

Ezetimibe inhibits the incorporation of dietary oxidized cholesterol into lipoproteins

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Abstract Oxidized cholesterol is present in significant quantities in the typical Western diet. When ingested, oxidized cholesterol is absorbed by the small intestine and incorporated into both chylomicrons and LDL, resulting in LDL that is more susceptible to further oxidation. Feeding studies in animal models and epidemiological studies in humans have suggested that oxidized cholesterol in the diet increases the development of atherosclerosis. In this study, we determined the effect of ezetimibe, a drug that inhibits small intestinal absorption of cholesterol, on the levels of oxidized cholesterol in the serum after a test meal containing oxidized cholesterol. We demonstrate that ezetimibe, 10 mg per day for 1 month, markedly reduced the levels (50% decrease) of oxidized cholesterol in the serum after feeding a test meal containing either α -epoxy cholesterol or 7-keto cholesterol, two of the predominant oxidized cholesterols found in the diet. Moreover, the decrease in oxidized cholesterol in the serum was attributable to a decrease in the incorporation of dietary oxidized cholesterol into both chylomicrons and LDL. Because there was no decrease in postprandial triglyceride levels, we conclude that this decrease in oxidized cholesterol levels in the serum is attributable to decreased absorption and not to enhanced clearance. Whether this decrease in oxidized cholesterol absorption prevents or delays the development of atherosclerosis remains to be determined.—Staprans, I., X-M. Pan, J. H. Rapp, A. H. Moser, and K. R. Feingold. **Ezetimibe inhibits the incorporation of dietary oxidized cholesterol into lipoproteins.** *J. Lipid Res.* 2006. 47: 2575–2580.

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There is strong evidence that oxidized lipoproteins play a key role in the pathogenesis of atherosclerosis (1, 2), but the site and mechanisms by which lipoproteins are oxidized are still not well understood. There are considerable data demonstrating that lipoproteins are oxidized locally in the intima of the arterial wall (1–4) as well as evidence that oxidized lipoproteins are present in human serum

(5, 6). These circulating oxidized lipoproteins can be taken up by the arterial wall and thereby contribute to the levels of oxidized lipoproteins in the intima. Moreover, these partially oxidized lipoproteins may be much more susceptible to further oxidation in the arterial wall (7). However, the source of oxidized lipoproteins in the serum is not known. We have been focusing our studies on the role of diet in contributing to the levels of oxidized lipoproteins in the circulation of humans.

The typical Western diet contains substantial quantities of oxidized cholesterol (8–13), and studies in humans have shown that this oxidized cholesterol is absorbed by the small intestine and then incorporated into lipoproteins (7). Moreover, we have demonstrated that oxidized cholesterol in the diet is incorporated not only into chylomicrons but also into endogenous LDL (7). Thus, the levels of oxidized cholesterol in the circulation are influenced by the quantity of oxidized cholesterol in the diet. Finally, studies in our laboratory have shown in mouse (14) and rabbit (15) models of atherosclerosis that the addition of oxidized cholesterol to the diet increases the severity of fatty streak lesions in the aorta. Thus, the development of treatment strategies that decrease the absorption of dietary oxidized cholesterol potentially could reduce the development of atherosclerosis.

Ezetimibe is a recently developed drug that reduces serum and LDL cholesterol levels by inhibiting the absorption of cholesterol in the small intestine by blocking the sterol transporter Niemann-Pick C1-Like 1 (16, 17). The purpose of this study was to determine whether ezetimibe would also decrease the absorption of oxidized cholesterol in the diet.

MATERIALS AND METHODS

Study subjects

This study was performed on seven subjects (four males and three females). All subjects were selected from volunteers em-

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ployed at the Department of Veterans Affairs Medical Center in San Francisco. All subjects were nonsmokers, were moderately active, and consumed a typical American diet. None of the subjects was on vitamin or antioxidant therapy. Blood was drawn from each subject after a 12 h fast (time 0) for measurement of fasting serum triglycerides, total cholesterol, HDL cholesterol, and calculated LDL cholesterol levels. Control subjects had normal serum triglyceride (<150 mg/dl) and cholesterol (250 mg/dl) levels. The average age was 57 years, and none of the subjects was obese (body mass index < 30) or had diabetes, congestive heart failure, or gastrointestinal disorders. Subjects were not taking any lipid-lowering medication. This study was approved by the Committee on Human Research at the University of California, San Francisco.

Study protocol

α -Epoxy cholesterol (cholestan-5 α ,6 α -epoxy-3 β -ol) and 7-keto cholesterol (5-cholesten-3 β -ol-7-one) were used as a source of oxidized cholesterol in a test meal. Both compounds were purchased from Steraloids, Inc. (Newport, RI) and were selected because they are the major oxidized cholesterol components formed in heated or stored foods (8–13) and they are efficiently absorbed, as indicated by studies in experimental animals (18) and humans (7).

After a 12 h fast, five subjects were given a dose of 400 mg of α -epoxy cholesterol dissolved in 50 ml of olive oil and added to 100 g of carbohydrate (mashed potatoes). In a separate study, four subjects were administered 7-keto cholesterol instead of α -epoxy cholesterol. The dose and experimental procedures were identical for both oxidized cholesterol studies. Two subjects participated in both the α -epoxy cholesterol and 7-keto cholesterol feeding studies. This absorption study was carried out in subjects before and after the administration of ezetimibe for 30 days (10 mg daily, including the day of the experiment). Thus, the subjects were given the test meal at day 0 and again at day 30, and ezetimibe was administered during the time period between day 0 and day 30. The oxidized cholesterol absorption after the same test meal was then compared before and after administration of the drug. Each subject served as his/her own control. The subjects tolerated the test meal well, and none had gastrointestinal symptoms. At 2, 4, 6, and 8 h after consumption of the test meal, 50 ml blood samples were obtained for the determination of serum triglycerides, cholesterol, and oxidized cholesterol. All serum samples were stored on ice and contained 10 μ M EDTA and 5 μ M butylated hydroxytoluene throughout the sample processing. The α -epoxy cholesterol and 7-keto cholesterol levels were measured in serum before and after ezetimibe administration, and the amount of oxidized cholesterol was expressed as μ g oxidized cholesterol/dl serum. The subjects were not permitted to consume any food for the 8 h test period. Water was allowed ad libitum.

Lipoprotein isolation and characterization

The oxidized cholesterol content was determined in chylomicron/remnant (CM/RM) and LDL fractions before and after the administration of ezetimibe. These lipoprotein fractions were isolated by density centrifugation as described by us previously (7).

Analytical methods

Total cholesterol, triglyceride, and HDL cholesterol levels in serum were measured using an Infinity kit from Thermo Electron (TR 13421; Louisville, CO). LDL was calculated using the Friedewald formula. α -Epoxy and 7-keto cholesterol in serum and serum lipoprotein fractions were determined by gas-liquid chromatography using the procedure described by Hughes et al. (19)

and previously by us (7, 14, 15). Lipid samples were derivatized with Sylon BIS (Trimethylsilyl) trifluoroacetamide chlorotrimethylsilane and injected into a gas chromatograph (5890; Hewlett-Packard, Palo Alto, CA) fitted with a DB-1 column (J&W Scientific, Folsom, CA). Standard α -epoxy and 7-keto cholesterol were obtained from Steraloids, Inc. Ezetimibe was obtained from Merck Research Laboratories (West Point, PA). The areas under the serum lipid clearance curves were determined using Macdraft 2.1 and were expressed in arbitrary area units.

Statistics

Data are presented as means \pm SEM. Mean differences between groups were assessed with a paired Student's *t*-test. Significance was expressed as $P < 0.05$.

RESULTS

Subjects

Table 1 summarizes lipid values of the subjects before and after the administration of ezetimibe 10 mg daily for 30 days. As expected, the serum cholesterol and LDL cholesterol levels were reduced in subjects who were taking ezetimibe. HDL levels increased in the ezetimibe group, but the difference did not reach statistical significance. No significant change was detected in triglyceride levels.

Dietary oxidized cholesterol incorporation into serum and serum lipoproteins in subjects before and after the administration of ezetimibe

We next examined the time course of the increase in serum α -epoxy cholesterol levels after feeding the test meal before and after the subjects were administered ezetimibe 10 mg daily for 30 days. As shown in **Fig. 1A**, after feeding a test meal containing α -epoxy cholesterol, the levels of α -epoxy cholesterol in the serum increased rapidly, reaching a peak value at 4 h, and remained high during the 8 h test period. These data are in agreement with our previously published results (7). In subjects who received ezetimibe for 30 days, serum oxidized cholesterol content was lower at all time points studied. When areas under the clearance curves were compared (**Fig. 1B**), a 51% reduction (53.2 ± 7.3 for controls and 25.9 ± 9.2 for ezetimibe-treated subjects, as expressed in area units) was observed in the ezetimibe-treated group ($P = 0.01$).

After the administration of the test meal, over an 8 h period, α -epoxy cholesterol was present in both CM/RM and LDL lipoprotein fractions. **Figure 2A** shows the time course of the appearance of α -epoxy cholesterol in serum

TABLE 1. Serum lipoprotein levels

Lipoprotein	Control Subjects (n = 7)	Ezetimibe-Treated Subjects (n = 7)	<i>P</i>
		μ g/dl	
Cholesterol	241 \pm 21	198 \pm 15	0.007
LDL cholesterol	165 \pm 20	122 \pm 16	0.012
HDL cholesterol	46 \pm 5	50 \pm 5	NS
Triglycerides	122 \pm 32	83 \pm 18	NS

All values are given as means \pm SEM.

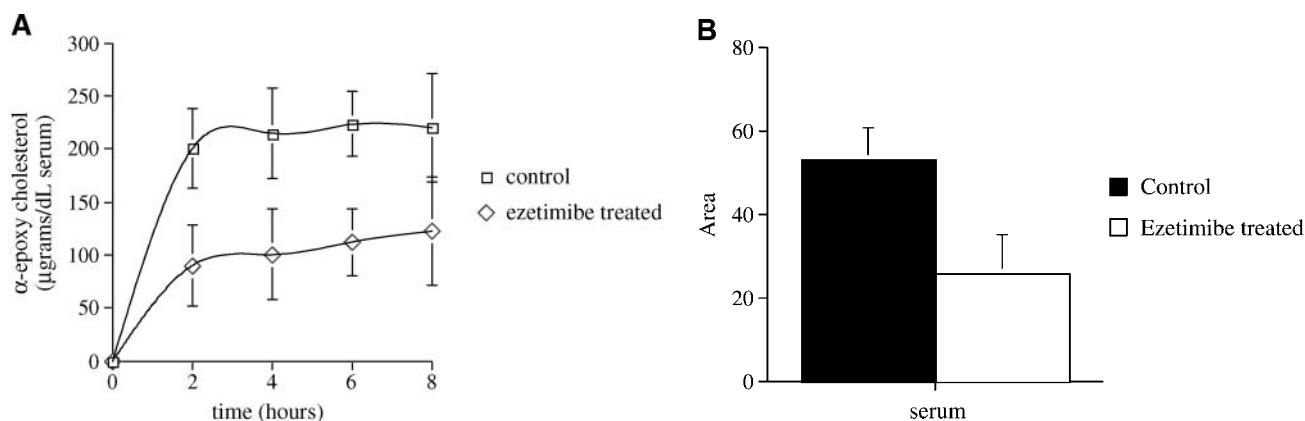


Fig. 1. Time course of α -epoxy cholesterol clearance in postprandial serum. A: Five control subjects were administered a test meal containing α -epoxy cholesterol (400 mg). The levels of α -epoxy cholesterol in serum were determined before and after treatment with ezetimibe (10 mg/day for 30 days). The levels of α -epoxy cholesterol in serum were measured using gas-liquid chromatography (GLC) and expressed as $\mu\text{g/dl}$ serum. B: The areas under the clearance curves were measured in arbitrary units, and the significance of the difference of areas under the curve was determined. $P = 0.010$. Data are expressed as means \pm SEM.

CM/RM after feeding α -epoxy cholesterol before and after ezetimibe administration. When the areas under the clearance curve were compared (Fig. 2B), there was a 64% decrease ($P = 0.019$) in α -epoxy cholesterol in subjects treated with ezetimibe (10.8 ± 1.4 for controls and 3.9 ± 1.8 for the ezetimibe-treated group). Similar results were found in the serum LDL fraction (Fig. 3A). As shown in Fig. 3B, the area under the clearance curve for the control subjects was decreased by 52% (13.8 ± 2.8 and 6.6 ± 1.7 for subjects treated with ezetimibe; $P = 0.048$).

Figure 4 shows that there was no change in the serum triglyceride levels over the 8 h study period, indicating that ezetimibe does not interfere with triglyceride absorption and CM/RM uptake by the liver.

We next determined the effect of ezetimibe on the serum levels of another oxidized cholesterol (7-keto cho-

lesterol) present in high quantities in the typical Western diet. Figure 5A demonstrates that, similar to α -epoxy cholesterol, there was a significant reduction in 7-keto cholesterol levels in the serum after the subjects were treated with ezetimibe for 30 days. The area under the clearance curve was reduced by 48% in the ezetimibe-treated group (49.9 ± 3.4 for controls and 25.9 ± 5.9 for ezetimibe-treated subjects; $P = 0.013$) (Fig. 5B).

DISCUSSION

Dietary cholesterol is easily oxidized, and oxidized cholesterol is a common component of most American diets (8–13). It has been established that cholesterol undergoes spontaneous autoxidation when exposed to air, heat, light,

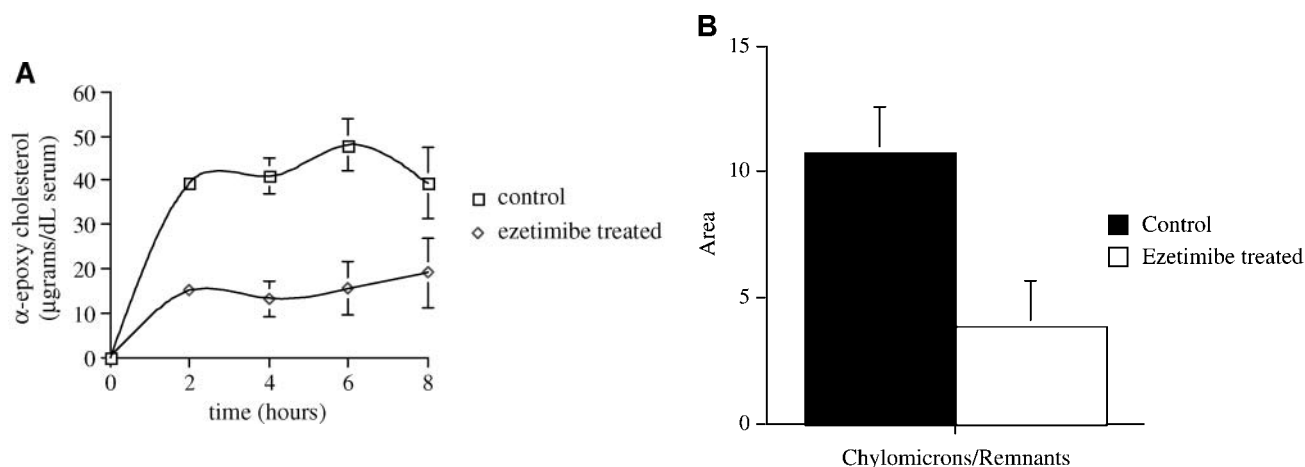


Fig. 2. Time course of α -epoxy cholesterol clearance in postprandial chylomicron/remnant (CM/RM) fraction. A: Five control subjects were administered a test meal containing α -epoxy cholesterol. The levels of α -epoxy cholesterol in CM/RM were determined before and after treatment with ezetimibe (10 mg/day for 30 days). CM/RM was isolated by sequential ultracentrifugation as described in Methods. The levels of α -epoxy cholesterol in serum were measured using GLC and expressed as $\mu\text{g/dl}$ serum. B: The areas under the clearance curves were measured in arbitrary units, and the significance of the difference of areas under the curve was determined. $P = 0.019$. Data are expressed as means \pm SEM.

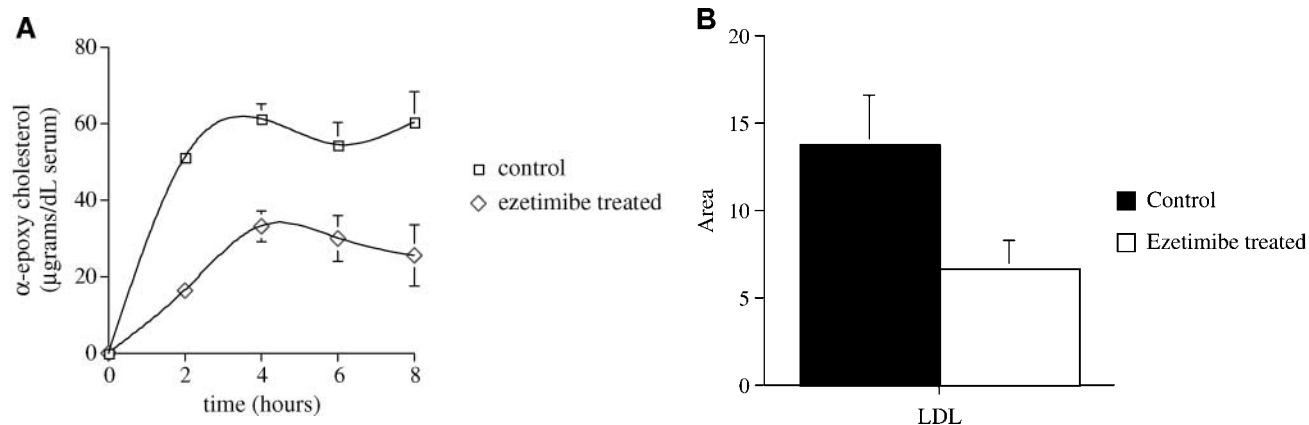


Fig. 3. Time course of α -epoxy cholesterol clearance in postprandial LDL fraction. A: Five control subjects were administered a test meal containing α -epoxy cholesterol. The levels of α -epoxy cholesterol in LDL were determined before and after treatment with ezetimibe (10 mg/day for 30 days). LDL from serum was isolated by sequential ultracentrifugation as described in Methods. The levels of α -epoxy cholesterol in LDL were measured using GLC and expressed as $\mu\text{g}/\text{dL}$ serum. B: The areas under the clearance curves were measured in arbitrary units, and the significance of the difference of areas under the curve was determined. $P = 0.048$. Data are expressed as means \pm SEM.

and oxidizing agents (20). The autoxidation of cholesterol results in the formation of a large number of oxidized sterols, but the major products are 7-oxygenated sterols (7 α - and 7 β -hydroxy cholesterol and 7-keto cholesterol) and 5,6-oxygenated sterols (5 α ,6 α -epoxy cholesterol, 5 β ,6 β -epoxy cholesterol, and 5 α ,6 β -dihydroxy cholesterol). The quantity of oxidized cholesterol has been measured in numerous foods that are commonly consumed as part of the typical American diet by several investigators (8–13). Oxidized cholesterol is particularly prevalent in fast and processed foods and foods subjected to high temperatures. For example, butter and French fried potatoes contain 0.41 mg (11) and 0.02 mg (13) of oxidized cholesterol per gram of food, respectively. In general, it has been estimated that a typical Western diet will on average

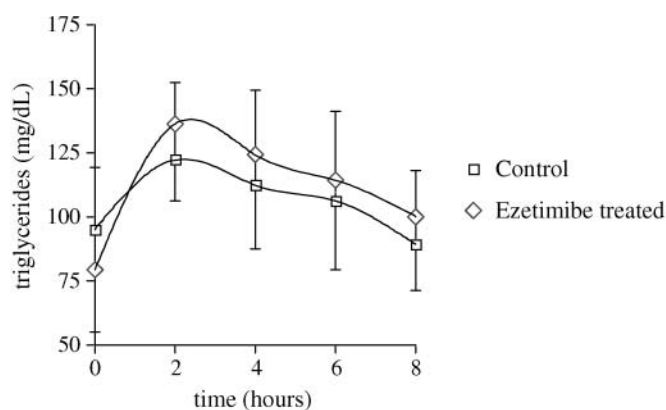


Fig. 4. Time course of triglyceride clearance in postprandial serum. Five subjects were administered a test meal containing α -epoxy cholesterol (400 mg) before and after the administration of ezetimibe (10 mg/day for 30 days), and the triglyceride levels in serum were measured at the indicated times. The levels of triglycerides were expressed as mg/dL serum. Data are expressed as means \pm SEM.

contain 1 to 5% of its cholesterol in the oxidized form. Thus, the levels of oxidized cholesterol in foods depend on the conditions of storage and the method of preparation.

Studies in both humans (7) and animals (14, 15, 21, 22) have demonstrated that the small intestine absorbs oxidized cholesterol in the diet. The absorbed oxidized cholesterol is incorporated into chylomicrons and secreted into the lymph. Chylomicrons containing oxidized cholesterol are metabolized similarly to “normal” chylomicrons, with the oxidized cholesterol being primarily delivered to the liver (23). Recent studies in our laboratory have shown that in humans, oxidized cholesterol in the diet is incorporated into postprandial chylomicrons (7). Moreover, we also found that oxidized cholesterol was present in endogenous lipoproteins, particularly LDL (7). Furthermore, the LDL containing oxidized cholesterol was more susceptible to further oxidation than “normal” LDL (7).

A number of studies in various animal models have examined the effect of oxidized cholesterol in the diet on atherosclerosis. In general, numerous studies have shown that adding oxidized cholesterol to the diet increases atherosclerosis in a variety of different animal models. For example, Jacobson et al. (24) and Peng et al. (25) demonstrated an increase in atherosclerotic lesion in the aortas of White Carneau pigeons and squirrel monkeys, respectively. In our studies, we demonstrated that oxidized cholesterol in the diet increased atherosclerosis when fed to rabbits (14), apolipoprotein E-deficient mice, and LDL receptor-deficient mice (15). In humans, there is a paucity of data on the effect of oxidized cholesterol in the diet on atherosclerosis. In the absence of other risk factors, Indians living in London have a higher incidence of atherosclerosis compared with non-Indians, and this increase has been proposed to be attributable to the ingestion of ghee (heated butter that is very rich in oxidized cholesterol, containing up to 12% of total cholesterol as oxysterols) (26). Similarly, in India, individuals who con-

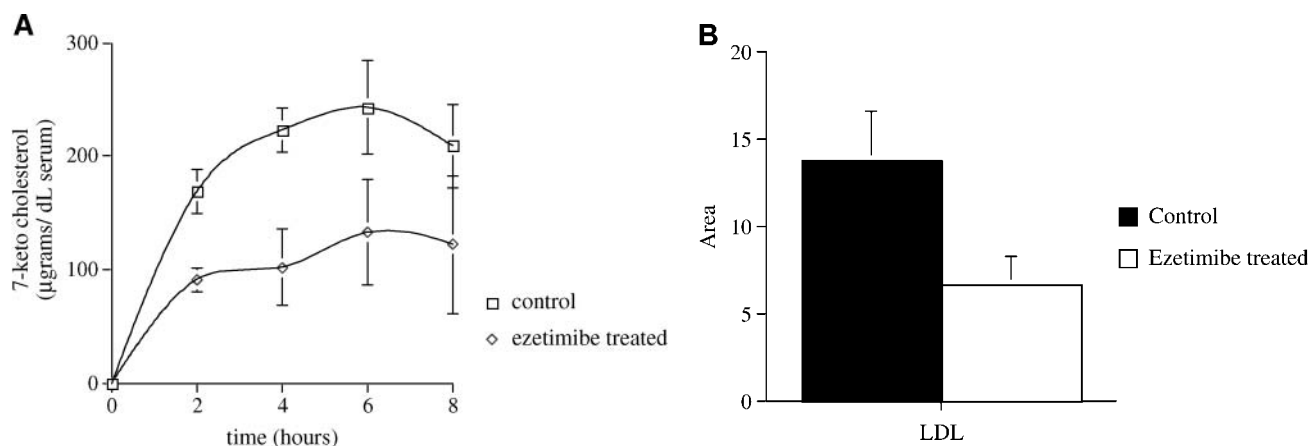


Fig. 5. Time course of 7-keto cholesterol clearance in postprandial serum. A: Four control subjects were administered a test meal containing 7-keto cholesterol, and the quantity of 7-keto cholesterol in serum was determined before and after treatment with ezetimibe (10 mg/day for 30 days). The levels of 7-keto cholesterol in serum were measured using GLC and are expressed as $\mu\text{g}/\text{dL}$ serum. B: The areas under the clearance curves were measured in arbitrary units, and the significance of the differences of areas under the curve was determined. $P = 0.013$. Data are expressed as means \pm SEM.

sume >1 kg of ghee per month had a 4-fold increase in the rate of heart disease (27). In addition to these studies, epidemiological studies have shown an association of oxidized cholesterol in the serum and atherosclerosis (28). It was shown that an increase in plasma 7β -hydroxy cholesterol in Lithuanian men compared with Swedish men was associated with a 4-fold increase in heart disease. Additionally, it has been demonstrated that coronary atherosclerosis is reflected by autoantibodies against oxidized LDL and oxidized cholesterol in the serum (29). Although these associations are supportive of the hypothesis that oxidized cholesterol in the diet predisposes to atherosclerosis, it is clear that other factors could be the basis for the increase in atherosclerosis in these studies. However, taking the animal data and human data in total, it appears that oxidized cholesterol in the diet might increase atherosclerosis.

Ezetimibe specifically inhibits the absorption of cholesterol and related plant sterols at the brush border of the small intestine by blocking the sterol transporter, Niemann-Pick C1-Like 1 (30). Studies have shown that ezetimibe 10 mg once per day reduces serum LDL levels by $\sim 20\%$ and causes minimal side effects (30). This inhibition is very specific, as ezetimibe does not inhibit the absorption of structurally similar compounds such as vitamin D, estrogens, bile acids, and progesterone (31).

In this study, we demonstrated that ezetimibe 10 mg per day for 1 month markedly reduced the levels of oxidized cholesterol in the serum after feeding a test meal containing either α -epoxy cholesterol or 7-keto cholesterol, two of the predominant oxysterols found in the diet. Moreover, the decrease in oxidized cholesterol in the serum was attributable to a decrease in the incorporation of dietary oxidized cholesterol into both chylomicrons and LDL. That this decrease in oxidized cholesterol in the serum was attributable to ezetimibe inhibiting the absorption of dietary oxidized cholesterol is suggested by the

absence of changes in postprandial triglyceride levels with ezetimibe treatment. If the marked reduction in oxidized cholesterol levels was caused by enhanced clearance, one would have expected a concomitant reduction in postprandial triglyceride levels.

In summary, this study demonstrates that ezetimibe markedly decreases the levels of oxidized cholesterol in lipoproteins after the ingestion of a diet that contains oxidized cholesterol. Whether this decrease in circulating oxidized cholesterol will prevent or delay the development of atherosclerosis remains to be determined.

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