerides and ApoB Cholesterol Concentrations in New Zealand Male Rabbits Fed a High Cholesterol Diet* at the Beginning (Day 28) and at the End (Day 56) of Drug Treatment**

	Triglycerides (mg/dL)		Apo B cholesterol (mg/dL)	
Day	28	56	28	56
CON	67 ± 17	271 ± 68**	635 ± 116	1581 ± 157**
SVT	59 ± 11	148 ± 28	660 ± 129	1101 ± 154

Data is presented as mean ± SEM; $n = 6$ in each treatment group.
*High cholesterol diet consists of 0.45% w/w cholesterol plus 12.75% Coconut Oil.
**$p < 0.05$ vs. Day 28.
ApoB, apolipoprotein B; TG, triglycerides; CON, control; SVT, simvastatin 3 mg/kg/day.

where D_o and A_t are the radioactivity's in the donor at time 0 and in the acceptor at time t, respectively. The constant k is the fraction of label transferred per unit time (t). Acceptor radioactivity in the absence of CETP (usually < 2–3%) is subtracted before calculating kt values. Calculations assume steady-state conditions where all lipid transfer is an exchange process. Plasma from rabbits were removed at the end of the study and used as the source of CETP to determine CETP activity. TP2, a monoclonal antibody raised up against CETP (purchased from the Ottawa Heart Institute), was used as a positive control. Rabbit plasma samples were spiked with TP2 (8 μg protein/mL; see Table 4) and CETP activity was determined.

Statistical Analysis

Results were expressed as mean ± standard error of the mean (SEM). Statistical analyses were conducted using an analysis of variance (PCANOVA; Human Dynamic Systems; GraphPad Inc., San Diego, CA) and assuming unequal variance (Newman Keuls post-hoc test; we assumed unequal variance). Variables compared among treatment groups include, weight gain, food intake, water intake, total plasma cholesterol, total plasma triglyceride, HDL-cholesterol, apoB cholesterol, and CETP activity. A p-value of less than 0.05 indicated a significant difference between treated and untreated groups.

RESULTS AND DISCUSSION

Total Body Weight, Food and Water Intake

No differences in weight gain, food, and water intake (data not shown) were observed for both groups throughout the duration of the study (data not shown). All rabbits were provided a set diet and received 110 g of food daily.

Total Cholesterol and Triglyceride Concentrations

The effects of simvastatin on plasma total cholesterol and triglyceride levels are reported in Tables 2 and 3. Simvastatin treatment significantly reduced the increase in total plasma cholesterol concentration by 56% between the beginning (day 28) and the end (day 56) of drug treatment compared to non-treated controls (Table 2). In addition, simvastatin treatment reduced the increase in total plasma triglyceride levels by 57% between the beginning (day 28) and the end (day 56) of drug treatment compared to non-treated controls (Table 3).

HDL and ApoB Cholesterol Concentrations

The effects of simvastatin on plasma HDL and apoB cholesterol concentrations are reported in Tables 2 and 3, respectively. No statistically significant differences in plasma HDL cholesterol levels were observed in the simvastatin group between the beginning (day 28) and the end (day 56) of drug treatment compared to non-treated controls (Table 2). Simvastatin treatment significantly reduced the increase in apoB cholesterol concentration by 53% between the

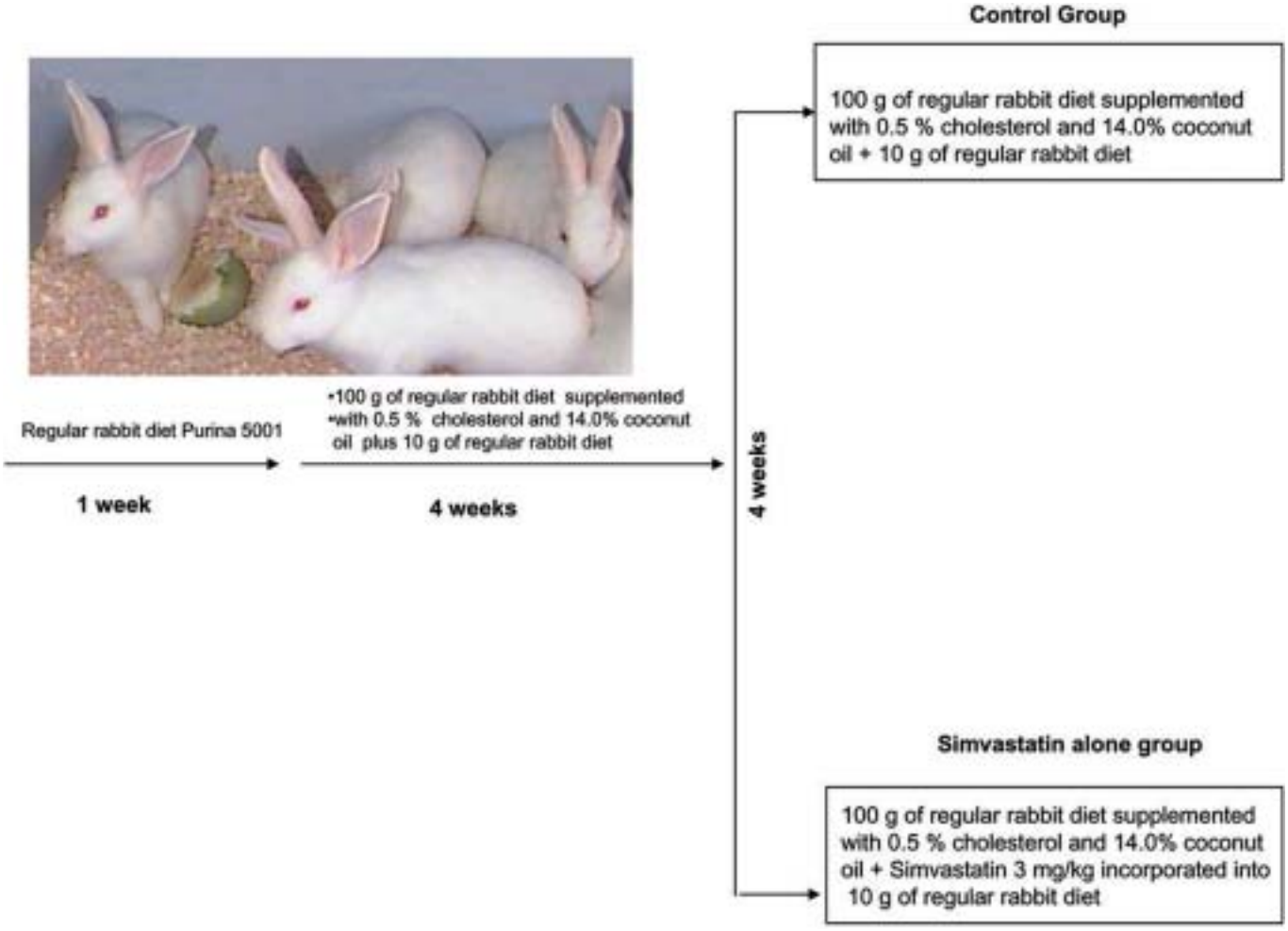

FIGURE 1 **Experimental Design of Rabbits on High Cholesterol/Fat Diet ± Simvastatin Lactone (3 mg/kg).**

TABLE 4 **Percent Transfer of Cholesteryl Ester (CE) from LDL to HDL in New Zealand Male Rabbits Fed a High Cholesterol Diet* at the Beginning (Day 28) and at the End (Day 56) of Drug Treatment**

Treatment	LDL to HDL %kt[a]
Control	
Day 28	22.5 ± 2.4
Day 56	20.4 ± 6.6
TP2 (Protein)	
Day 28	1.8 ± 0.2**
Day 56	7.3 ± 1.9**
Simvastatin	
Day 28	20.4 ± 3.4
Day 56	23.4 ± 7.0

A monoclonal antibody (TP2; 8 ug protein/mL) directed against CETP in rabbit plasma serves as a positive control.

*High cholesterol diet consists of 0.45% w/w cholesterol plus 12.75% coconut oil.

**$p < 0.05$ vs. control using PCANOVA.

Data presented as mean ± SEM; $n = 6$ for control and simvastatin; $n = 3$ for TP2.

beginning (day 28) and the end (day 56) of drug treatment compared to non-treated controls (Table 3).

Plasma CETP Activity

The effects of simvastatin on plasma CETP activity is reported in Table 4. No statistically significant differences in plasma CETP activities were observed in the simvastatin group between the beginning (day 28) and the end (day 56) of drug treatment compared to non-treated controls (Table 4).

The purpose of this study was to assess the lipid lowering and plasma CETP activity of simvastatin following administration to rabbits on a high fat/cholesterol diet. Previous groups reported on the effect of different HMG-CoA reductase inhibitors on CETP activity in animals and humans. Ahnadi and colleagues reported over a decade ago that

simvastatin administration decreased the transfer of cholesterol esters from HDL to VLDL and LDL in normolipidemic patients by lowering of plasma CETP activity (Ahnadi et al., 1993). Homma et al. investigated the effects of simvastatin on CETP activity in 28 patients with type II hyperlipoproteinemia. They observed that although CETP activity was reduced by approximately 30%, reductions in CETP activity and in lipoprotein cholesterol levels were not correlated. The authors suggested that changes in lipoprotein cholesterol levels were not primarily due to suppression in CETP activity (Homma et al., 1995). Meijer and co-workers reported that rabbits fed a high cholesterol diet found decreased serum CETP activity levels following simvastatin treatment may contribute to lowering of cholesterol apoB-containing lipoproteins (Meijer et al., 1998).

However, in our study, although simvastatin significantly reduced total and apoB cholesterol compared to controls, unlike the Meijer study, the drug did not significantly decrease plasma CETP activity. A possible explanation for the discrepancy is that in our study the rabbits were administered an extremely high fat/cholesterol diet (0.45% w/v cholesterol and 12.5% w/v coconut oil) while the concentration of the cholesterol diet used in the Meijer study was 0.1% w/v (24). These findings suggest that in extreme cases of dyslipidemia, statins may decrease plasma lipids primarily by inhibiting HMG-CoA reductase activity.

In conclusion, we observed that simvastatin significantly reduced total plasma cholesterol, triglyceride, and apoB cholesterol compared to non-treated controls. Simvastatin treatment did not alter plasma CETP activity compared to non-treated controls. These findings suggest that decreasing plasma lipid levels by treatment with simvastatin is not due to changes in serum CETP activity in rabbits fed a high fat/cholesterol diet.

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